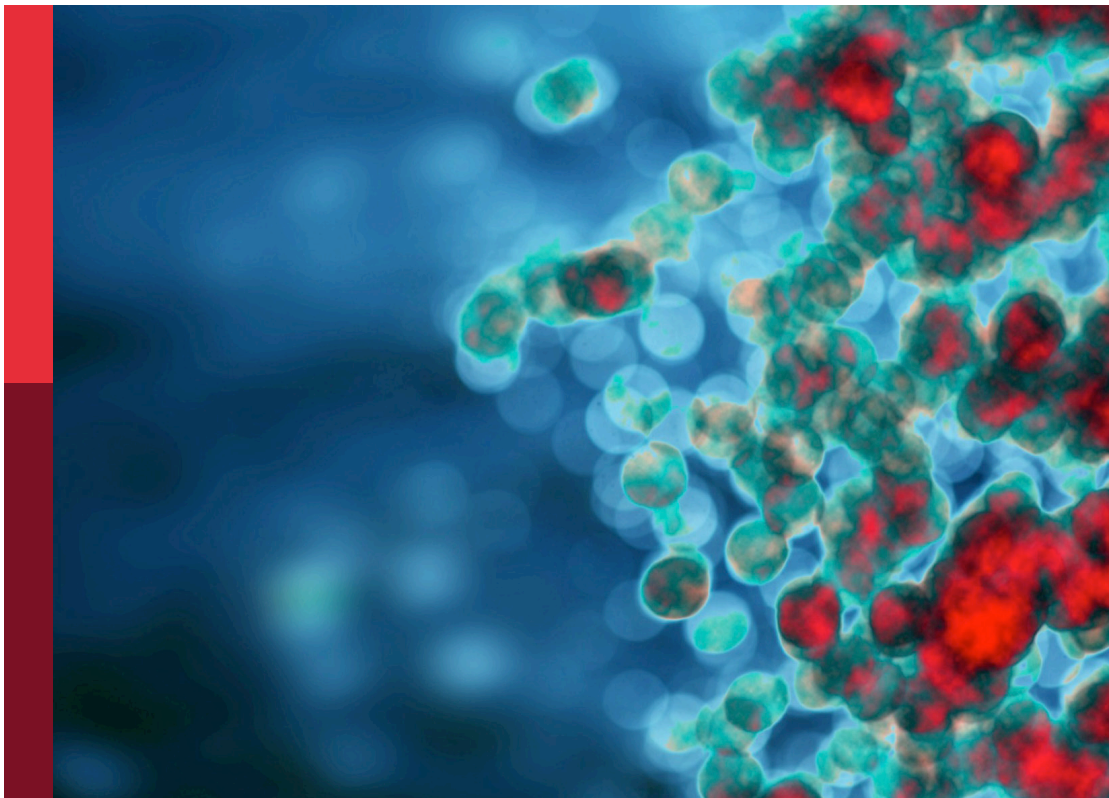
frontiers | Research Topics

Immunology at the feto-maternal interface

Edited by
Mahmood Jeddi-Tehrani, Amir-Hassan Zarnani,
Maria Laura Zenclussen and Marie-Pierre Piccinni

Published in
Frontiers in Immunology



FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-8325-6748-7
DOI 10.3389/978-2-8325-6748-7

Generative AI statement
Any alternative text (Alt text) provided alongside figures in the articles in this ebook has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Immunology at the feto-maternal interface

Topic editors

Mahmood Jeddi-Tehrani — Avicenna Research Institute, Academic Center for Education, Culture and Research, Iran

Amir-Hassan Zarnani — Tehran University of Medical Sciences, Iran

Maria Laura Zenclussen — National Scientific and Technical Research Council (CONICET), Argentina

Marie-Pierre Piccinni — University of Florence, Italy

Citation

Jeddi-Tehrani, M., Zarnani, A.-H., Zenclussen, M. L., Piccinni, M.-P., eds. (2025). *Immunology at the feto-maternal interface*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-6748-7

Table of contents

06 Editorial: Immunology at the feto-maternal interface
Amir-Hassan Zarnani, Mahmood Jeddi-Tehrani, Marie-Pierre Piccinni and Maria Laura Zenclussen

10 Uterine Nodal expression supports maternal immunotolerance and establishment of the FOXP3+ regulatory T cell population during the preimplantation period
Sarah Yull, Shiva Shafiei, Craig B. Park, Parinaz Kazemi, Emily B. Tiemann, Marie-Hélène Godin Pagé and Daniel Dufort

27 Endometrial immune profiling as a new tool for preconceptional assessment in patients with systemic autoimmune diseases
Juan J. Fierro, Jelmer R. Prins, Svenja Henning, Hendrika Bootsma, Johanna Westra and Karina de Leeuw

32 Melatonin: the placental antioxidant and anti-inflammatory
Tyana T. Joseph, Viviane Schuch, Daniel J. Hossack, Rana Chakraborty and Erica L. Johnson

44 Protective role of complement factor H against the development of preeclampsia
Hadida Yasmin, Chiara Agostinis, Miriam Toffoli, Tamali Roy, Silvia Pegoraro, Andrea Balduit, Gabriella Zito, Nicoletta Di Simone, Giuseppe Ricci, Taruna Madan, Uday Kishore and Roberta Bulla

56 Decidual γδT cells of early human pregnancy produce angiogenic and immunomodulatory proteins while also possessing cytotoxic potential
Jasper Nörenberg, Péter Vida, Isabell Bösmeier, Barbara Forró, Anna Nörenberg, Ágnes Buda, Diana Simon, Szabina Erdő-Bonyár, Pál Jáksó, Kálmán Kovács, Éva Mikó, Tímea Berki, Emese Mezősi and Alíz Barakonyi

68 Placenta: an old organ with new functions
Sara Khorami-Sarvestani, Negar Vanaki, Sorour Shojaeian, Kayhan Zarnani, Allan Stensballe, Mahmood Jeddi-Tehrani and Amir-Hassan Zarnani

91 CD4+ T cell heterogeneity in gestational age and preeclampsia using single-cell RNA sequencing
Sayaka Tsuda, Shigeyuki Shichino, Tamara Tilburgs, Tomoko Shima, Keiko Morita, Akemi Yamaki-Ushijima, Krishna Roskin, Michio Tomura, Azusa Sameshima, Shigeru Saito and Akitoshi Nakashima

103 Palmitate and group B *Streptococcus* synergistically and differentially induce IL-1β from human gestational membranes
Jennifer A. Gaddy, Rebecca E. Moore, Jonathan S. Lochner, Lisa M. Rogers, Kristen N. Noble, Ayush Giri, David M. Aronoff, David Cliffel and Alison J. Eastman

116 IL-1 and TNF mediates IL-6 signaling at the maternal-fetal interface during intrauterine inflammation
Pietro Presicce, Cynthia Roland, Paranthaman Senthamaraikannan, Monica Cappelletti, McKensie Hammons, Lisa A. Miller, Alan H. Jobe, Claire A. Chougnet, Emily DeFranco and Suhas G. Kallapur

128 Endocytosis at the maternal-fetal interface: balancing nutrient transport and pathogen defense
Mingming Fan, Hongyu Wu, Amanda N. Sferruzzi-Perri, Yan-Ling Wang and Xuan Shao

142 Emerging role of C5aR2: novel insights into the regulation of uterine immune cells during pregnancy
Fenna Froehlich, Konstanze Landerholm, Johanna Neeb, Ann-Kathrin Meß, Daniel Leonard Seiler, Tamara Tilburgs and Christian Marcel Karsten

156 Chronic endometritis and recurrent reproductive failure: a systematic review and meta-analysis
Carlo Ticconi, Annalisa Inversetti, Serena Marraffa, Luisa Campagnolo, Jephtah Arthur, Enrica Zambella and Nicoletta Di Simone

173 The role of pyroptosis in the occurrence and development of pregnancy-related diseases
Jiahui Li, Min Wang, Haiyan Zhou, Zhong Jin, Haonan Yin and Shuli Yang

185 *In-utero* transfer of decidualized endometrial stromal cells increases the frequency of regulatory T cells and normalizes the abortion rate in the CBA/J × DBA/2 abortion model
Kayhan Zarnani, Kimia Zarnani, Nasim Maslehat-Lay, Bahman Zeynali, Sedigheh Vafaei, Mohammad-Reza Shokri, Negar Vanaki, Haleh Soltanghoraee, Ebrahim Mirzadegan, Haleh Edalatkhah, Mohammad-Mehdi Naderi, Ali Sarvari, Farnoosh Attari, Mahmood Jeddi-Tehrani and Amir-Hassan Zarnani

200 B cells: roles in physiology and pathology of pregnancy
Jin-Chuan Liu, Qunxiong Zeng, Yong-Gang Duan, William S. B. Yeung, Raymond H. W. Li, Ernest H. Y. Ng, Ka-Wang Cheung, Qingqing Zhang and Philip C. N. Chiu

217 Recurrent reproductive failure and celiac genetic susceptibility, a leading role of gluten
Eduardo de la Fuente-Munoz, Miguel Fernández-Arquero, Nabil Subbhi-Issa, Kissy Guevara-Hoyer, Lydia Pilar Suárez, Raquel Gil Laborda, Marina Sánchez, Juliana Ochoa-Grullón, María Guzmán-Fulgencio, Ángela Villegas, María Dolores Mansilla, Noelia Pérez, Ricardo Savirón Cornudella, Teresa Gastañaga-Holguera, Marta Calvo Urrutia, Ignacio Cristóbal García and Silvia Sánchez-Ramón

224 Circulating extracellular vesicles and neutrophil extracellular traps contribute to endothelial dysfunction in preeclampsia
Alex Ramos, Lina Youssef, Patricia Molina, Sergi Torramadé-Moix, Julia Martinez-Sanchez, Ana Belen Moreno-Castaño, Miquel Blasco, Elena Guillén-Olmos, Blanca De Moner, Marc Pino, Marta Tortajada, Marta Camacho, Maria Borrell, Francesca Crovetto, Maria Jose Ramirez-Bajo, Pedro Ventura-Aguiar, Elisenda Banon-Maneus, Jordi Rovira, Gines Escolar, Enric Carreras, Eduard Gratacos, Maribel Diaz-Ricart, Fatima Crispi and Marta Palomo

237 Tacrolimus (FK506) promotes placentation and maternal-fetal tolerance through modulating FASN-CEACAM1 pathway
Xinhang Meng, Minfeng Shi, Jinfeng Qian, Yujie Luo, Liyuan Cui, Dajin Li and Songcun Wang

248 Endometrial immune profiling and precision therapy increase live birth rate after embryo transfer: a randomised controlled trial
Nathalie Lédée, Marie Petitbarat, Geraldine Dray, Lucie Chevrier, Alaa Kazhalawi, Mona Rahmati, Eric Vicaut, Abdourahmane Diallo, Nino Guy Cassuto, Lea Ruoso and Laura Prat-Ellenberg

263 Epigenetic regulation in oogenesis and fetal development: insights into m6A modifications
Lusheng Liu, Danxia Ge, Yumeng Lin, Zhongyu Han, Heng Zhao, Liqin Cao, Xi Wu and Guizhi Ma

frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 12 August 2025
DOI 10.3389/fimmu.2025.1667375

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
David Cameron Wraith,
University of Birmingham, United Kingdom

*CORRESPONDENCE
Amir-Hassan Zarnani
zarnania@tums.ac.ir
Maria Laura Zenclussen
mzenclussen@gmail.com

RECEIVED 16 July 2025
ACCEPTED 29 July 2025
PUBLISHED 12 August 2025

CITATION
Zarnani A-H, Jeddi-Tehrani M, Piccinni M-P and Zenclussen ML (2025) Editorial: Immunology at the feto-maternal interface. *Front. Immunol.* 16:1667375. doi: 10.3389/fimmu.2025.1667375

COPYRIGHT
© 2025 Zarnani, Jeddi-Tehrani, Piccinni and Zenclussen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Editorial: Immunology at the feto-maternal interface

Amir-Hassan Zarnani[1,2,3]*, Mahmood Jeddi-Tehrani[4], Marie-Pierre Piccinni[5] and Maria Laura Zenclussen[6]*

[1]Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, [2]Reproductive Immunology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran, [3]Department of Tissue Engineering and Regenerative Medicine, Nanobiotechnology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran, [4]Monoclonal Antibody Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran, [5]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy, [6]Instituto de Salud y Ambiente del Litoral (ISAL), Universidad Nacional del Litoral (UNL) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Bioquímica y Ciencias Biológicas (FBCB), UNL, Santa Fe, Argentina

KEYWORDS

reproduction, immunoregulation, tolerance, endometrium, placenta, inflammation, decidualization

Editorial on the Research Topic
Immunology at the feto-maternal interface

Reproductive biology serves as the foundation for other biological disciplines, as reproduction has been the key driver of evolution in both simple and complex organisms. Evolutionary success is ultimately measured by biological fitness, which hinges on successful reproduction. Consequently, reproductive sciences have become a major focus of research. In particular, reproductive immunology has gained unprecedented attention, driven by societal industrialization, delayed marriages, and declining fertility rates. Despite advancements in assisted reproductive technologies (ART), the low success rate of embryo transfer remains a major bottleneck in *in vitro* fertilization (IVF). Thus, deciphering the immunological mechanisms of embryo implantation—particularly how maternal immune cells at the pregnancy site foster tolerance to fetal antigens—is critical.

The female reproductive tract undergoes immunological changes from the first encounter with seminal fluid, triggering molecular and cellular adaptations that enhance conception and pregnancy by modulating inflammatory responses and inducing paternal antigen-specific regulatory T cells (Tregs) (1). Eutherian implantation evolved from an ancestral inflammatory response to embryo attachment in therian mammals. This explains the dual role of inflammation in human pregnancy—it is essential for both implantation and labor, yet poses a risk to pregnancy maintenance if uncontrolled (2). Thus, inflammation is neither inherently good nor bad—its effects depend on the stage of pregnancy, where it can be either beneficial or harmful. In this regard, many studies have attempted to clarify how inflammation influences the onset of pregnancy and its possible connection to complications like recurrent miscarriage (3). Indeed, several attempts have been made to take advantage of mechanical injury of endometrium to enhance uterine receptivity (4). Nonetheless, controlling inflammation at the feto-maternal interface is a hallmark of a successful pregnancy and in this context both endometrial and placental-

derived factors play a role, as highlighted in the reviews by Khorami-Sarvestani et al. and Joseph et al. Placenta, maternal immune cells, and endometrium interact dynamically to adapt the maternal immune system to the genetically distinct fetus and to control inflammatory reactions towards the fetal antigens. Changes in the placental proteome profile may serve as early diagnostic biomarkers for pregnancy disorders. Intriguingly, placenta and cancer development share striking similarities in metabolic activity, cellular behavior, molecular signatures, signaling pathways, and tissue microenvironment. This overlap has led to the theory of "cancer as ectopic trophoblastic cells", as pointed out by Khorami-Sarvestani et al. Interestingly, the placenta can independently produce melatonin, reducing reliance on the pineal gland. During pregnancy, melatonin safeguards maternal-fetal health by combating oxidative stress via free radical neutralization and modulating inflammation, thereby preserving placental integrity. It also helps regulate maternal immune responses to accommodate the developing fetus throughout gestation, as described by Joseph et al. While acute inflammation initiates implantation and labor, chronic inflammation—linked to conditions such as endometriosis, and chronic endometritis—can lead to cell senescence, infertility, or miscarriage as reviewed by Ticconi et al.. This is mostly driven by the pro-inflammatory cytokines, IL-6 and IL-1, as shown by Presicce et al. Activation of inflammasome by various factors, including pro-inflammatory cytokines, can trigger pyroptosis, an innate immune response leading to activation of apoptotic pathways and perforation of cell membrane. Pyroptosis is known to be activated in pregnancy-related complications such as congenital Zika virus infection, preterm labor, preeclampsia and intrauterine growth restriction as pointed out by Li et al. During late pregnancy, bacterial infections—particularly group B Streptococcus—in the upper reproductive tract frequently trigger chorioamnionitis and inflammatory responses. These infections are a predominant cause of serious pregnancy complications, notably preterm labor and premature delivery. Obese individuals with higher circulating palmitate levels exhibit higher rates of group B Streptococcus colonization compared to those with normal weight. Notably, palmitate and group B streptococcus synergistically and differentially induce IL-1β from human gestational membranes, which would predispose pregnant people to a greater likelihood of pregnancy complications, as highlighted in the work by Gaddy et al.. Bacteria, viruses and parasites could cross the fetal membranes by different endocytotic mechanisms. Given that the maternal-fetal interface must carefully regulate nutrient uptake through endocytosis while preventing harmful substances and drugs from crossing to support healthy fetal development, it is crucial to understand the precise balance between nutrient transport and protection against pathogens, as pointed out in the review by Fan et al..

For a successful human pregnancy, maternal immune tolerance to the semi-allogeneic fetus is essential. This tolerance is controlled by genetic and epigenetic regulations, as described by Liu et al., and is established through a sophisticated interplay of decidual immune cells, cytokines, chemokines, steroid hormones, adhesion molecules, and immune-regulatory factors from both maternal and fetal sources, operating both before and throughout gestation. The maternal-fetal interface hosts a diverse array of immune cells, including decidual natural killer (dNK) cells, macrophages, T cells, and dendritic cells and a minor population of B cells, and NKT cells. These cells interact with decidual stromal cells and trophoblasts, forming an intricate network of cellular communication. Disruptions in this immunological balance can contribute to pregnancy complications such as recurrent miscarriage, preeclampsia, preterm birth, intrauterine growth restriction, and infections (5). Uterine natural killer (uNK) cells, the most abundant immune cells in the pregnant endometrium, are essential for successful pregnancy. Unlike their cytotoxic peripheral counterparts, uNK cells exhibit unique regulatory properties, specializing in tissue homeostasis, vascular remodeling, and immune tolerance. By clearing senescent cells, they maintain decidualization and uterine receptivity. Functional impairment of uNK cells can disrupt endometrial function, contributing to recurrent pregnancy loss. Regulatory T cells (Tregs) suppress inflammatory responses and promote tolerance by inhibiting effector T cells and secreting anti-inflammatory cytokines, IL-10, TGF-β. Their expansion is crucial for preventing fetal rejection. While the roles of steroid hormones, TGFβ and related signaling molecules in induction of Tregs and pregnancy-related immune regulation have been extensively studied, recent studies highlight the potential involvement of uterine Nodal in this process. Previous studies have characterized the expression dynamics, regulatory mechanisms, and functional role of Nodal - a TGFβ superfamily morphogen - during mouse pregnancy. Recently, it was shown that conditional knockout of Nodal in the mouse female reproductive tract caused significant subfertility associated with dysregulation of key inflammatory cytokines, uterine infiltration of pro-inflammatory macrophages and complete absence of CD4+FOXP3+ regulatory T cells during the preimplantation window, as shown in the work by Yull et al..

While B cells and γδ T cells represent a relatively small proportion of decidual immune cells, their critical contributions to sustaining pregnancy cannot be overlooked. γδ T cells have garnered increasing attention for their potential functions in pregnancy. Recognized for their role in monitoring tissue integrity at barrier sites, these cells are more abundant in the decidua among CD3+ lymphocytes than in peripheral blood. Decidual γδ T cell populations - comprising Vδ1+, Vδ2+, and double-negative (Vδ1-/Vδ2-) subsets - differentially express HLA-specific regulatory receptors including NKG2C, NKG2A, ILT2, and KIR2DL4 and secrete key cytokines (G-CSF, FGF2) and cytotoxic factors (Granulysin, IFN-γ), indicating their dual roles in supporting angiogenesis, placental development and providing antimicrobial protection, as highlighted in the work of Nörenberg et al. B cells play an essential yet understudied role in pregnancy, maintaining a delicate equilibrium between immune tolerance for fetal acceptance and protective immunity against infections. Although their importance is recognized, the precise mechanisms by which B cells influence pregnancy outcomes remain poorly understood. Regulatory B cells (Bregs) and their production of IL-10 are pivotal in sustaining fetal tolerance, illustrating the body's intricate adaptation to prevent rejection of the semi-allogeneic fetus.

Conversely, aberrant autoantibody production by specific B cell subsets reveals the fine line between immune regulation and dysfunction, where disruptions can lead to adverse gestational outcomes, as reviewed in the article by Liu et al..

The endometrial immune system, along with other cells not primarily involved in immune functions, releases numerous soluble and regulatory molecules that play a key role in promoting maternal tolerance during pregnancy. These include but are not limited to galectins, indoleamine 2,3-dioxygenase (IDO), and complement regulatory proteins. During pregnancy, complement protein levels increase, but their activity is balanced by high concentrations of regulatory proteins like factor H, which inhibits the alternative C3 convertase and decay-accelerating factor (DAF/CD55), which prevents C3 convertase formation and suppresses downstream complement activation. When the complement system is dysregulated, it can contribute to complications such as pre-eclampsia and intrauterine growth restriction (6). A recent study showed that in the first trimester human placenta, factor H is largely produced by decidual spiral arteries and syncytiotrophoblasts. Notably, placental expression and plasma levels of factor H in preeclampsia women was found comparatively lower than control counterparts, as described in the research article by Yasmin et al.. The activity of complement proteins is related to the receptor they bind. For example, C5a the most powerful anaphylatoxin, triggers diverse immune responses by binding to two transmembrane receptors, C5aR1 and C5aR2. C5aR1 is known for its pro-inflammatory effects, while, C5aR2 KO mice exhibit fewer implantation sites, along with elevated mRNA levels of pro-inflammatory cytokines, IL-12, IL-18, and IFN-γ at the maternal-fetal interface. Additionally, maternal C5aR2 deficiency leads to reduced uterine NK cell infiltration, suggesting a key regulatory role for this receptor in pregnancy maintenance, as pointed out by Froehlich et al..

While a variety of factors and mediators—acting at both endometrial and systemic levels—work in precise harmony to foster immune tolerance at the feto-maternal interface, the critical immunomodulatory role of endometrial cells has often been underestimated. The endometrium is essential for embryo implantation and development, guiding early embryo attachment, supporting placental formation and invasion, and creating a protective environment for fetal growth. A crucial prerequisite for human embryo implantation is the transformation of endometrial stromal cells (ESCs) into a decidualized state. This process involves complex morphological and functional changes in ESCs, accompanied by significant shifts in their epigenetic, metabolomic, transcriptomic, and proteomic profiles. Disruptions in decidualization have been linked to pregnancy loss (7). Decidual stromal cells (DSCs) play a major role in immune regulation, orchestrating, sustaining, and modulating immune responses. They facilitate the recruitment of peripheral blood NK cells (pbNKs), Th1, and Tc lymphocytes, promote the differentiation of uterine NK (uNK) cells, drive monocytes toward an M2-like phenotype, induce tolerogenic dendritic cells, stimulate regulatory T cell (Treg) development, and promote a type 2 immune bias (8). The pivotal role of decidualization in regulating uterine immune milieu has been recently addressed in CBA/J × DBA/2 mouse model. Although this model has long been used to support classical immune tolerance theories during pregnancy and with this notion in mind, a variety of immunomodulators including Tacrolimus has been used to alleviate abortion in this model, like in the article by Meng et al., new findings indicate that immune imbalance is not the primary cause of fetal loss. Instead, the authors demonstrated that impaired endometrial decidualization likely triggers immune dysregulation at the maternal-fetal interface, ultimately resulting in pregnancy failure, as shown by Zarnani et al..

Alongside the well-established role of immunoregulation in fostering maternal tolerance to the feto-placental unit, immune dysregulation has also been widely investigated in the context of pregnancy complications. Pregnancy-related disorders arise from diverse and intricate pathogenic mechanisms, with immune-mediated processes playing a central role in nearly all cases. Understanding the precise contribution of the immune system in these conditions is therefore critical. While past research primarily relied on reductionist methods—examining isolated or limited disease-associated factors—advances in high-throughput technologies now allow for a comprehensive, systems-level approach. This shift enables more precise immunodiagnosis and targeted therapies for women with pregnancy-related complications (9). Among these emerging strategies, endometrial immune profiling stands out as a key tool for characterizing the endometrial milieu including immune cells and soluble immunoregulatory proteins before or during pregnancy. This approach focuses on deciphering localized immune responses within the endometrium to uncover their role in reproductive success or pathology. This approach has been the focus of recent papers for preconceptional immunodiagnosis of different diseases including systemic autoimmune diseases (as highlighted by Fierro et al.) and preeclampsia (as demonstrated by Tsuda et al.). It has also been suggested by Lédée et al., that this approach could enhance the overall performance of assisted reproductive therapies.

This Research Topic in Frontiers in Immunology mainly covers fundamental aspects of the immunoregulatory processes within the innate and adaptive immune systems that facilitate successful embryo implantation and placental formation. It also explores the immunopathology that leads to adverse pregnancy outcomes, as detailed in the articles by Ramos et al. and by de la Fuente-Munoz et al.. Furthermore, it sheds light on the emerging technologies for the immunodiagnosis of pregnancy-related disorders.

Author contributions

A-HZ: Conceptualization, Writing – review & editing, Writing – original draft. MJ-T: Writing – review & editing. M-PP: Writing – review & editing. MZ: Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Robertson SA, Prins JR, Sharkey DJ, Moldenhauer LM. Seminal fluid and the generation of regulatory T cells for embryo implantation. *Am J Reprod Immunol N Y N 1989*. (2013) 69:315–30. doi: 10.1111/aji.12107

2. Griffith OW, Chavan AR, Protopapas S, Maziarz J, Romero R, Wagner GP. Embryo implantation evolved from an ancestral inflammatory attachment reaction. *Proc Natl Acad Sci*. (2017) 114:E6566–75. doi: 10.1073/pnas.1701129114

3. PrabhuDas M, Bonney E, Caron K, Dey S, Erlebacher A, Fazleabas A, et al. Immune mechanisms at the maternal-fetal interface: perspectives and challenges. *Nat Immunol*. (2015) 16:328–34. doi: 10.1038/ni.3131

4. Dekel N, Gnainsky Y, Granot I, Mor G. Inflammation and implantation. *Am J Reprod Immunol N Y N 1989*. (2010) 63:17–21. doi: 10.1111/j.1600-0897.2009.00792.x

5. Arck PC, Hecher K. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat Med*. (2013) 19:548–56. doi: 10.1038/nm.3160

6. Abu-Raya B, Michalski C, Sadarangani M, Lavoie PM. Maternal immunological adaptation during normal pregnancy. *Front Immunol*. (2020) 11–2020. doi: 10.3389/fimmu.2020.575197

7. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev*. (2014) 35:851–905. doi: 10.1210/er.2014-1045

8. Llorca T, Ruiz-Magaña MJ, Abadía AC, Ruiz-Ruiz C, Olivares EG. Decidual stromal cells: fibroblasts specialized in immunoregulation during pregnancy. *Trends Immunol*. (2025) 46:138–52. doi: 10.1016/j.it.2024.12.007

9. Zarnani AH. Two paths to discovery: bridging outside-in high throughput technologies and inside-out artificial intelligence for biological decoding of women reproductive failure. *J Reprod Infertil*. (2024) 25:242–4. doi: 10.18502/jri.v25i4.18121

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 30 October 2023
DOI 10.3389/fimmu.2023.1276979

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Rosanna Ramhorst,
University of Buenos Aires, Argentina
Panicos Shangaris,
King's College London, United Kingdom

*CORRESPONDENCE
Daniel Dufort
daniel.dufort@mcgill.ca

RECEIVED 13 August 2023
ACCEPTED 17 October 2023
PUBLISHED 30 October 2023

CITATION
Yull S, Shafiei S, Park CB, Kazemi P, Tiemann EB, Pagé M-HG and Dufort D (2023) Uterine Nodal expression supports maternal immunotolerance and establishment of the FOXP3$^+$ regulatory T cell population during the preimplantation period.
Front. Immunol. 14:1276979.
doi: 10.3389/fimmu.2023.1276979

COPYRIGHT
© 2023 Yull, Shafiei, Park, Kazemi, Tiemann, Pagé and Dufort. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Uterine Nodal expression supports maternal immunotolerance and establishment of the FOXP3$^+$ regulatory T cell population during the preimplantation period

Sarah Yull[1,2], Shiva Shafiei[2,3], Craig B. Park[1], Parinaz Kazemi[2,3], Emily B. Tiemann[4], Marie-Hélène Godin Pagé[1,2] and Daniel Dufort[1,2,3,4]*

[1]Division of Experimental Medicine, McGill University, Montreal, QC, Canada, [2]Child Health and Human Development Program, Research Institute of the McGill University Health Centre, Montreal, QC, Canada, [3]Department of Obstetrics and Gynecology, McGill University, Montreal, QC, Canada, [4]Department of Biology, McGill University, Montreal, QC, Canada

Pregnancy success is dependent on the establishment of maternal tolerance during the preimplantation period. The immunosuppressive function of regulatory T cells is critical to limit inflammation arising from implantation of the semi-allogeneic blastocyst. Insufficient maternal immune adaptations to pregnancy have been frequently associated with cases of female infertility and recurrent implantation failure. The role of Nodal, a secreted morphogen of the TGFβ superfamily, was recently implicated during murine pregnancy as its conditional deletion (Nodal$^{\Delta/\Delta}$) in the female reproductive tract resulted in severe subfertility. Here, it was determined that despite normal preimplantation processes and healthy, viable embryos, Nodal$^{\Delta/\Delta}$ females had a 50% implantation failure rate compared to Nodal$^{loxP/loxP}$ controls. Prior to implantation, the expression of inflammatory cytokines MCP-1, G-CSF, IFN-γ and IL-10 was dysregulated in the Nodal$^{\Delta/\Delta}$ uterus. Further analysis of the preimplantation leukocyte populations in Nodal$^{\Delta/\Delta}$ uteri showed an overabundance of infiltrating, pro-inflammatory CD11b^{high} Ly6C$^+$ macrophages coupled with the absence of CD4$^+$ FOXP3$^+$ regulatory T cells. Therefore, it is proposed that uterine Nodal expression during the preimplantation period has a novel role in the establishment of maternal immunotolerance, and its dysregulation should be considered as a potential contributor to cases of female infertility and recurrent implantation failure.

KEYWORDS

Nodal, pregnancy, female infertility, recurrent implantation failure, preimplantation period, inflammation, maternal tolerance, regulatory T cells

Introduction

Female infertility is defined as the inability to establish or maintain pregnancy and affects approximately 15% of women of reproductive age. As the age women attempt to conceive their first child steadily increases, consequences of advanced maternal age including a higher incidence of infertility and a greater reliance on assisted reproductive technologies (ART) have become unavoidable (1). Infertility can arise from defects at any of the critical events during early reproduction such as irregular or failed ovulation, tubal obstruction, reproductive tract dysfunction or pathological conditions like endometriosis (2, 3). However, unexplained infertility still accounts for a substantial portion of cases (2). Although several forms of female infertility can be overcome with *in vitro* fertilization, recurrent implantation failure is not easily circumvented with ART (4). Importantly, these underlying reproductive conditions as well as the use of ART pose a significant risk for later pregnancy complications like preeclampsia or preterm labor (5–7). Therefore, elucidating mechanisms that contribute to reproductive pathologies and infertility is fundamental for the improvement of maternal and fetal health outcomes.

Pregnancy is established once the embryo implants into the uterine wall after oocyte fertilization and transport through the fallopian tube (oviduct). The uterine lumen and endometrium are conditioned by ovarian steroid hormones into a receptive and competent state required for embryo implantation. Precisely orchestrated and reciprocal signaling between the receptive uterus and the free-floating blastocyst mediates the apposition, attachment and invasion of the embryo into the uterine endometrium. Numerous factors such as cytokines, growth factors and morphogens have been implicated in the molecular crosstalk of implantation but the precise role of many of these components remains undefined (8–12).

An integral concept of pregnancy is the dynamics of the maternal immune system in response to the semi-allogeneic fetus. Implantation and the early events of placentation are considered pro-inflammatory, as the breakdown of the uterine epithelium, invasion of the blastocyst and vasculature remodeling is mediated by the infiltration and activation of leukocytes to assist in endometrial repair. This local inflammation in the uterus is controlled by regulatory T cells (Tregs) which have anti-inflammatory and immunosuppressive functions to support maternal tolerance while preventing the rejection of the fetus. Balance between these two states is necessary for successful implantation and pregnancy maintenance, therefore any dysregulation or challenge from external inflammation could risk the viability of pregnancy (13–17). Indeed, reproductive pathologies that present across all stages of gestation have been extensively correlated with inadequate maternal immune adaptations to pregnancy. For cases of unexplained infertility and specifically recurrent implantation failure Tregs are a commonly implicated population (18, 19).

The expression, regulation and function of Nodal, a morphogen of the TGFβ superfamily, was previously described during murine pregnancy. Nodal is expressed throughout the uterine glandular epithelium during the preimplantation period following mating. At the time of embryo apposition and attachment, Nodal is expressed exclusively in the areas between pre-emptive implantation sites and is embryo-dependent, implying a critical function for Nodal during the crosstalk of implantation (20). The contribution of TGFβ and other superfamily members to the molecular and immunomodulatory events of pregnancy has been well characterized (19, 21, 22), but the specific function of uterine Nodal in facilitating successful reproduction remains unknown. Previously, the generation of a maternal reproductive tract-specific Nodal knockout mouse strain ($Nodal^{\Delta/\Delta}$) demonstrated multiple reproductive phenotypes including a reduced pregnancy rate at term, smaller litter size and pups with intrauterine growth restriction (23). Interestingly, at later stages of pregnancy heterozygous $Nodal^{\Delta/+}$ uteri had premature elevation of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, and increased infiltration of decidual macrophages. This premature pro-inflammatory response during the expected stage of sustained anti-inflammatory tolerance caused a greater susceptibility for LPS-induced preterm birth, and it was proposed that uterine Nodal expression supported an anti-inflammatory state during the later stages of pregnancy (24).

Despite significant subfertility in $Nodal^{\Delta/\Delta}$ females the role of Nodal during early reproduction has not been considered. Therefore, the focus of this study is implantation and the establishment of pregnancy using the $Nodal^{\Delta/\Delta}$ model. Here, a novel role for uterine Nodal expression in supporting maternal immunotolerance during the preimplantation period is proposed. $Nodal^{\Delta/\Delta}$ females experience implantation failure which is largely attributed to the lack of a $CD4^+$ $FOXP3^+$ Treg population in the uterus prior to implantation.

Results

Nodal deficient females are subfertile and have implantation failure

The generation of a reproductive-tract specific deletion of Nodal using the *Pgr*-Cre and $Nodal^{loxP/loxP}$ strains was previously described (23). Nodal deficiency in both $Nodal^{\Delta/+}$ and $Nodal^{\Delta/\Delta}$ female mice resulted in significant subfertility, which was more drastic in $Nodal^{\Delta/\Delta}$ females (23, 24). In order to understand the pathophysiology of this reduced fertility, the pregnancy status of $Nodal^{loxP/loxP}$ controls, $Nodal^{\Delta/+}$ heterozygotes and $Nodal^{\Delta/\Delta}$ knockout females was determined across multiple stages of gestation. Mice were mated overnight with wild-type CD1 males and the presence of a copulatory plug the following morning indicated day 0.5 post coitum (d0.5). The pregnancy rate was evaluated by independent dissection experiments on d3.5 (confirmed by the presence of embryos), d5.5 and d10.5, or the birth of a litter at term on d19.5 (Figure 1A). On d3.5, flushed uteri of all females contained embryos with a normal morphology. Following implantation, $Nodal^{loxP/loxP}$ and $Nodal^{\Delta/+}$ mice showed a similar pregnancy rate ranging between 80-90% at each gestational stage assessed. However, the pregnancy rate of $Nodal^{\Delta/\Delta}$ females was

considerably decreased to 50% on d5.5 (Figure 1B). Whole mount uteri from plugged and pregnant d5.5 Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/+}$ females showed implantation sites as visible swellings along each uterine horn (Figure 1C, black arrows). Surprisingly, among the 50% of Nodal$^{\Delta/\Delta}$ females that could progress past implantation there was no difference in the number of implantation sites seen on d5.5 (Nodal$^{loxP/loxP}$ 9.5 ± 0.5, Nodal$^{\Delta/+}$ 10.8 ± 0.5, Nodal$^{\Delta/\Delta}$ 10.8 ± 0.4 sites/female) (Figure 1D). Although pregnant Nodal$^{\Delta/\Delta}$ females too had numerous, visible implantation sites, the total absence of sites in 50% of plugged mice suggested "all or nothing" implantation.

A six-month fertility trial showed consistencies across all Nodal$^{\Delta/\Delta}$ pregnancies. Females in each group were paired with a wild-type CD1 male for the duration of the breeding trial, and after confirmation of the first plug the pregnancy rate at term was found to be 44% in Nodal$^{\Delta/\Delta}$ females (Figure 2A). The average number of pups in the first litter was significantly less in both Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice compared to Nodal$^{loxP/loxP}$ controls, as previously reported for this strain (23, 24) (Nodal$^{loxP/loxP}$ 9.33 ± 0.2, Nodal$^{\Delta/+}$ 5.67 ± 1.5, Nodal$^{\Delta/\Delta}$ 5.50 ± 1.3 pups/litter) (Figure 2B). The 56% of plugged Nodal$^{\Delta/\Delta}$ mice that did not deliver as expected on d19.5 instead delivered a litter 26-30 days from the first observed plug (Figure 2C). Overall, there was a reduction in the total number of litters delivered by Nodal-deficient mice across six months (Nodal$^{loxP/loxP}$ 7.0 ± 0.3, Nodal$^{\Delta/+}$ 5.40 ± 0.5, Nodal$^{\Delta/\Delta}$ 5.56 ± 0.5 litters/female) (Figure 2D). While the pregnancy rate of Nodal$^{\Delta/+}$ females was normal, there was still post-implantation fetal loss similar to Nodal$^{\Delta/\Delta}$ pregnancies as reflected by both a significantly reduced average number of pups per litter and total number of pups delivered during the six-month trial (Figures 2E, F). Although Nodal$^{loxP/loxP}$ females continued to deliver a normal-sized litter at an advanced age, the Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice showed an earlier decline in fertility (Figure 2G). By the fifth parity, the percentage of

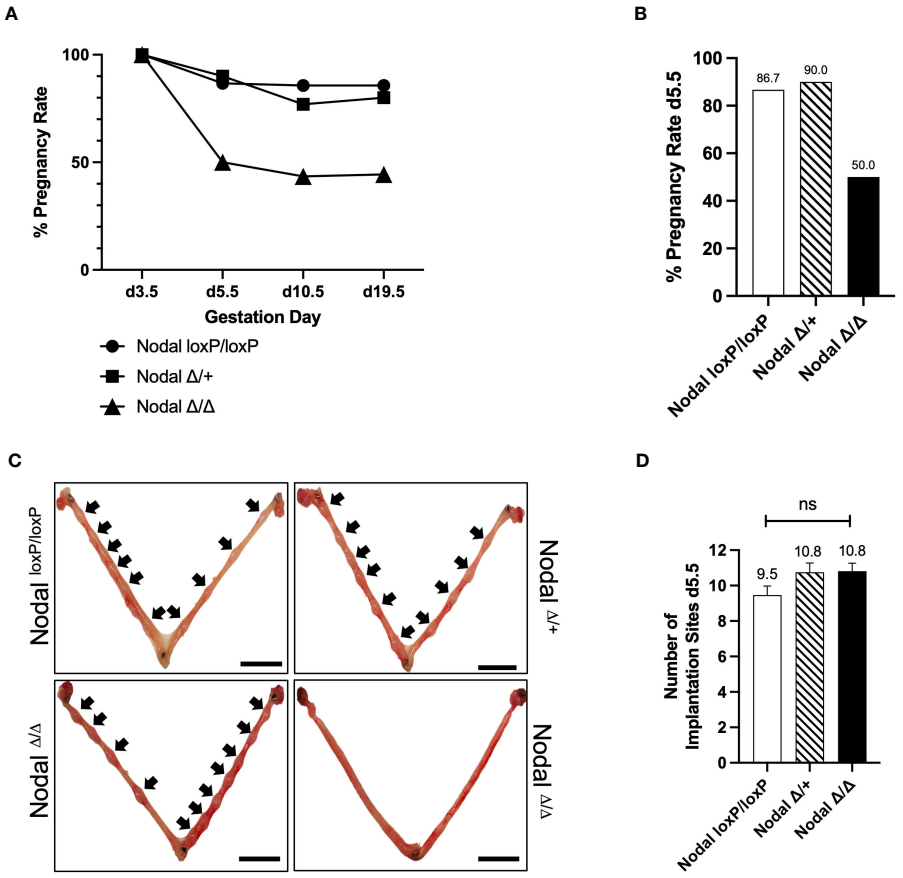


FIGURE 1
Nodal$^{\Delta/\Delta}$ females have implantation failure. **(A, B)** The pregnancy rate of Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ females during independent dissection experiments across multiple gestation days. Nodal$^{\Delta/\Delta}$ mice have a significant reduction in pregnancy rate between d3.5 and d5.5 (d3.5 Nodal$^{loxP/loxP}$ n=6, Nodal$^{\Delta/+}$ n=2, Nodal$^{\Delta/\Delta}$ n=9, confirmed by the presence of embryos after flushing; d5.5 Nodal$^{loxP/loxP}$ 87.6% (n=15), Nodal$^{\Delta/+}$ 90.0% (n=10), Nodal$^{\Delta/\Delta}$ 50.0% (n=22); d10.5 Nodal$^{loxP/loxP}$ 85.7% (n=7), Nodal$^{\Delta/+}$ 76.9% (n=13), Nodal$^{\Delta/\Delta}$ 43.5% (n=23), d19.5 Nodal$^{loxP/loxP}$ 85.7% (n=7), Nodal$^{\Delta/+}$ 80.0% (n=5), Nodal$^{\Delta/\Delta}$ 44.4% (n=9)). **(C)** Representative whole mount d5.5 uteri show implantation sites (black arrows) of pregnant Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ females. Nodal$^{\Delta/\Delta}$ mice that were plugged but not pregnant have no implantation sites on d5.5, demonstrating complete implantation failure. Scale bars indicate 1 cm. **(D)** Nodal$^{\Delta/\Delta}$ females that had visible implantation sites on d5.5 had a similar number of sites compared to Nodal$^{loxP/loxP}$ controls (Nodal$^{loxP/loxP}$ n=13, Nodal$^{\Delta/+}$ n=8, Nodal$^{\Delta/\Delta}$ n=11). Data shows mean ± SEM.

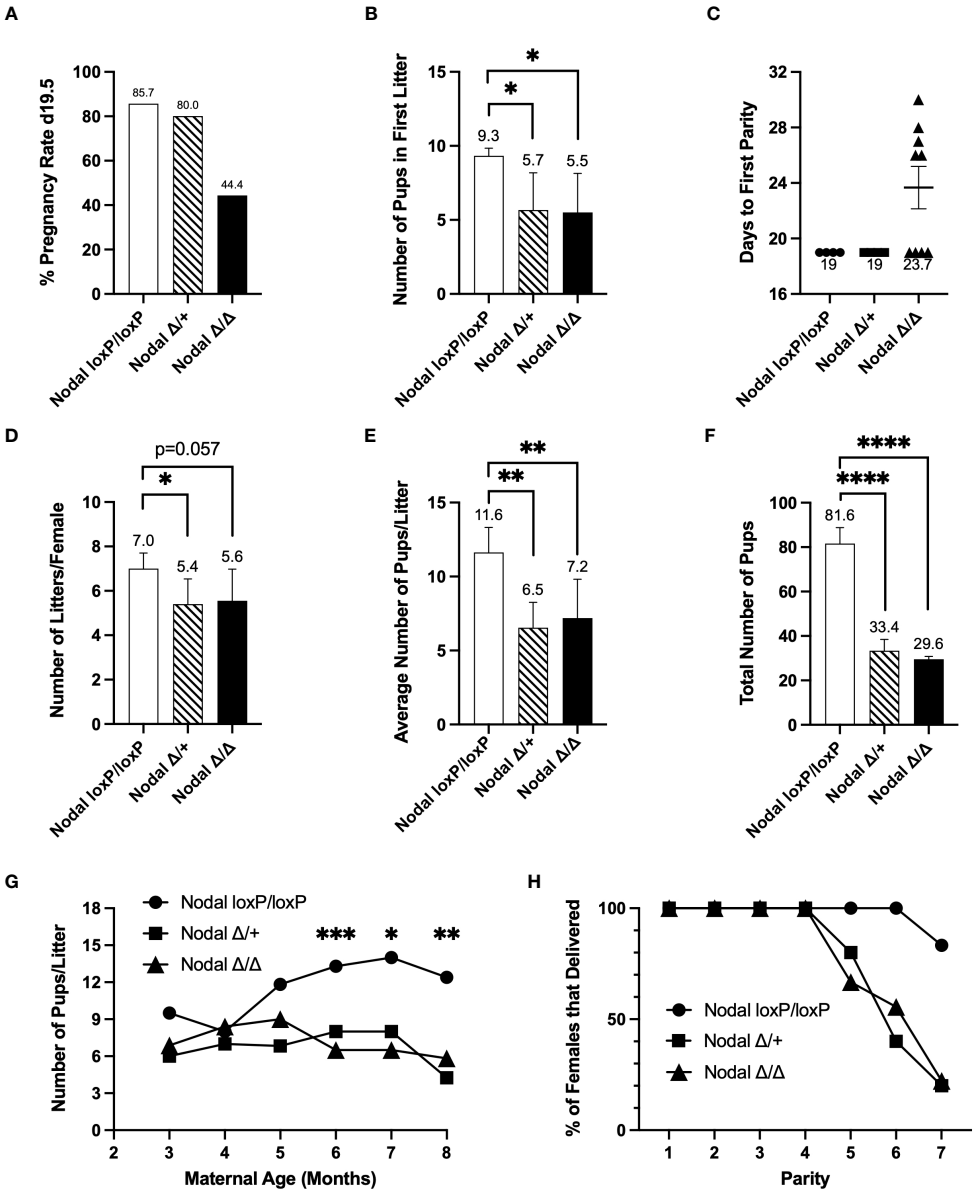


FIGURE 2
Nodal-deficient females are subfertile. Eight-week-old Nodal females were mated and housed with wildtype CD1 males to be observed during a six-month breeding period. After confirmation of the first copulatory plug (d0.5), **(A)** the initial pregnancy rate of Nodal$^{\Delta/\Delta}$ mice, defined as the percentage of females that delivered a litter on d19.5, was 44.4% (Nodal$^{loxP/loxP}$ n=7, Nodal$^{\Delta/+}$ n=5, Nodal$^{\Delta/\Delta}$ n=9). **(B)** On average, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ females gave birth to less pups in the first litter (Nodal$^{loxP/loxP}$ n=6, Nodal$^{\Delta/+}$ n=3, Nodal$^{\Delta/\Delta}$ n=4). **(C)** Of the five remaining plugged Nodal$^{\Delta/\Delta}$ mice which did not deliver a litter 19 days after the first observed plug, there was a delay of approximately 8 days before the first parity. **(D)** The average number of litters delivered by each female during the six-month trial was reduced in both Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice (Nodal$^{loxP/loxP}$ n=5, Nodal$^{\Delta/+}$ n=5, Nodal$^{\Delta/\Delta}$ n=9). **(E, F)** The average number of pups delivered per litter (Nodal$^{loxP/loxP}$ n=5, Nodal$^{\Delta/+}$ n=5, Nodal$^{\Delta/\Delta}$ n=9), and total number of pups born across all litters (Nodal$^{loxP/loxP}$ n=5, Nodal$^{\Delta/+}$ n=5, Nodal$^{\Delta/\Delta}$ n=8) was significantly less in Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ females. **(G)** As maternal age increased, the number of pups per litter in Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice was significantly less than Nodal$^{loxP/loxP}$ mice. **(H)** As the Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mothers reached a later parity, less percentage of females were able to deliver a litter. By parity seven, 20% of Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice delivered a litter. Data shows mean ± SEM. *P<0.05, **P<0.01, ****P<0.0001.

Nodal$^{\Delta/\Delta}$ females that delivered a litter decreased to 67% and was further reduced to 22% by the seventh parity (Figure 2H). Although beyond the scope of this study, it is suggested that advanced maternal age heightens the decline in fertility due to the deletion of Nodal in the reproductive tract. In conclusion, due to the inability of most young Nodal$^{\Delta/\Delta}$ mice to show signs of pregnancy after the time of implantation, it is likely that an initial subfertility can be attributed to implantation failure.

Estrous cycling and reproductive tract morphology are unaffected in Nodal$^{\Delta/\Delta}$ mice

The cyclic variation of estrogen and progesterone in the murine reproductive tract occurs during the estrous cycle and is divided into four stages: proestrus, estrus, metestrus and diestrus. Specifically, receptivity to mating and ovulation coincide during estrus and

therefore is the only stage when pregnancy can occur (25, 26). To monitor progression of the estrous cycle, Nodal$^{\Delta/\Delta}$ females were vaginally smeared daily for eighteen days. Each individual stage of the estrous cycle was morphologically distinct, exhibiting the characteristic ratios of nucleated epithelial cells (proestrus), cornified epithelial cells (estrus/metestrus) or leukocytes (diestrus) (Supplementary Figure 1A). The average cycle length was similar to Nodal$^{loxP/loxP}$ controls (4.8 ± 0.8, Nodal$^{\Delta/\Delta}$ 4.0 ± 0.2 days) (Supplementary Figure 1B) and the reported average of four to five days in wildtype mice (27). As a standard indicator of the regular hormonal control of estrous cycling, Nodal$^{\Delta/\Delta}$ females showed a comparable plugging efficiency when mated overnight with wild-type CD1 males (Nodal$^{loxP/loxP}$ 52%, Nodal$^{\Delta/\Delta}$ 45%) (Supplementary Figure 1C). Since many knockout strains with uterine gland deletions or reduced morphogenesis are infertile (28–31), uteri and ovaries from d3.5 subfertile Nodal$^{\Delta/\Delta}$ females were examined. Histologically, Nodal$^{\Delta/\Delta}$ uteri appeared normal with abundant glands and luminal epithelial cells lining the uterine cavity (Supplementary Figure 1D). Furthermore, the number of corpora lutea within each ovary was counted to show successful ovulation and luteogenesis (Nodal$^{loxP/loxP}$ 13.67 ± 2.2, Nodal$^{\Delta/\Delta}$ 14.33 ± 2.2 C.L./female) (Supplementary Figure 1E). To summarize, reproductive tract histology, estrous cycling and corpora lutea formation were normal in females deficient in uterine Nodal expression.

Embryos derived from Nodal$^{\Delta/\Delta}$ females are abundant and viable

Implantation success is dependent on the precise synchronization between the competent blastocyst and receptive endometrium (8, 10). Embryonic abnormalities such as aneuploidy or impaired hatching from the zona pellucida are a major factor in cases of recurrent implantation failure in humans (32). Therefore, to assess oocyte quality and fertilization efficiency prior to implantation in the Nodal-deficient uterus, oocytes were isolated on d0.5. Following fertilization there was a similar number of oocytes present in the oviduct (Nodal$^{loxP/loxP}$ 6.6 ± 1.8, Nodal$^{\Delta/\Delta}$ 5.3 ± 0.8 ova/female) (Supplementary Figure 1F). Additionally, there was a high rate of fertilization indicated by the presence of two pronuclei or a second polar body (Nodal$^{loxP/loxP}$ 96.9%, Nodal$^{\Delta/\Delta}$ 92.5%) (Supplementary Figure 1G). When the oviduct and uterus were flushed on d3.5 there was no difference in the number of embryos isolated between groups (Nodal$^{loxP/loxP}$ 5.5 ± 0.7, Nodal$^{\Delta/\Delta}$ 4.8 ± 0.9 embryos/female) (Supplementary Figure 1H).

Embryo viability independent of the Nodal-deficient uterine environment was evaluated by transferring Nodal$^{\Delta/\Delta}$ derived zygotes into the oviducts of d0.5 pseudopregnant CD1 wild-type recipients. Of 39 zygotes introduced, 24 live and healthy term pups were born (data not shown), correlating to the expected efficiency of transfer experiments (33). Conversely, the pregnancy rate was assessed following the transfer of seven wild-type blastocysts into one uterine horn of d2.5 pseudopregnant Nodal$^{\Delta/\Delta}$ females (Supplementary Figure 1I). It was found that uteri deficient in Nodal signaling had a reduced pregnancy rate on d7.5 or complete implantation failure. The pregnancy rate of Nodal$^{\Delta/\Delta}$ females after transfer with wild-type embryos was 33% in comparison to 66% of Nodal$^{loxP/loxP}$ controls (Supplementary Figure 1J). Therefore, since Nodal$^{\Delta/\Delta}$ derived zygotes were inherently viable and developed normally in a wild-type uterine environment, but wild-type embryos failed to implant into the Nodal$^{\Delta/\Delta}$ uterus, it was conclusive that the Nodal-deficient uterus bore responsibility for implantation failure.

Differential expression of cytokines and receptivity factors in Nodal$^{\Delta/\Delta}$ uteri

The window of implantation in mice occurs between d3.5 and d4.5 as it coincides with strictly regulated changes in uterine signaling. A state of maternal endometrial receptivity is initially achieved by the transition of an estrogen-dominant proliferative state into a progesterone-responsive state. Subsequent expression of cytokines, growth factors and other signaling molecules is critical for the success of implantation (11, 12, 31). Here, expression of receptivity factors in the uterus was assessed on d3.5 by qPCR. Genes expressed downstream of ovarian hormones including *Ihh*, *Lif* and *Muc1* were similar between Nodal$^{\Delta/\Delta}$ and Nodal$^{loxP/loxP}$ females. *Nr2f2* (COUP-TFII) and *Hoxa10* were significantly reduced and *Msx1* was elevated in Nodal$^{\Delta/\Delta}$ uteri (Figure 3A). Conditional ablation of these genes in the uterus has been previously shown to cause implantation failure or infertility in mice (28, 34–38), which confirmed the necessity for synchronous, timed gene expression during the window of implantation.

Notably, in addition to regulating the changes in gene expression preceding implantation ovarian hormones regulate the infiltration and activation of leukocytes in the uterus, which contributes substantially to the state of receptivity. In turn, the expression of many pro-inflammatory cytokines from both endometrial stromal cells and immune cells are increased prior to implantation (39, 40). On d3.5 the expression of *Il-1β*, *Il-6* and *Tnf-α* in the mouse uterus was too low to be detected by qPCR, however multiplex ELISA demonstrated no difference in protein abundance between controls and conditional knockouts (data not shown). The level of other inflammatory cytokines IFN-γ, IL-10 and MCP-1 (CCL2) were significantly decreased in Nodal$^{\Delta/\Delta}$ uteri. Interestingly, G-CSF was increased in Nodal$^{\Delta/\Delta}$ mice despite it being considered a pro-implantation factor (Figure 3B) (41). Nodal signaling appeared to have dual roles influencing both uterine gene expression and leukocyte-derived factors during the preimplantation period, the latter was of novel interest and encouraged further investigation.

Localization and abundance of infiltrating leukocytes in the Nodal-deficient preimplantation uterus is similar to Nodal$^{loxP/loxP}$ controls

The leukocyte population within the uterus is highly dynamic throughout pregnancy as it facilitates close interactions between the maternal endometrial and semi-allogeneic fetal cells. Precise balance of this immunological landscape (cell type, abundance

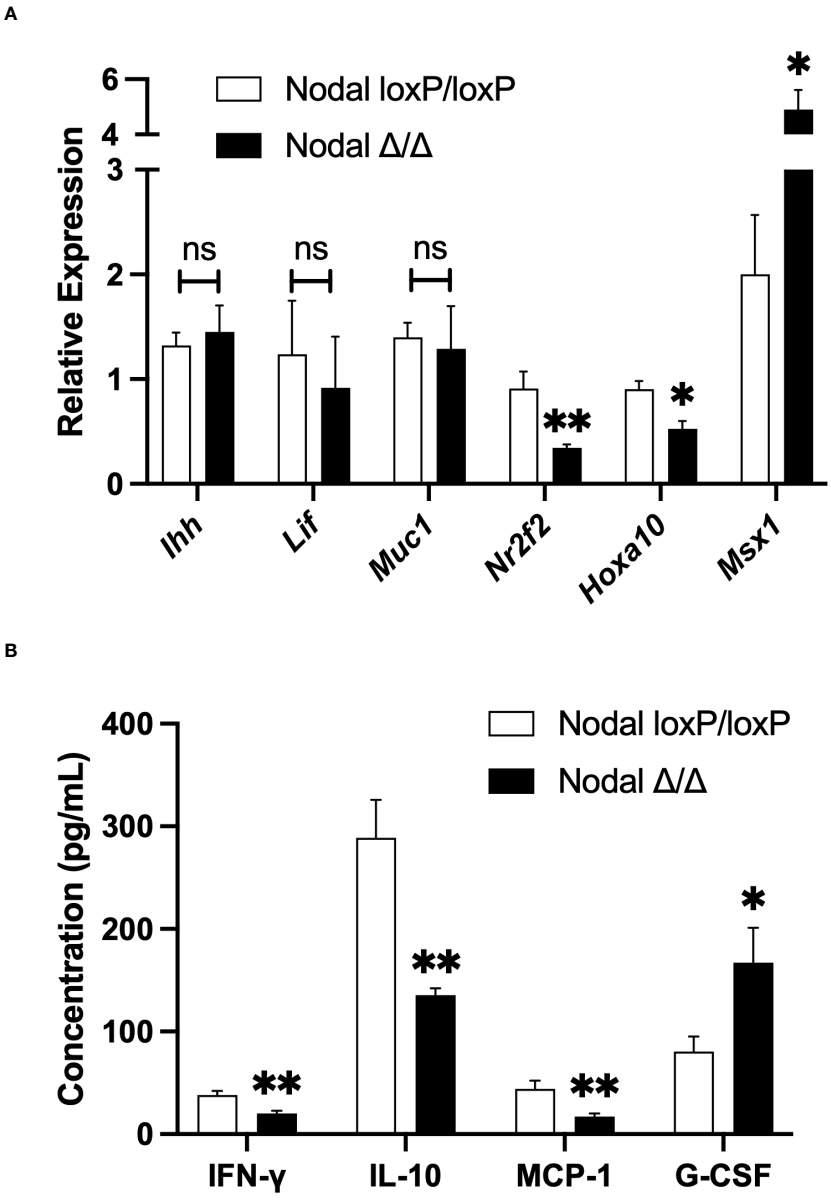


FIGURE 3
Differential expression of cytokines and receptivity factors in Nodal$^{\Delta/\Delta}$ uteri. **(A)** Relative expression of genes in the d3.5 uterus by quantitative-PCR demonstrated no difference in *Ihh, Lif* or *Muc1* expression in Nodal$^{\Delta/\Delta}$ uteri (n=7) compared to Nodal$^{loxP/loxP}$ controls (n=5). However, the expression of *Nr2f2* and *Hoxa10* was significantly reduced, and *Msx1* increased in Nodal$^{\Delta/\Delta}$ females. **(B)** Due to low mRNA expression, the protein abundance of inflammatory cytokines in the d3.5 uterus was determined by multiplex ELISA. IFN-γ, IL-10 and MCP-1 were significantly decreased in Nodal$^{\Delta/\Delta}$ mice while G-CSF was increased (Nodal$^{loxP/loxP}$ n=16, Nodal$^{\Delta/\Delta}$ n=14). Data shows mean ± SEM. *P<0.05, **P<0.01.

and the magnitude of factors produced) is a critical determinant for initiating a healthy pregnancy. Specifically, during the preimplantation period the female reproductive tract is exposed to factors within the seminal fluid which trigger the uterine immune response to prepare for implantation (15, 42, 43). As an immunomodulatory role for maternal Nodal during late pregnancy was previously proposed (24), it was hypothesized that Nodal is also involved in the establishment of the uterine immune landscape during the preimplantation and implantation period.

Visualization of immune cells within the d3.5 uterus by immunofluorescence staining revealed CD45$^+$ leukocyte populations within the endometrium, both layers of myometrium and with more visual frequency at the mesometrial pole and endometrial-myometrial junction in all groups (Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$) (Figure 4A). Utilizing flow cytometry as a precise quantification method and gating strategies previously described for similar tissue types (44), it was confirmed there was no difference in the number (data not shown) or overall percentage of live, single CD45$^+$ cells isolated from d3.5 uteri (Nodal$^{loxP/loxP}$ 5.4%, Nodal$^{\Delta/+}$ 5.2%, Nodal$^{\Delta/\Delta}$ 5.9%) (Figure 4B).

Uterine natural killer (uNK) cells are a prominent immune population during spiral artery remodeling and placentation but are uncommon in the preimplantation mouse uterus, beginning to accumulate during decidualization and peaking on d10.5 at the

maternal-fetal interface (45). To definitively exclude this population in the context of murine implantation failure Periodic-acid Schiff (PAS) staining was performed. Glycoprotein-rich PAS$^+$ uNKs were absent from all d3.5 uterine sections as expected (Figure 4C) but abundant within the d10.5 implantation site in addition to glycogen-containing PAS$^+$ trophoblast cells (46). Since the total number of CD45$^+$ cells within the preimplantation uterus was unaffected in Nodal$^{\Delta/\Delta}$ mice, a more meaningful determinant of maternal immune activation prior to implantation would be to consider the composition of the myeloid and lymphocyte subpopulations.

Significant increase in the proportion of neutrophils and macrophages in d3.5 Nodal$^{\Delta/\Delta}$ uteri

In response to seminal TGFβ, neutrophils infiltrate the uterus to clear excess sperm, fluid and restore microbial balance (47). Macrophages and dendritic cells are also recruited and present antigens to naïve T cells in the draining lymph node, eventually establishing a residential uterine regulatory T cell (Treg) population and maternal tolerance (43, 48, 49). Alternatively, pro-inflammatory macrophages mediate the controlled breakdown of the uterine epithelium and tissue remodeling during blastocyst attachment (50). Depletion of CD11b$^+$ macrophages during early pregnancy was reported to cause complete implantation failure in mice (51), while an excess of inflammatory macrophages has been implicated in cases of recurrent implantation failure and spontaneous abortion in humans (52–55). Therefore, a balanced macrophage reaction is essential for initiating the functional immune response at implantation.

To determine the composition of the myeloid population residing in the preimplantation uterus, immunofluorescence staining was performed using CD64 to identify monocytes and macrophages (Figure 5A). CD64$^+$ cells were observed in all layers of the d3.5 uterus (endometrium, myometrium and perimetrium) in all groups. Although the localization of the CD64$^+$ cells within the uterus was similar between groups, the frequency of these cells seemed much higher in the d3.5 Nodal$^{\Delta/\Delta}$ uteri specifically towards

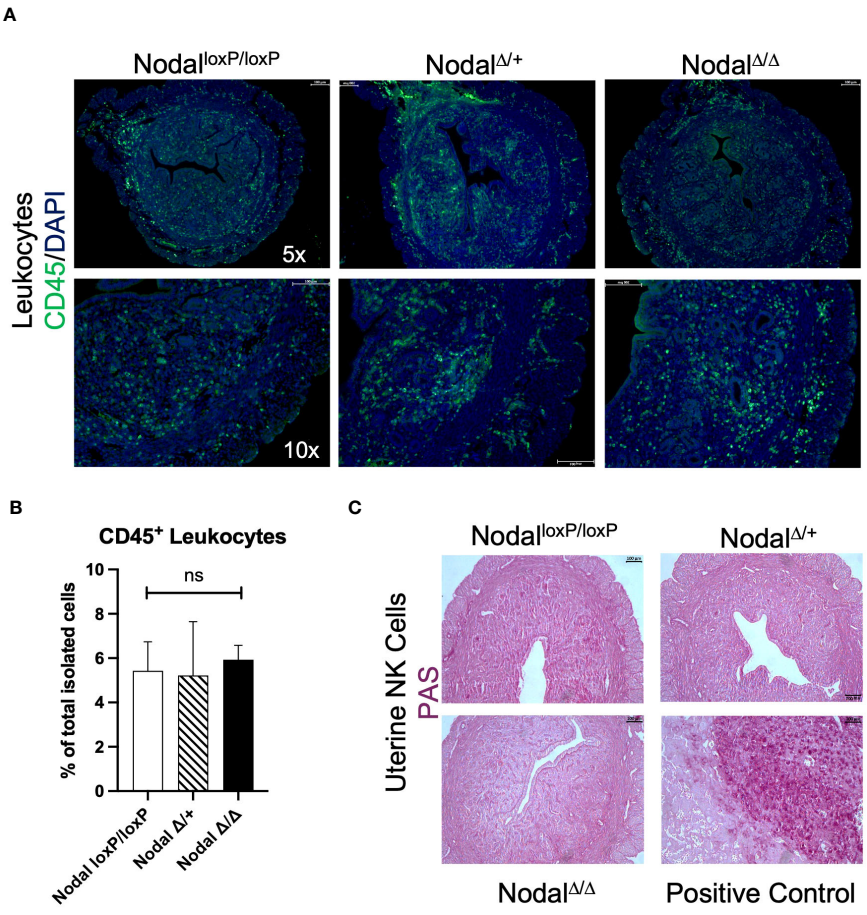


FIGURE 4
Distribution and quantity of leukocytes within the preimplantation uterus is consistent across groups. **(A)** Immunofluorescence staining revealed equal distribution of CD45$^+$ leukocytes (green) within the d3.5 uterus. **(B)** Quantification of CD45$^+$ cells by flow cytometry showed no difference in the percentage of leukocytes isolated (Nodal$^{loxP/loxP}$ n=7, Nodal$^{\Delta/+}$ n=4, Nodal$^{\Delta/\Delta}$ n=10). **(C)** The presence of PAS$^+$ uNKs in the uterus is not detected prior to implantation (d3.5), as the expansion of this population begins during decidualization. Positive control shows the maternal decidua on d10.5 where uNKs and glycogen trophoblast cells are PAS$^+$. Data shows mean ± SEM, scale bars indicate 100 μm.

the uterine periphery in comparison to Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/+}$ mice.

Isolated cell suspensions from d3.5 flushed Nodal uteri were stained using a panel of antibodies designed for classifying general leukocyte populations. Using CD11b as a marker for myeloid cell migration and adhesion (44, 56), three populations were gated as CD11b^{-} (non-myeloid), CD11b$^{low-mid}$ (resident) or CD11b^{high} (infiltrating) (Figure 5B) using flow cytometry. The non-myeloid CD11b^{-} population (which includes the CD3^{+} and CD19^{+} lymphocytes) was significantly less in Nodal$^{\Delta/\Delta}$ preimplantation uteri (4.8%, Nodal$^{loxP/loxP}$ 11.4%, Nodal$^{\Delta/+}$ 10.8% of leukocytes) (Figure 5C). Intermediary CD11b$^{low-mid}$ residential myeloid cells were found to be more numerous only in Nodal$^{\Delta/+}$ females when compared to knockout mice (20.1%, Nodal$^{loxP/loxP}$ 16.4%, Nodal$^{\Delta/\Delta}$ 7.6% of leukocytes) (Figure 5C). The CD11b^{high} infiltrating cells were substantially increased from 66.4% in Nodal$^{loxP/loxP}$ mice to

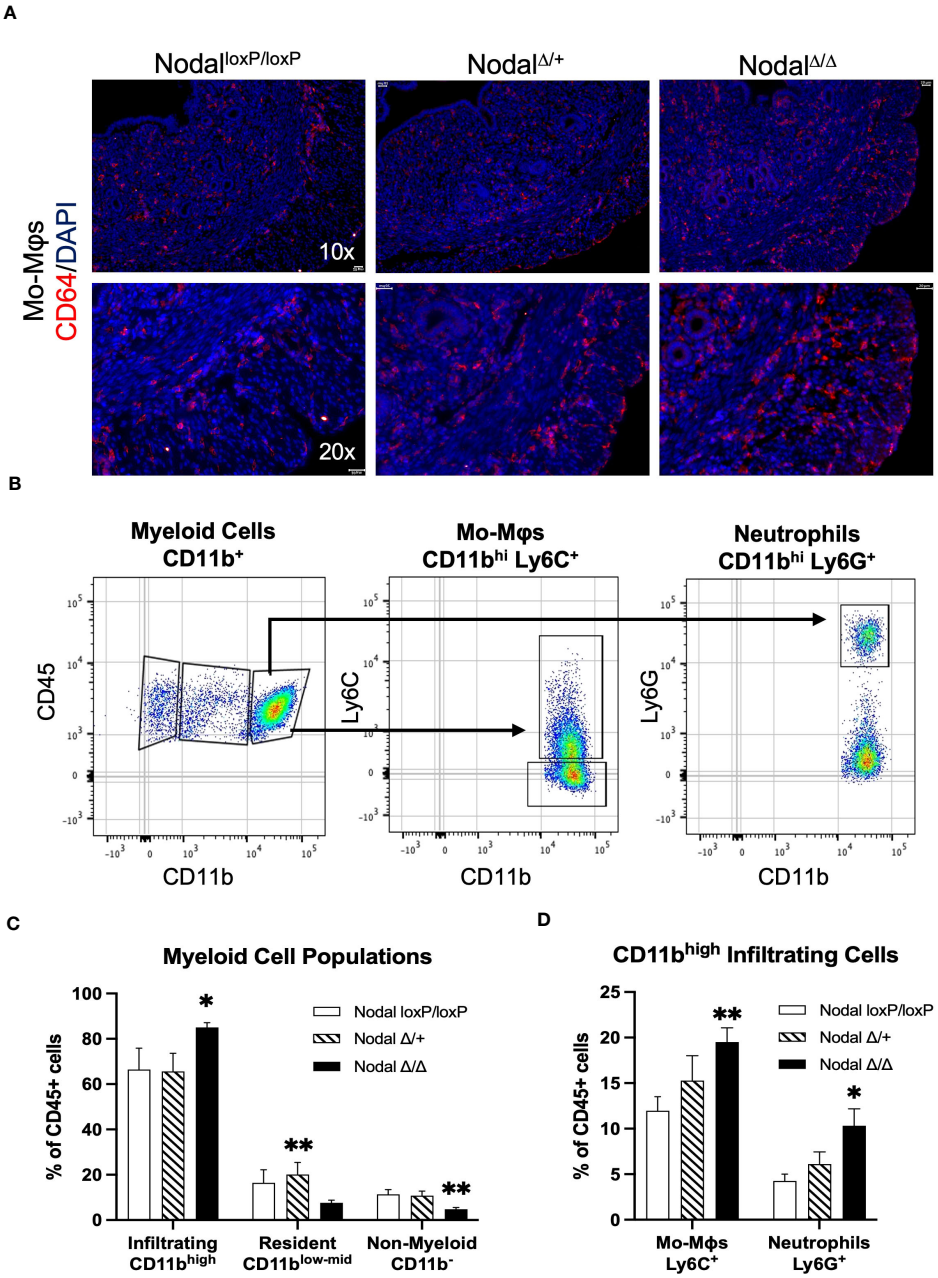


FIGURE 5
Increased abundance of CD11b^{high} myeloid cells in the Nodal$^{\Delta/\Delta}$ preimplantation uterus. **(A)** Immunofluorescence staining shows similar localization but higher visual frequency of CD64^{+} immune cells (monocytes and macrophages) within the d3.5 uteri of Nodal$^{\Delta/\Delta}$ females compared to Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/+}$ females. Scale bars indicate 100 µm. **(B)** Myeloid-derived cells in the d3.5 uterus were gated from the CD45^{+} live cell population based on expression of CD11b, and then with further lineage markers of Ly6C (monocyte-derived macrophages, Mo-Mφ) and Ly6G (neutrophils). **(C)** Quantification of CD11b^{+} populations in the d3.5 preimplantation uterus (Nodal$^{loxP/loxP}$ n=7, Nodal$^{\Delta/+}$ n=4, Nodal$^{\Delta/\Delta}$ n=9) showed a significant increase in the number of infiltrating myeloid cells in Nodal$^{\Delta/\Delta}$ mice, and a decrease in the number of non-myeloid CD11b^{-} leukocytes. **(D)** Both Ly6C^{+} macrophages and Ly6G^{+} neutrophils are increased in Nodal$^{\Delta/\Delta}$ uteri. Data shows mean ± SEM. *P<0.05, **P<0.01.

85.0% in Nodal$^{\Delta/\Delta}$ mice and was determined to be the major leukocyte population present prior to implantation (Figure 5C). Further analysis of the infiltrating CD11b^{high} leukocytes using Ly6C, a marker for monocyte-derived pro-inflammatory macrophages (Mo-Mϕ) (57), revealed a considerable increase in macrophage abundance with almost double the proportion observed in Nodal$^{\Delta/\Delta}$ uteri (Nodal$^{loxP/loxP}$ 12.0%, Nodal$^{\Delta/+}$ 15.3%, Nodal$^{\Delta/\Delta}$ 19.5% of leukocytes). Similarly, the amount of Ly6G$^+$ neutrophils was doubled in Nodal$^{\Delta/\Delta}$ mice compared to Nodal$^{loxP/loxP}$ controls (Nodal$^{loxP/loxP}$ 4.3%, Nodal$^{\Delta/+}$ 6.1%, Nodal$^{\Delta/\Delta}$ 10.3% of leukocytes) (Figure 5D). Evidentially, the myeloid response in Nodal$^{\Delta/\Delta}$ uteri was overwhelmingly increased in magnitude compared to Nodal$^{loxP/loxP}$ mice.

Nodal$^{\Delta/\Delta}$ females lack FOXP3$^+$ regulatory T cells during the preimplantation period

Counteractive to the macrophage response is the activity of Tregs which function by promoting the immunosuppressive maternal uterine environment required for implantation of the semi-allogeneic embryo (43, 49). This is mediated through the production of cytokines that polarize anti-inflammatory "M2" macrophages, the regulation of T effector cell types and the support of maternal vascularization (19). Current research has emphasized the role of Tregs during implantation as insufficiencies in both overall number and function, with a subsequent increase in T effector types, has been observed in various reproductive pathologies (18, 19, 58, 59).

It was indicated that the CD11b$^-$ population was affected in Nodal$^{\Delta/\Delta}$ females (Figure 5C), so CD3 and CD19 were first used to characterize the uterine lymphocyte populations. The CD3$^+$ T cell population was significantly reduced in the d3.5 Nodal$^{\Delta/\Delta}$ uteri, amounting to 2.3% of total leukocytes and about half the number present in controls (Nodal$^{loxP/loxP}$ 4.0%, Nodal$^{\Delta/+}$ 3.8% of leukocytes). The portion of CD19$^+$ B cells was not significantly different across groups but trended towards a decrease in the Nodal$^{\Delta/\Delta}$ females (Figure 6A). CD3$^+$ T cells could be further classified into subpopulations of activated T effectors based on the expression of CD8 (cytotoxic T) or CD4 (T helper, Th). There was no difference in the total percentage of CD8$^+$, CD4$^+$ or the subset of CD4$^+$ IFN-γ^+ (Th1) cells across groups (data not shown). CD4$^+$ IL-17$^+$ (Th17) cells had no statistical difference but showed a strong trending decrease in Nodal$^{\Delta/\Delta}$ uteri (Nodal$^{loxP/loxP}$ 13.4%, Nodal$^{\Delta/+}$ 11.6%, Nodal$^{\Delta/\Delta}$ 6.7% of CD4$^+$ leukocytes) (Figure 6B). Strikingly, the CD4$^+$ FOXP3$^+$ Treg population, which is necessary for maternal tolerance during implantation, was completely non-existent in the d3.5 uterus of Nodal$^{\Delta/\Delta}$ females (Figures 6C, D). Although rare and in low abundance, Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/+}$ mice had obvious CD4$^+$ FOXP3$^+$ populations (Nodal$^{loxP/loxP}$ 8.2%, Nodal$^{\Delta/+}$ 4.8% of CD4$^+$ leukocytes) (Figure 6D). The absence of FOXP3$^+$ Tregs in uteri of Nodal$^{\Delta/\Delta}$ females during the preimplantation period provided justification for the observed implantation failure. Therefore, it is proposed that uterine Nodal signaling during the preimplantation period is important for the development of the FOXP3$^+$ Treg population and the establishment of maternal immunotolerance to pregnancy.

Discussion

Within the last decade very few studies have investigated the function of Nodal during reproduction beyond its usual role in embryonic development. In comparison to other TGFβ superfamily members uterine Nodal expression is not well characterized, however the initiation of Nodal expression shortly after mating in the adult mouse uterus provided a strong indication for its role during pregnancy (20). The generation of the conditional knockout Nodal$^{\Delta/\Delta}$ strain proved that Nodal was necessary for successful reproduction since all Nodal-deficient females showed severe subfertility, seen as a reduced pregnancy rate at term and smaller litter size (23). Although only later stages of gestation were considered, it was revealed that uterine Nodal expression promoted an anti-inflammatory state before parturition (24). Acknowledging the dynamics of the maternal immune response to pregnancy and how dysregulation at the earliest stages could result in later pathological pregnancy complications, it was hypothesized that uterine Nodal contributed to the immunotolerant environment established during the preimplantation period.

Despite normal preimplantation processes and viable embryos (Supplementary Figure 1), Nodal$^{\Delta/\Delta}$ females were shown to have a 50% implantation failure rate (Figures 1A, B). Similarly, previous *in vivo* gene transfer experiments of exogenous Lefty (the inhibitor of Nodal signaling) into the preimplantation uterus of wild-type CD1 females significantly decreased implantation efficiency. Both the use of a Lefty retroviral expression vector system or liposome-mediated introduction of a Lefty expression vector showed either a reduced number of embryos on d9.5 or complete implantation failure (60). Nodal$^{\Delta/\Delta}$ females demonstrated a very similar phenotype to both Lefty overexpression experiments, therefore either directly knocking out Nodal from the uterus or overexpressing the inhibitor Lefty throughout the preimplantation period can lead to implantation failure. These independently generated results provided an intriguing correlation given the intricacies of the Nodal signaling pathway and crosstalk with other TGFβ superfamily members. Many mouse models with conditional genetic deletions of TGFβ family ligands, receptors or signaling components share reproductive phenotypes throughout gestation, but the precise signaling pathways of each factor remains difficult to interpret due to redundancies and potential compensatory functions within the superfamily (21, 61, 62). This could explain implantation success for a portion of the Nodal$^{\Delta/\Delta}$ females. Outside of the TGFβ superfamily, TGFβ signaling has been shown to regulate the Wnt and hedgehog pathways which are fundamental in preparing the uterus for embryo implantation (8, 62). Wnt and hedgehog pathways are upstream of the endometrial receptivity factors *Nr2f2* (COUP-TFII), *Hoxa10* and *Msx1* that were shown to be dysregulated during the window of implantation in Nodal$^{\Delta/\Delta}$ mice (Figure 3A). Although beyond the scope of this study, it is not inconceivable that Nodal too is involved in the complexities of

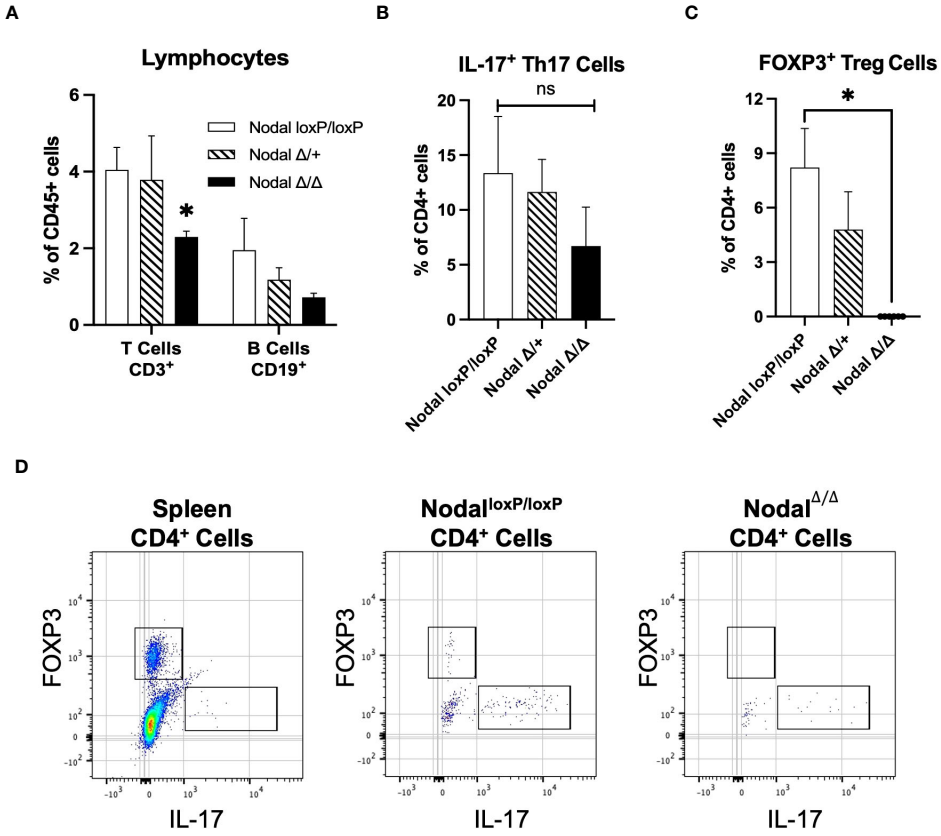


FIGURE 6
Significant reduction in the frequency of T cells accompanied by a lack of Treg cells in the preimplantation uterus of Nodal$^{\Delta/\Delta}$ females. **(A)** Quantification of CD3$^+$ T cell populations in the preimplantation uterus (Nodal$^{loxP/loxP}$ n=7, Nodal$^{\Delta/+}$ n=4, Nodal$^{\Delta/\Delta}$ n=10) showed a significant decrease in the number of T cells in Nodal$^{\Delta/\Delta}$ mice, and a trending decrease in the number of CD19$^+$ B cells. **(B)** Further characterization of the CD3$^+$ CD4$^+$ population showed a non-significant but trending reduction in the percentage of IL-17$^+$ Th17 cells in Nodal$^{\Delta/\Delta}$ uteri (Nodal$^{loxP/loxP}$ n=10, Nodal$^{\Delta/+}$ n=8, Nodal$^{\Delta/\Delta}$ n=8). **(C)** Analysis of the FOXP3$^+$ Treg population showed that although rare in Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/+}$ females, a clear population could be defined. However, there was a complete absence of FOXP3$^+$ Treg cells in the Nodal$^{\Delta/\Delta}$ d3.5 uterus. **(D)** Gating strategy to determine FOXP3$^+$ and IL-17$^+$ CD4$^+$ populations in the preimplantation uterus (concatenated) based on marker expression in the spleen. Data shows mean ± SEM. *P<0.05.

uterine receptivity through perhaps an unknown interaction with TGFβ signaling or Wnt/hedgehog pathways. In either case, uninterrupted uterine Nodal signaling is imperative for efficient embryo implantation.

The continued housing of breeding pairs allowed for the observation of Nodal$^{\Delta/\Delta}$ females natural mating behaviors. Interestingly, the plugged Nodal$^{\Delta/\Delta}$ females that experienced complete implantation failure after the first mating were eventually successful in delivering a litter, however delayed 7-11 days when compared to those that delivered on time after the first mating (Figure 2C). Nodal$^{\Delta/\Delta}$ mice were classified as having delayed pregnancy as opposed to delayed implantation as the pregnancy rate did not improve at later timepoints of dissection (Figure 1A). Essentially, if mating did not result in fertilization or implantation, the corpora lutea regressed and the next estrus cycle followed after 10-12 days (63, 64). Sustained pairing of mice permitted a second mating within this period and ultimate success of the second Nodal$^{\Delta/\Delta}$ pregnancy. Repeated exposure to seminal fluid over the course of multiple mating cycles with the same partner in both mouse and human studies was shown to increase the capacity of the maternal immune response to tolerate future pregnancies, in addition to reducing the risk of developing preeclampsia (43, 65, 66). Furthermore, *in vitro* fertilization treatments co-treated with seminal fluid during the time of embryo transfer significantly increased the rate of clinical pregnancy (67), confirming the importance of paternal (fetal) antigen conditioning within the uterus for maternal tolerance. Together, the occurrence of implantation failure, delayed pregnancy or further reproductive challenges during mid-gestation for those Nodal$^{\Delta/\Delta}$ females with implantation success overwhelmingly supported the argument for a dysregulated maternal immune response in the Nodal-deficient preimplantation uterus.

Inflammatory environments during pregnancy can be classified based on the dominance of either pro-inflammatory, classical "M1" or anti-inflammatory, alternatively activated "M2" macrophages. Though this is an oversimplification of concepts and perhaps more representative of *in vitro* conditions (57), the dynamic polarization of macrophages in response to the *in vivo* uterine microenvironment at different stages of gestation is imperative for a healthy pregnancy (68, 69). MCP-1 (CCL2) is a driver of myeloid cell recruitment into the uterus, and in response to seminal factors during mating MCP-1 expression increases during the preimplantation period and window

of implantation. This is coupled with an increase in the number of M1-polarized infiltrating macrophages (70). Although MCP-1 was decreased in the d3.5 Nodal$^{\Delta/\Delta}$ uteri (Figure 3B), the proportion of CD11b^{high} myeloid cells (Figure 5C) and CD11b^{high} Ly6C^{+} infiltrating pro-inflammatory macrophages (Figures 5A, D) was almost doubled. While the M1 state is more prevalent at implantation, the magnitude of the inflammatory response still needs to be appropriate since a maternal environment that is excessively pro-inflammatory and hostile would not be favorable for implantation. Therefore, perhaps reduced expression of MCP-1 with an increase of G-CSF (Figure 3B) before implantation in Nodal$^{\Delta/\Delta}$ females was a mechanism to preserve the integrity of the uterus in response to the overwhelming infiltration of pro-inflammatory macrophages. These findings during early pregnancy in Nodal$^{\Delta/\Delta}$ mice mirror those from previous studies of later pregnancy, as increased infiltration of decidual macrophages and expression of pro-inflammatory cytokines were seen in the Nodal$^{\Delta/+}$ uterus before parturition and led to the increased susceptibility for LPS-induced preterm labor. Complementary *in vitro* experiments were performed using both bone marrow-derived macrophages and RAW264.7 cell lines, where upon pre-treatment with recombinant Nodal protein (rNodal) before LPS the level of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α was significantly reduced (24), corroborating other reports of rNodal polarizing primary mouse macrophages into an M2 state (71). Together, there is compelling evidence that uterine Nodal is an anti-inflammatory mediator of macrophage responses throughout pregnancy. Although there was no difference in the level of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α on d3.5 (data not shown), this could be due to the inclusion of both structural uterine cells and leukocytes in the samples and is a limitation of this study. More definitive relationships between specific immune populations and their secreted factors in a Nodal-deficient environment could be proven by cell sorting prior to analysis, single-cell sequencing or flow cytometry panels with additional M1/M2 intracellular cytokine markers. The dendritic cell population which shares similar but distinct functions to macrophages during early pregnancy should also be addressed in the Nodal$^{\Delta/\Delta}$ model. Unfortunately, few studies consider the mouse preimplantation innate myeloid response, as more emphasis has been placed on understanding post-implantation decidualization, placentation and parturition processes. Current research in reproductive immunology concerning implantation and infertility has instead focused on the adaptive T cell responses. As the CD11b^{-} (Figure 5C) and CD3^{+} (Figure 6A) was significantly less in d3.5 Nodal$^{\Delta/\Delta}$ uteri, there was indication that the responding lymphocyte population was also impacted by the deletion of uterine Nodal.

Mild inflammation generated by stromal and myeloid cells within the uterus recruits and induces CD4^{+} T cells into the proper effector phenotype required for implantation, including Th1, Th17 and Treg responses (72). IFN-γ is the major cytokine produced by activated Th1 cells which contributes to the dominant pro-inflammatory state at implantation. The protein level of uterine IFN-γ was significantly decreased in Nodal$^{\Delta/\Delta}$ females on d3.5 (Figure 3B) however no difference was observed in the number of CD4^{+} IFN-γ^{+} Th1 cells (data not shown). Excessive Th1 immunity is correlated with recurrent implantation failure, recurrent pregnancy loss and miscarriage (73, 74) and was expected to be prevalent in the Nodal$^{\Delta/\Delta}$ implantation failure model. Alternatively, while the myeloid source of IFN-γ during implantation has been debated (75) some studies have shown that it can be produced by macrophages and assist in Th1 polarization (76), so maybe differential levels of IFN-γ in the preimplantation Nodal$^{\Delta/\Delta}$ uterus implicates the immunoreactivity of M1 macrophages. IL-17 producing Th17 cells have been shown to be elevated in the peripheral blood of women with recurrent implantation failure and pregnancy loss (58, 77). Similar to the Th1 response, this was expected to be elevated in Nodal$^{\Delta/\Delta}$ females but was instead almost significantly decreased (Figure 6B). Conversely, Tregs function by limiting excessive inflammation while suppressing these effector T cell responses to fetal antigens and sustaining maternal tolerance (19). Reduced levels of uterine IL-10 (Figure 3B) could be due to the absence of a FOXP3^{+} Treg population in d3.5 Nodal$^{\Delta/\Delta}$ uteri (Figures 6C, D). Overall, Treg deficiencies have been causal in numerous mouse and human studies of infertility (19, 78). The interplay and plasticity of Th1, Th17 and Treg lineages from the naïve CD4^{+} state in the presence or absence of Nodal signaling encourages further investigation.

It is unclear if the lack of uterine FOXP3^{+} Tregs on d3.5 in Nodal$^{\Delta/\Delta}$ females is the direct result of failed induction of naïve CD4^{+} T cells at any point during the preimplantation period, or possibly just the loss of proliferation and maintenance of these Tregs after an initial wave of recruitment into the uterus. It should be noted that the lack of uterine Nodal was shown to effect only the FOXP3^{+} Tregs, and any impact on the FOXP3^{-} population is still undetermined. Since the number of Tregs in the preimplantation mouse uterus is very limited, complementary *in vitro* experiments would also be beneficial. It has been well established that TGFβ is an inducer of Tregs during normal immune functions and pathological conditions (79). Activin A (a TGFβ superfamily member) was previously shown to promote the conversion of CD4^{+} CD25^{-} T cells into induced FOXP3^{+} Tregs in a dose-dependent manner with TGFβ *in vitro*. Although Activin A alone was able to induce a moderate level of conversion, the overall effect was additive when using Activin A and low concentrations of TGFβ1 together (80). Interestingly, Activin A and Nodal share the same membrane receptor (ALK4) and activate intracellular SMAD2/3 signaling pathways. Since redundancy and interactions between TGFβ superfamily members was previously highlighted (21), it is now hypothesized that Nodal acts similarly to Activin A by directly promoting the induction of Tregs *in vitro*. Alternatively, indirect mechanisms of Nodal signaling supporting maternal tolerance during pregnancy could be through the polarization of macrophages as previously reported *in vitro* (24, 71). Current work is therefore focused on *in vitro* and *ex vivo* assays to determine the precise contribution of Nodal signaling to the regulation and interaction of decidual macrophage and T cell populations. Results from these ongoing assays would elevate *in vivo* data and identify a new role for Nodal in the support of maternal tolerance during the preimplantation period, and this mechanism could be relevant to the later gestational phenotypes seen in Nodal$^{\Delta/\Delta}$ mice.

Finally, from human studies involving non-pregnant women it was shown that Nodal and its inhibitor Lefty had opposing phase-dependent expression patterns throughout the menstrual cycle.

Uterine Nodal expression steadily increased throughout the early- to late-proliferative phases and into the early-secretory phase but dropped during the mid-secretory phase and menses. This was particularly interesting as the shift between Nodal or Lefty dominance occurred during the mid-secretory phase when the uterus was receptive to implantation (81, 82). Indeed, Lefty misexpression has been implicated in cases of unexplained infertility (83). More recently, the association between uterine inflammatory environments and Nodal expression in later human reproductive pathologies was proposed. In a Dutch cohort of familial, intrauterine growth restriction-complicated preeclampsia the Nodal "H165R" single nucleotide polymorphism (SNP) was present in all affected women and reduced Nodal activity by 50% (84). This same SNP was also a significant risk factor for preterm labor in a separate retrospective study, but only if there was existing placental inflammation (defined as membrane inflammation, funisitis and/or umbilical cord vasculitis). Similarly, other Nodal SNPs were a risk factor for delivering preterm if the woman had bacterial vaginosis (85). Evidentially, the connection between uterine Nodal signaling and inflammatory environments during mouse pregnancy is conserved in human pregnancies. Based on the main findings from the Nodal$^{\Delta/\Delta}$ mouse model in combination with evidence of disrupted Nodal-Lefty signaling in women with fertility complications, current work is concentrated on the potential association between Nodal SNPs and immune profiles of women with recurrent implantation failure. Broadly, the intriguing relevance of Nodal signaling on the modulation of inflammatory states could be applicable to other clinical cases beyond reproduction.

In conclusion, initial dysregulation of the maternal immune landscape during the preimplantation period of Nodal$^{\Delta/\Delta}$ females has negative impacts on the establishment and progression of pregnancy. Here, during the preimplantation period the overabundance of CD11b^{high} Ly6C^{+} pro-inflammatory macrophages combined with the absence of CD4^{+} FOXP3^{+} Tregs in Nodal-deficient mice was detrimental to embryo implantation in 50% of cases. This suggests other mechanisms are involved, and the extent of maternal Nodal signaling in the preparation for implantation remains to be defined. A poor maternal immune response during the preimplantation period is predicted to amplify over the course of gestation and complicate later processes like placentation and sustained tolerance that are extremely dependent and vulnerable to immunomodulation. Therefore, it is proposed that uterine Nodal signaling during the preimplantation period has a novel role in supporting the initiation of maternal tolerance to pregnancy, and its dysregulation should be emphasized as a potential contributor to cases of female infertility and recurrent implantation failure.

Materials and methods

Generation and maintenance of Nodal conditional heterozygous and knockout mice

Experimental protocols in this study were in accordance with regulations established by the Canadian Council on Animal Care and were reviewed by the Animal Care Committee of the McGill University Health Centre. Animals were housed according to the rodent husbandry standard operating procedure #508 of the Animal Resources Division at the Research Institute of the McGill University Health Centre. The generation of these mice has been previously described (23). Mice with loxP sites flanking exons 2 and 3 of the *Nodal* gene (Nodal$^{loxP/loxP}$) on a mixed background were kindly donated by E.J. Robertson (University of Oxford) (86). Progesterone receptor (*Pgr*)-Cre females (*Pgr*$^{Cre/+}$) on a C57BL6/129 background were generously provided by F.J. DeMayo and J.P. Lydon (Baylor College of Medicine) (87). Both strains have been previously reported to demonstrate normal fertility, and *Pgr*$^{Cre/+}$ mice are a standard model to investigate uterine-specific gene functions (36, 88). Nodal$^{loxP/loxP}$ and *Pgr*$^{Cre/+}$ strains were crossed, and the offspring were genotyped by PCR. In this study, 8–12-week-old *Nodal* floxed control (Nodal$^{loxP/loxP}$/*Pgr*$^{+/+}$ - denoted Nodal$^{loxP/loxP}$), *Nodal* conditional heterozygous (Nodal$^{loxP/+}$/*Pgr*$^{Cre/+}$ - Nodal$^{\Delta/+}$) and *Nodal* conditional knockout (Nodal$^{loxP/loxP}$/*Pgr*$^{Cre/+}$ - Nodal$^{\Delta/\Delta}$) females were used as experimental mice.

Fertility trial

To assess the pregnancy rate of Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice, eight-week-old virgin, littermate females from each group were mated and housed with a wild-type CD1 male. The presence of a copulatory plug indicated successful mating and it was considered day 0.5 of pregnancy (d0.5). Females were monitored at the expected time of delivery (d19.5) for the birth of a litter to determine initial pregnancy rate as well as the number of pups delivered. Each female was continuously housed with a paired wild-type male so normal fertility data could be recorded for exactly six months from the date of the first plug.

Embryo flushing

Oviducts and uterine horns, separated from the ovaries and cervix of Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ females on d3.5 were dissected. Oviduct and uterine horns were flushed from both ends using Hanks' Balanced Salt Solution (HBSS) into a tissue culture dish. The isolated embryos were counted and staged. Flushed uterine horns were used for DNA, RNA, and protein extraction or enzymatic digestion to generate single cell suspensions.

Vaginal smearing and estrous staging

Vaginal smears were collected daily at 10:00 am for eighteen days from five Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/\Delta}$ females. The vaginal cavity was rinsed with PBS and wet-mount slides were immediately prepared and examined under a light microscope. The estrous stage was determined by the relative ratio of cells observed: diestrus; primarily leukocytes, proestrus; only nucleated epithelial cells, estrus; predominately large cornified epithelial cells, metestrus;

moderate leukocytes with remaining cornified cells evident (89). Slides were dried overnight to promote cell adherence, washed, counter-stained with Nuclear Fast Red, dehydrated with an increasing ethanol gradient and mounted for imaging.

Embryo transfer

Nodal$^{loxP/loxP}$ and Nodal$^{\Delta/\Delta}$ females were mated with wild-type CD1 males and the oviducts from plugged females were dissected on d0.5 into M2 media. The cumulus oocyte complexes were released by gently tearing open the infundibulum. Ova were isolated by brief incubation in hyaluronidase (300 µg/mL) to digest the cumulus mass, and fertilization of individual ova was determined by the presence of two pronuclei or a second polar body. Fertilized zygotes from either Nodal$^{\Delta/\Delta}$ or Nodal$^{loxP/loxP}$ females were pooled and 19-20 zygotes were transferred into the oviducts of anesthetized, d0.5 pseudopregnant CD1 recipient females. Recipients were allowed to recover, and pregnancy was monitored until parturition when delivered pups were quantified.

The reciprocal transfer to determine implantation efficiency of Nodal$^{\Delta/\Delta}$ or Nodal$^{loxP/loxP}$ uteri was achieved by mating the respective females with vasectomized CD1 males. On day 2.5 of pseudopregnancy, blastocysts generated from naturally mated, wild-type CD1 females (d3.5) were transferred directly into one uterine horn of anesthetized Nodal$^{\Delta/\Delta}$ or Nodal$^{loxP/loxP}$ recipients. The contralateral horn was utilized as a negative control by injecting the same volume of KSOM embryo media. The experimental and control females were allowed to recover, and uteri were removed on d7.5 to determine the conceptus site number and calculate implantation efficiency.

Tissue processing, paraffin embedding, and sectioning

Paraffin embedding and tissue histology methods were employed as previously described (90). Briefly, dissected samples were collected in PBS and fixed in 10% neutral buffered formalin for a minimum of 48 hours at 4 °C. Samples were dehydrated in increasing concentrations of ethanol and cleared in xylene before embedded into paraffin wax (Tissue Tek). Blocks were slowly solidified on a cold plate for one hour before transfer to a -20 °C freezer until sectioning. Using the Leica RM2145 microtome, 7 µm transverse sections were cut and mounted onto Fisher Superfrost plus slides and dried. Slides were either used for immunofluorescence, PAS staining or Hematoxylin and Eosin staining (90). To count absolute corpora lutea number serial sections of the complete ovary were prepared and counter-stained with Nuclear Fast Red.

Immunofluorescence

Uteri from d3.5 Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ females were dissected, fixed, embedded and sectioned as described above. Immunofluorescence staining was conducted as previously described (90). Incubation with the primary antibody CD45 (BioLegend Cat. No. 103102, 1:100) or CD64 (BioLegend Cat. No. 161002, 1:100) occurred at 4 °C overnight. Following washes with 0.1% TBS-Tween 20, slides were incubated with the appropriate secondary antibody; donkey α-rat Alexa Fluor 594 (Invitrogen Cat. No. A21209, 1:300) or goat α-rat Alexa Fluor 488 (Invitrogen Cat. No. A11006, 1:300) and DAPI (1:500) for two hours at room temperature. Slides were washed and mounted.

PAS staining

Slides from d3.5 uteri were stained using Periodic-acid Schiff (PAS) by the Histopathology Platform at the Research Institute of the McGill University Health Centre following standard protocols without counterstaining.

Quantitative PCR

Isolated tissues were immediately frozen on dry ice and stored at -80 °C. Total RNA extraction was conducted using Trizol (Invitrogen Cat. No. 15596018) and the RNeasy Mini Kit (Qiagen Cat. No. 74104). QuantiTect Reverse Transcription Kit (Qiagen Cat. No. 205311) was used for cDNA synthesis. Quantitative PCR (qPCR) was performed using the Rotor-Gene SYBR Green PCR Kit (Qiagen Cat. No. 204074) following the manufacturer's protocol. Each biological replicate was performed in technical triplicate. Samples were run on the Corbett Rotor-Gene 6000 thermocycler and analyzed using the Rotor-gene 6000 software. The primers used are listed in Supplementary Table 1 and were designed using the NCBI primer-blast tool. The relative expression of the gene of interest was calculated by the ΔΔCt method, where fold changes in gene expression were normalized to an internal control (*Gapdh*) and relative to one sample (calibrator). Each qPCR run underwent melt curve analysis to confirm the presence of one peak.

Protein extraction and multiplex ELISA

Uterine horns from d3.5 mice were flushed as described before. Samples were immediately frozen on dry ice and stored at -80 °C until protein extraction. Tissue was weighed and homogenized in 1 mL lysis buffer per 50-100 mg tissue (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20, 1x AEBSF protease inhibitor (Sigma Cat. No. 30827-99-7)). After centrifugation, the total protein in the supernatant was quantified using the BCA Protein Assay Kit (Thermo Scientific Cat. No. 23227) and Tecan Infinite M200 Pro plate reader. Protein concentrations were determined by comparing absorbance values to a standard BSA curve and equalized to 2 mg/mL prior to the assay. The concentrations of G-CSF, GM-CSF, IL-1β, IL-6, IL-10, IL-15, IFN-γ, LIF, M-CSF, MCP-1 (CCL2), MIP-1β (CCL4) and TNF-α were measured by multiplex ELISA using a custom Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore Cat. No. MCYTOMAG-70K) according to the

manufacturer's instructions. Each biological replicate was performed in technical duplicate. The multiplex plate was read on the Luminex 200 System (Millipore) and the data was analyzed with the Belysa (Millipore Version 1.1.0) and Microsoft Excel software, fitting absorbance values to a standard curve.

Flow cytometry

Flushed d3.5 uterine horns from each group were weighed and digested by Liberase TM (Roche Cat. No. 5401119001) in HBSS supplemented with 2% FBS (25 µg Liberase per 0.1 g of tissue). Digestion occurred for 45 minutes at 37 °C, with agitation every 10 minutes, and products were filtered through a 70 µm cell strainer. To generate a single-cell suspension from the spleen, tissue was pushed through the cell strainer using a syringe plug and treated with 5 mL ACK buffer pH 7.2 (150 mm/L NH_4Cl, 10 mm/L $KHCO_3$, 0.1 mm/L Na_2EDTA) for 30 seconds. 5 mL cold PBS was added to stop the lysis reaction. When necessary for intracellular cytokine staining, cells were stimulated with Cell Activation Cocktail (phorbol-12-myristate 13-acetate and ionomycin, BioLegend Cat. No. 423301) following manufacturers protocol for 4 hours in HBSS/2% FBS. Monensin (BioLegend Cat. No. 420701) transport inhibitor was added during the last hour of incubation. 1 million cells/sample were blocked using the FcγR antibody for 10 minutes (BD Biosciences Cat. No. 553142). The panel of fluorophore-conjugated antibodies used to identify immune cell populations residing in the uterus is listed in Supplementary Table 2 and specific T cell subpopulations in Supplementary Table 3. Samples were stained for 30 minutes and fixed using either 4% PFA or the FoxP3/Transcription Factor Staining Buffer Set when detecting intracellular cytokines (Invitrogen Cat. No. 00-5523-00). 30 µL of CountBright Absolute Counting Beads (Invitrogen Cat. No. C36950) were added to the final 300 µL single cell suspension for quantification of total cell number. Compensation was performed using UltraComp eBeads (Invitrogen Cat. No. 01-2222-42). FMO controls were used during initial panel validation and again as necessary, with the inclusion of an unstimulated control during intracellular cytokine staining. Samples were processed on the BD Biosciences LSRFortessa X-20 at the Immunophenotyping Platform at the Research Institute of the McGill University Health Centre. Flow cytometry data was analyzed using the FlowJo software (BD Biosciences Version 10.8.1).

Statistical analysis

Data shown represents mean ± SEM of independent samples. Statistical analysis comparing experimental groups was performed first by the removal of outliers (ROUT, Q=1%), and either ordinary one-way analysis of variance (ANOVA), Tukey's multiple comparisons test or two-tailed unpaired Student t-tests as fit, using GraphPad Prism (Version 9.4.1) software. P-values less than 0.05 were considered statistically significant.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Animal Care Committee of the McGill University Health Centre, #MUHC 5261. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

DD: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. SY: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. SS: Investigation, Methodology, Writing – review & editing. CP: Investigation, Writing – review & editing. PK: Investigation, Writing – review & editing. ET: Investigation, Writing – review & editing. MP: Investigation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by a grant from the Canadian Institutes of Health Research #PJT-159788 and the Montreal Children's Hospital Foundation awarded to DD.

Acknowledgments

We would like to thank Dr. Laurie Pinel, Elaine Lee and Neha Kamath for their critical review of the manuscript. In addition, Dr. Fernando Alvarez and Dr. Ciriaco Piccirillo for their expertise in flow cytometry and T cell biology. Finally, we thank the staff of the Immunophenotyping, Histopathology and Animal Resource Division platforms at the Research Institute of the McGill University Health Centre. SY was in part supported by a scholarship from the Research Institute of the McGill University Health Centre.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1276979/full#supplementary-material

References

1. Bushnik T, Cook JL, Yuzpe AA, Tough S, Collins J. Estimating the prevalence of infertility in Canada. *Hum Reprod* (2012) 27(3):738–46. doi: 10.1093/humrep/der465

2. Quaas A, Dokras A. Diagnosis and treatment of unexplained infertility. *Rev Obstet Gynecol* (2008) 1(2):69–76.

3. Bulletti C, Coccia ME, Battistoni S, Borini A. Endometriosis and infertility. *J Assisted Reprod Genet* (2010) 27(8):441–7. doi: 10.1007/s10815-010-9436-1

4. Margalioth EJ, Ben-Chetrit A, Gal M, Eldar-Geva T. Investigation and treatment of repeated implantation failure following IVF-ET. *Hum Reprod* (2006) 21(12):3036–43. doi: 10.1093/humrep/del305

5. Dunietz GL, Holzman C, Mckane P, Li C, Boulet SL, Todem D, et al. Assisted reproductive technology and the risk of preterm birth among primiparas. *Fertil Steril* (2015) 103(4):974–9.e1. doi: 10.1016/j.fertnstert.2015.01.015

6. He M, Sun X, Wang C, Sui Y. Analysis of the risk of complications during pregnancy in pregnant women with assisted reproductive technology: a retrospective study using registry linkage from 2013 to 2018 in Shanghai, China. *BMC Pregnancy Childbirth* (2022) 22(1). doi: 10.1186/s12884-022-04846-1

7. Zhu L, Zhang Y, Liu Y, Zhang R, Wu Y, Huang Y, et al. Maternal and live-birth outcomes of pregnancies following assisted reproductive technology: A retrospective cohort study. *Sci Rep* (2016) 6(1):35141. doi: 10.1038/srep35141

8. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* (2006) 7(3):185–99. doi: 10.1038/nrg1808

9. Singh M, Chaudhry P, Asselin E. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. *J Endocrinol* (2011) 210(1):5–14. doi: 10.1530/JOE-10-0461

10. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. *Hum Reprod Update* (2006) 12(6):731–46. doi: 10.1093/humupd/dml004

11. Robertshaw I, Bian F, Das SK. Mechanisms of uterine estrogen signaling during early pregnancy in mice: an update. *J Mol Endocrinol* (2016) 56(3):R127–R38. doi: 10.1530/JME-15-0300

12. Wang X, Wu S-P, Demayo FJ. Hormone dependent uterine epithelial-stromal communication for pregnancy support. *Placenta* (2017) 60:S20–S6. doi: 10.1016/j.placenta.2017.07.003

13. Abu-Raya B, Michalski C, Sadarangani M, Lavoie PM. Maternal immunological adaptation during normal pregnancy. *Front Immunol* (2020) 11. doi: 10.3389/fimmu.2020.575197

14. Förger F, Villiger PM. Immunological adaptations in pregnancy that modulate rheumatoid arthritis disease activity. *Nat Rev Rheumatol* (2020) 16(2):113–22. doi: 10.1038/s41584-019-0351-2

15. McMaster MT, Newton RC, Dey SK, Andrews GK. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *J Immunol* (1992) 148(6):1699–705. doi: 10.4049/jimmunol.148.6.1699

16. Mor G, Cardenas I. The immune system in pregnancy: A unique complexity. *Am J Reprod Immunol* (2010) 63(6):425–33. doi: 10.1111/j.1600-0897.2010.00836.x

17. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann New York Acad Sci* (2011) 1221 (1):80–7. doi: 10.1111/j.1749-6632.2010.05938.x

18. Jasper MJ, Tremellen KP, Robertson SA. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. *Mol Hum Reprod* (2006) 12(5):301–8. doi: 10.1093/molehr/gal032

19. Robertson SA, Care AS, Moldenhauer LM. Regulatory T cells in embryo implantation and the immune response to pregnancy. *J Clin Invest* (2018) 128 (10):4224–35. doi: 10.1172/JCI122182

20. Park CB, Dufort D. Nodal expression in the uterus of the mouse is regulated by the embryo and correlates with implantation. *Biol Reprod* (2011) 84(6):1103–10. doi: 10.1095/biolreprod.110.087239

21. Monsivais D, Matzuk MM, Pangas SA. The TGF-β Family in the reproductive tract. *Cold Spring Harbor Perspect Biol* (2017) 9(10):a022251. doi: 10.1101/cshperspect.a022251

22. Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-β superfamily expression and actions in the endometrium and placenta. *Reproduction* (2006) 132(2):217–32. doi: 10.1530/rep.1.01076

23. Park CB, DeMayo FJ, Lydon JP, Dufort D. NODAL in the uterus is necessary for proper placental development and maintenance of pregnancy. *Biol Reprod* (2012) 86 (6):194. doi: 10.1095/biolreprod.111.098277

24. Ayash TA, Starr LM, Dufort D. Nodal is required to maintain the uterine environment in an anti-inflammatory state during pregnancy. *Biol Reprod* (2020) 102 (6):1340–50. doi: 10.1093/biolre/ioaa037

25. Lovick TA, Zangrossi H Jr. Effect of estrous cycle on behavior of females in rodent tests of anxiety. *Front Psychiatry* (2021) 12:711065. doi: 10.3389/fpsyt.2021.711065

26. McLean AC, Valenzuela N, Fai S, Bennett SA. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J Vis Exp* (2012) 67:e4389. doi: 10.3791/4389-v

27. Coleman DL, Dagg CP, Fuller JL, Green MC, Kaliss N, Russel ES, et al. *Biology of the Laboratory Mouse. 2 ed*. Green EL, editor. New York: Dover Publications Inc (1966).

28. Daikoku T, Cha J, Sun X, Tranguch S, Xie H, Fujita T, et al. Conditional deletion of MSX homeobox genes in the uterus inhibits blastocyst implantation by altering uterine receptivity. *Dev Cell* (2011) 21(6):1014–25. doi: 10.1016/j.devcel.2011.09.010

29. Dunlap KA, Filant J, Hayashi K, Rucker EB, Song G, Deng JM, et al. Postnatal deletion of Wnt7a inhibits uterine gland morphogenesis and compromises adult fertility in mice1. *Biol Reprod* (2011) 85(2):386–96. doi: 10.1095/biolreprod.111.091769

30. Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, et al. Endometrial glands are required for preimplantation conceptus elongation and survival1. *Biol Reprod* (2001) 64(6):1608–13. doi: 10.1095/biolreprod64.6.1608

31. Monsivais D, Nagashima T, Prunskaite-Hyyryläinen R, Nozawa K, Shimada K, Tang S, et al. Endometrial receptivity and implantation require uterine BMP signaling through an ACVR2A-SMAD1/SMAD5 axis. *Nat Commun* (2021) 12(1). doi: 10.1038/s41467-021-23571-5

32. Das M, Holzer HEG. Recurrent implantation failure: gamete and embryo factors. *Fertil Steril* (2012) 97(5):1021–7. doi: 10.1016/j.fertnstert.2012.02.029

33. Cui L, Zhang Z, Sun F, Duan X, wang M, Di K, et al. Transcervical embyro transfer in mice. *J Am Assoc Lab Anim Sci* (2014) 53(3):228–31.

34. Kurihara I, Lee D-K, Petit FG, Jeong J, Lee K, Lydon JP, et al. COUP-TFII mediates progesterone regulation of uterine implantation by controlling ER activity. *PloS Genet* (2007) 3(6):e102. doi: 10.1371/journal.pgen.0030102

35. Satokata I, Benson G, Maas R. Sexually dimorphic sterility phenotypes in HoxalO-deficient mice. *Nature* (1995) 374(6521):460–3. doi: 10.1038/374460a0

36. Lee K, Jeong J, Kwak I, Yu C-T, Lanske B, Soegiarto DW, et al. Indian hedgehog is a major mediator of progesterone signaling in the mouse uterus. *Nat Genet* (2006) 38 (10):1204–9. doi: 10.1038/ng1874

37. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* (1992) 359(6390):76–9. doi: 10.1038/359076a0

38. Surveyor GA, Gender SJ, Pemberton L, Das SK, Chakraborty I, Julian J, et al. Expression and steroid hormonal control of Muc-1 in the mouse uterus. *Endocrinology* (1995) 136(8):3639–47. doi: 10.1210/endo.136.8.7628404

39. Park D-W, Yang K-M. Hormonal regulation of uterine chemokines and immune cells. *Clin Exp Reprod Med* (2011) 38(4):179. doi: 10.5653/cerm.2011.38.4.179

40. Schjenken JE, Glynn DJ, Sharkey DJ, Robertson SA. TLR4 signaling is a major mediator of the female tract response to seminal fluid in mice1. *Biol Reprod* (2015) 93 (3):1–13. doi: 10.1095/biolreprod.114.125740

41. Davari-tanha F, Tehraninejad ES, Ghazi M, Shahraki Z. The role of G-CSF in recurrent implantation failure: A random double blind placebo control trial. *Int J Reprod BioMed* (2016) 14(12):737–42.

42. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res* (2005) 322(1):43–52. doi: 10.1007/s00441-005-1127-3

43. Schjenken JE, Robertson SA. The female response to seminal fluid. *Physiol Rev* (2020) 100(3):1077–117. doi: 10.1152/physrev.00013.2018

44. Zhao H, Kalish F, Schulz S, Yang Y, Wong RJ, Stevenson DK. Unique roles of infiltrating myeloid cells in the murine uterus during early to midpregnancy. *J Immunol* (2015) 194(8):3713–22. doi: 10.4049/jimmunol.1401930

45. Yadi H, Burke S, Madeja Z, Hemberger M, Moffett A, Colucci F. Unique receptor repertoire in mouse uterine NK cells. *J Immunol* (2008) 181(9):6140–7. doi: 10.4049/jimmunol.181.9.6140

46. Tesser RB, Scherholz PLA, Nascimento L, Katz SG. Trophoblast glycogen cells differentiate early in the mouse ectoplacental cone: putative role during placentation. *Histochem Cell Biol* (2010) 134(1):83–92. doi: 10.1007/s00418-010-0714-x

47. Taylor NJ. Investigation of sperm-induced cervical leucocytosis by a double mating study in rabbits. *Reproduction* (1982) 66(1):157–60. doi: 10.1530/jrf.0.0660157

48. Kachkache M, Acker GM, Chaouat G, Noun A, Garabédian M. Hormonal and local factors control the immunohistochemical distribution of immunocytes in the rat uterus before conceptus implantation: Effects of ovariectomy, fallopian tube section, and injection. *Biol Reprod* (1991) 45:860–8. doi: 10.1095/biolreprod45.6.860

49. Moldenhauer LM, Diener KR, Thring DM, Brown MP, Hayball JD, Robertson SA. Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy. *J Immunol* (2009) 182(12):8080–93. doi: 10.4049/jimmunol.0804018

50. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and apoptotic cell clearance during pregnancy. *Am J Reprod Immunol* (2004) 51(4):275–82. doi: 10.1111/j.1600-0897.2004.00156.x

51. Care AS, Diener KR, Jasper MJ, Brown HM, Ingman WV, Robertson SA. Macrophages regulate corpus luteum development during embryo implantation in mice. *J Clin Invest* (2013) 123(8):3472–87. doi: 10.1172/JCI60561

52. Mrozikiewicz AE, Ożarowski M, Jędrzejczak P. Biomolecular markers of recurrent implantation failure—a review. *Int J Mol Sci* (2021) 22(18):10082. doi: 10.3390/ijms221810082

53. Tsao F-Y, Wu M-Y, Chang Y-L, Wu C-T, Ho H-N. M1 macrophages decrease in the deciduae from normal pregnancies but not spontaneous abortions or unexplained recurrent spontaneous abortions. *J Formosan Med Assoc* (2018) 117(3):204–11. doi: 10.1016/j.jfma.2017.03.011

54. Guenther S, Vrekoussis T, Heublein S, Bayer B, Anz D, Knabl J, et al. Decidual macrophages are significantly increased in spontaneous miscarriages and over-express FasL: A potential role for macrophages in trophoblast apoptosis. *Int J Mol Sci* (2012) 13 (7):9069–80. doi: 10.3390/ijms13079069

55. Lambropoulou M, Tamiolakis D, Venizelos J, Liberis V, Galazios G, Tsikouras P, et al. Imbalance of mononuclear cell infiltrates in the placental tissue from foetuses after spontaneous abortion versus therapeutic termination from 8th to 12th weeks of gestational age. *Clin Exp Med* (2006) 6(4):171–6. doi: 10.1007/s10238-006-0111-x

56. Springer TA. Adhesion receptors of the immune system. *Nature* (1990) 346 (6283):425–34. doi: 10.1038/346425a0

57. Li Y-h, Zhang Y, Pan G, Xiang L-x, Luo D-c, Shao J-z. Occurrences and functions of Ly6Chi and Ly6Clo macrophages in health and disease. *Front Immunol* (2022) 13. doi: 10.3389/fimmu.2022.901672

58. Lee S, Kim J, Hur S, Kim C, Na B, Lee M, et al. An imbalance in interleukin-17-producing T and Foxp3+ regulatory T cells in women with idiopathic recurrent pregnancy loss. *Hum Reprod* (2011) 26(11):2964–71. doi: 10.1093/humrep/der301

59. Yang H, Qiu L, Chen G, Ye Z, Lü C, Lin Q. Proportional change of CD4+ CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. *Fertil Steril* (2008) 89(3):656–61. doi: 10.1016/j.fertnstert.2007.03.037

60. Tang M, Taylor HS, Tabibzadeh S. *In vivo* gene transfer of lefty leads to implantation failure in mice. *Hum Reprod* (2005) 20(7):1772–8. doi: 10.1093/humrep/deh849

61. Schier AF. *Nodal Morphogens* Vol. 1. Long Island, NY, USA: Cold Spring Harbor Perspectives in Biology (2009). p. a003459–a.

62. Guo X, Wang X-F. Signaling cross-talk between TGF-β/BMP and other pathways. *Cell Res* (2009) 19(1):71–88. doi: 10.1038/cr.2008.302

63. Deanesly R. The corpora lutea of the mouse, with special reference to fat accumulation during the estrus cycle. *R Soc* (1930) 106(747):578–95.

64. Smith MS, Neil JD. Termination at midpregnancy of the two daily surges of plasma prolactin initiated by mating in the rat. *Endocrinology* (1976) 98(3):696–701. doi: 10.1210/endo-98-3-696

65. Robertson SA, Prins JR, Sharkey DJ, Moldenhauer LM. Seminal fluid and the generation of regulatory T cells for embryo implantation. *Am J Reprod Immunol* (2013) 69(4):315–30. doi: 10.1111/aji.12107

66. Saftlas AF, Rubenstein L, Prater K, Harland KK, Field E, Triche EW. Cumulative exposure to paternal seminal fluid prior to conception and subsequent risk of preeclampsia. *J Reprod Immunol* (2014) 101–102:104-10. doi: 10.1016/j.jri.2013.07.006

67. Crawford G, Ray A, Gudi A, Shah A, Homburg R. The role of seminal plasma for improved outcomes during in *vitro* fertilization treatment: review of the literature and meta-analysis. *Hum Reprod Update* (2014) 21(2):275–84. doi: 10.1093/humupd/dmu052

68. Brown MB, Mv C, AB A, Reyes L. M1/M2 macrophage polarity in normal and complicated pregnancy. *Front Immunol* (2014) 5. doi: 10.3389/fimmu.2014.00606

69. Yao Y, Xu X-H, Jin L. Macrophage polarization in physiological and pathological pregnancy. *Front Immunol* (2019) 10. doi: 10.3389/fimmu.2019.00792

70. Jaiswal MK, Mallers TM, Larsen B, Kwak-Kim J, Chaouat G, Gilman-Sachs A, et al. V-ATPase upregulation during early pregnancy: a possible link to establishment of an inflammatory response during preimplantation period of pregnancy. *Reproduction* (2012) 143(5):713–25. doi: 10.1530/REP-12-0036

71. Wang X-F, Wang H-S, Zhang F, Guo Q, Wang H, Wang K-F, et al. Nodal promotes the generation of M2-like macrophages and downregulates the expression of IL-12. *Eur J Immunol* (2014) 44(1):173–83. doi: 10.1002/eji.201343535

72. Wang W, Sung N, Gilman-Sachs A, Kwak-Kim J. T helper (Th) cell profiles in pregnancy and recurrent pregnancy losses: Th1/Th2/Th9/Th17/Th22/Tfh cells. *Front Immunol* (2020) 11. doi: 10.3389/fimmu.2020.02025

73. Kwak-Kim J, Chung-Bang HS, Ng SC, Ntrivalas EI, Mangubat CP, Beaman KD, et al. Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF. *Hum Reprod* (2003) 18(4):767–73. doi: 10.1093/humrep/deg156

74. Makhseed M, Raghupathy R, Azizieh F, Omu A, Al-Shamali E, Ashkanani L. Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and subsequent abortions. *Hum Reprod* (2001) 16(10):2219–26. doi: 10.1093/humrep/16.10.2219

75. Bogdan C, Schleicher U. Production of interferon-γ by myeloid cells – fact or fancy? *Trends Immunol* (2006) 27(6):282–90. doi: 10.1016/j.it.2006.04.004

76. Darwich L, Coma G, Peña R, Bellido R, Blanco EJJ, Este JA, et al. Secretion of interferon-γ by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology* (2009) 126(3):386–93. doi: 10.1111/j.1365-2567.2008.02905.x

77. Guo L, Guo A, Yang F, Li L, Yan J, Deng X, et al. Alterations of cytokine profiles in patients with recurrent implantation failure. *Front Endocrinol* (2022) 13. doi: 10.3389/fendo.2022.949123

78. Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, et al. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. *J Reprod Immunol* (2010) 85:121–9. doi: 10.1016/j.jri.2010.02.006

79. Wan YY, Flavell RA. 'Yin–Yang' functions of transforming growth factor-β and T regulatory cells in immune regulation. *Immunol Rev* (2007) 220(1):199–213. doi: 10.1111/j.1600-065X.2007.00565.x

80. Huber S, Stahl FR, Schrader J, Luth S, Presser K, Carambia A, et al. Activin A promotes the TGF-B induced conversion of CD4+ CD25- T cells into FoxP3+ induced regulatory T cells. *J Immunol* (2009) 182(8):4633–40. doi: 10.4049/jimmunol.0803143

81. Papageorgiou I, Nicholls PK, Wang F, Lackmann M, Makanji Y, Salamonsen LA, et al. Expression of nodal signalling components in cycling human endometrium and in endometrial cancer. *Reprod Biol Endocrinol* (2009) 7 (1):122. doi: 10.1186/1477-7827-7-122

82. Park CB, Dufort D. NODAL signaling components regulate essential events in the establishment of pregnancy. *Reproduction* (2013) 145(2):R55–64. doi: 10.1530/REP-12-0103

83. Tabibzadeh S. Isolation, characterization, and function of EBAF/LEFTY B: role in infertility. *Ann New York Acad Sci* (2011) 1221(1):98–102. doi: 10.1111/j.1749-6632.2010.05944.x

84. Thulluru HK, Park C, Dufort D, Kleiverda G, Oudejans C, van Dijk M. Maternal Nodal inversely affects NODAL and STOX1 expression in the fetal placenta. *Front Genet* (2013) 4:170. doi: 10.1016/j.placenta.2013.06.251

85. Starr LM, Ayash TA, Dufort D. Evidence of a gene-environment interaction of NODAL variants and inflammation in preterm birth. *J Perinatol* (2018) 38(5):482–8. doi: 10.1038/s41372-018-0073-3

86. Lu CC, Robertson EJ. Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior-posterior patterning. *Dev Biol* (2004) 273 (1):149–59. doi: 10.1016/j.ydbio.2004.06.004

87. Soyal SM, Mukherjee A, Lee KY-S, Li J, Li H, Demayo FJ, et al. Cre-mediated recombination in cell lineages that express the progesterone receptor. *genesis* (2005) 41 (2):58–66. doi: 10.1002/gene.20098

88. Mukherjee A, Amato P, Allred DC, Demayo FJ, Lydon JP. Steroid receptor coactivator 2 is required for female fertility and mammary morphogenesis: insights from the mouse, relevance to the human. *Nucl Recept Signaling* (2007) 5(1):nrs.05011. doi: 10.1621/nrs.05011

89. Caligioni CS. Assessing reproductive status/stages in mice. *Curr Protoc Neurosci* (2009) 48(1). doi: 10.1002/0471142301.nsa04is48

90. Shafiei S, Dufort D. Maternal Cripto is critical for proper development of the mouse placenta and the placental vasculature. *Placenta* (2021) 107:13–23. doi: 10.1016/j.placenta.2021.02.016

frontiers | Frontiers in Immunology

TYPE Opinion
PUBLISHED 05 January 2024
DOI 10.3389/fimmu.2023.1334231

Check for updates

OPEN ACCESS

EDITED BY
Marie-Pierre Piccinni,
University of Florence, Italy

REVIEWED BY
Kailash Singh,
Uppsala University, Sweden

*CORRESPONDENCE
Juan J. Fierro
j.j.fierro.martinez@umcg.nl

RECEIVED 06 November 2023
ACCEPTED 15 December 2023
PUBLISHED 05 January 2024

CITATION
Fierro JJ, Prins JR, Henning S, Bootsma H, Westra J and de Leeuw K (2024) Endometrial immune profiling as a new tool for preconceptional assessment in patients with systemic autoimmune diseases.
Front. Immunol. 14:1334231.
doi: 10.3389/fimmu.2023.1334231

COPYRIGHT
© 2024 Fierro, Prins, Henning, Bootsma, Westra and de Leeuw. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Endometrial immune profiling as a new tool for preconceptional assessment in patients with systemic autoimmune diseases

Juan J. Fierro[1,2*], Jelmer R. Prins[3], Svenja Henning[1], Hendrika Bootsma[1], Johanna Westra[1] and Karina de Leeuw[1]

[1]Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Center Groningen, Groningen, Netherlands, [2]Grupo Reproducción, Departamento de Microbiología y Parasitología, Universidad de Antioquia UdeA, Medellín, Colombia, [3]Department of Obstetrics and Gynecology, University of Groningen, University Medical Center Groningen, Groningen, Netherlands

KEYWORDS

endometrial immune profiling, systemic lupus erythematosus, antiphospholipid syndrome, Sjögren's disease, pregnancy outcomes

Introduction

Systemic autoimmune diseases such as systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), and Sjögren's disease (SjD) affect predominantly women of childbearing age. SLE is a heterogeneous autoimmune disease characterized by interferon upregulation, production of autoantibodies and systemic symptoms (1). Patients with APS have thrombosis and/or pregnancy morbidity associated with persistent positivity of antiphospholipid antibodies (aPL) (2), whereas SjD is characterized by exocrine glandular lymphocytic infiltration, sicca symptoms, extra glandular manifestations such as arthritis, and sometimes presence of auto-antibodies, especially against SSA and SSB (3). These autoimmune diseases have been associated with a higher risk of adverse pregnancy outcomes (APO) like intrauterine fetal death, fetal growth restriction (FGR), preterm birth, low birth weight, and preeclampsia (2–4). Type I interferon signature and complement activation in peripheral blood have been linked to this increased APO risk (5–7).

The menstrual cycle is governed by a sophisticated interaction involving endometrial cells, immune cells, cytokines and sex hormones (8). It is known that the endometrial immune environment prepares to accept the embryo and facilitates implantation (9). Receptive endometrium is characterized by a pro-inflammatory response, complement activation, and an adequate interaction between extracellular vesicles, endometrial epithelial cells and the blastocyst (10). Therefore, an optimal balance between pro-inflammatory factors and the tolerogenic adaptive immune response in the endometrial tissue is pivotal for proper embryo implantation (11, 12).

Preconceptional disease activity can predict APO in patients with systemic autoimmune diseases, which stresses the importance of remission prior to conception.

However, not all APO can be predicted by these disease-related factors (7, 13). Local immune changes in the endometrial environment might also contribute to this increased risk for APO. Endometrial immune profiling is a new method to analyze the immune cell distribution and cytokine production in endometrial tissue samples or menstrual blood. Different techniques are used such as multiparameter flow cytometry or gene expression analysis (14). It has already been suggested as a new screening strategy for personalized care for couples with repeated embryo implantation failures using assisted-reproductive therapy (ART) (15). In this paper, we describe the endometrial immune environment, the method of endometrial immune profiling and hypothesize that this technique might be a valuable tool to assess local immune changes that possibly play a role in development of APO in women with systemic autoimmune diseases.

Endometrial immune environment

Immune cells in the endometrium/decidua are essential during implantation and placentation and immune imbalances have been associated with subsequent placental development failure (16). Uterine natural killer cells (uNK), CD4+ T regulatory cells and different subsets of CD68+ macrophages play a pivotal role in decidualization (maturation of the endometrium) and immune tolerance maintenance throughout pregnancy (12).

Early in pregnancy, uNK account for 70% of decidual lymphocytes and are involved in decidualization, trophoblast cell invasion, uterine vascular remodeling and immune tolerance (17). Notably, using a bioinformatics approach, it has been shown that impaired uNK function and defective endometrium maturation during early pregnancy preceded the development of preeclampsia (18). Decidua natural killer cells (dNK) express an inhibitor profile during gestation regulated by maternal HLA-C alleles through inhibitory receptors such as killer cell immunoglobulin-like receptors (KIRs), natural killer cell receptor NKG2A, and leukocyte immunoglobulin-like receptor B1 (LILRB1) (17). Interestingly, higher endometrial levels of transforming growth factor-beta (TGF-ß) have been associated with dNK-impaired maturation in patients with preeclampsia (19).

Other maternal leukocytes are also involved in decidualization processes and induce immune tolerance of the semi-allogenic fetus during early stages of pregnancy. It has been shown that levels of CD4+ CD25+ Foxp3+ T regulatory cells in peripheral blood fluctuate during the menstrual cycle, possibly preparing for pregnancy (20). During pregnancy, CD4+ T regulatory cells support maternal vascular adaptation and prevent placental inflammatory pathology (21). A lower number of these cells and/or impaired function have been associated with defective placentation and subsequent development of preeclampsia (22). The local T helper cell phenotype varies throughout pregnancy. During the implantation window, a Th1 response is crucial in inducing low-grade inflammation, immune cell recruitment, and tissue remodeling (23). Later on, production of IL-4, IL-5, IL-10 and IL-13 is promoted by a shift towards Th2 phenotype induced by decidual dendritic cells and the increased production of human chorionic gonadotropin (hCG), progesterone and estradiol during pregnancy (24). T helper cell imbalances with a predominant Th1 phenotype and a lower count of IL-10 producing CD8+ T cells have been associated with implantation failure and pregnancy loss (25). Interestingly, Th17 phenotype polarization and lower levels of Foxp3 T regulatory cells have been identified in blood from women with early onset preeclampsia compared with normotensive controls (26). Decidual CD8+ T cells are involved in antiviral protection and fetal tolerance due to an immunosuppressive phenotype through T-cell immunoglobulin mucin-3 (Tim-3), programmed cell death 1 (PD-1), and Cytotoxic T-Lymphocyte associated protein 4 (CTLA-4) inhibitory pathways. Notably, blockage of these pathways was correlated with fetal loss in animal models (27, 28).

A higher proportion of CD20+ B cells has been identified in decidua from patients with recurrent pregnancy loss compared to controls (29). Although B cells make up a small proportion of decidual lymphocytes, a regulatory B cell subset has been linked to fetal tolerance and Th1 suppression, preventing allogeneic responses against the fetus through IL-10 production (24).

Endometrial CD68+ macrophages also play an essential role in the menstrual cycle, restoring the endometrial integrity in preparation for pregnancy (30). Alterations in proportions of endometrial classical activated macrophages (M1) and alternatively activated macrophages (M2) have been described as predictors of implantation failure in patients under ART (31). Notably, a higher prevalence of CD163+ M2 type macrophages has been described during the proliferative phase in endometrial tissue, and was related to adverse implantation outcomes (31).

In conclusion, a complex immunological endometrial environment is linked to decidualization and subsequent healthy pregnancy development in the general population, and immune imbalances have been detected in patients with APO, especially preeclampsia, fetal loss and infertility. Nevertheless, there is no information concerning endometrial immune imbalances in patients with SLE, APS or SjD.

Endometrial immune profiling: from reproductive medicine to systemic autoimmune diseases

Lédée et al. proposed endometrial immune profiling based on RT-qPCR analysis of endometrial biopsies, collected by aspiration during mid-luteal phase, focusing on CD56+ uNK levels, Interleukin-18/Tumor necrosis factor-like weak inducer of apoptosis (IL-18/TWEAK) and Interleukin 15/Fibroblast growth factor-inducible molecule (IL-15/Fn-14) mRNA ratios as key factors involved in uterine embryo receptivity (14, 15). The IL-18/TWEAK mRNA ratio reflects the Th1/Th2 balance and local angiogenesis, while the IL-15/Fn-14 mRNA ratio reflects uNK maturation. An overactive endometrial immune environment in biopsies during mid-luteal phase was established in the presence of

high ratios or high uNK count, while low endometrial immune activation corresponded to low ratios and/or low uNK count (15).

This method was used in infertility patients as a preconception tool to assess suitability of the uterine immunological environment for embryo implantation, and results were used for personalized treatment decisions (32). Treatment modifications differed between patients classified as overactive and those with low endometrial immune activation levels, but overall an increase in live birth rate was achieved in analyzed and treated patients compared to non-analyzed (32). In brief, overactive patients received high-dose luteal hormonal support, oral estradiol supplementation, 20mg of corticosteroids, and vitamin E. At the same time, in those with low endometrial immune activation levels, an endometrial scratching or other local injury was performed and accompanied by hCG supplementation and standard-dose luteal hormonal support (32).

In subsequent studies by Lédée et al., higher pregnancy rates were achieved in patients under ART with a history of repeated implantation failures or recurrent pregnancy loss after endometrial immune profiling assessment (14). Patients were classified as having no dysregulation, low immune activation, over immune activation or mixed profile and received personalized treatment accordingly. Those patients with over-immune activation or mixed profile received immunotherapy with corticosteroids adjunction or low molecular weight heparin in case of resistance to corticosteroids. In contrast, those with low immune activation received endometrial scratching (14). Overall, some infertility patients undergo uterine immune dysregulations and immune profiling might aid in optimizing care and outcomes in these patients (14, 33).

Marron, et al. performed endometrial analysis of luteal-phase biopsies focusing on lymphocyte subsets with a flow cytometry panel assessing natural killer cells (NKs) subsets (uNK, peripheral NKs and T NKs), CD19+ B lymphocytes, CD8+ T lymphocytes, CD4+ T lymphocytes and their Th1, Th2, Th17 and regulatory subsets. Notably, higher concentrations of peripheral NKs (CD16+, $CD56^{dim}$) and CD19+ B lymphocytes were identified in patients with a history of infertility or recurrent pregnancy loss compared to healthy controls (34). Furthermore, commercial endometrial immune profiling tests using endometrial tissue samples have been introduced in clinical practice for patients under ART (35). In those patients, a high expression of B-cell CLL/Lymphoma 6 (BCL6) by immunohistochemistry was identified as a marker for implantation failure or pregnancy loss (36).

Assessment of uNK, plasma cells, CD68+ macrophages, CXC-motif ligand 1 (CXCL1), CXC-motif receptor 2 (CXCR2), syndecan-1 and Vascular Endothelial Growth Factor A (VEGF-A) with immunohistochemistry has been used to map the endometrial immune environment in infertility patients with endometriosis. Interestingly, more macrophages were found in patients with endometriosis than those without and macrophage count was negatively correlated with uNK count in these patients (37).

So different techniques are described to assess the endometrium immune balance. We acknowledge that sample collection is an issue of matter since endometrial biopsy is an invasive procedure with inherent risks. Recent studies confirmed that menstrual blood collected during the first twenty-four hours of menstruation resemble the endometrial environment and can be used as an alternative, easier-to-get sample (38, 39). Recently, multiparameter flow cytometry panels have analyzed lymphocyte subpopulations from menstrual blood samples. Notably, subpopulations differed from peripheral blood samples and between patients with recurrent pregnancy loss and healthy controls (39, 40). Although it has been described that the cytokine profile of menstrual blood differed from the peripheral blood with a higher expression of IL-6, IL-1ß and CXCL8 in healthy controls, there is no information concerning cytokine profile in menstrual blood in patients with systemic autoimmune diseases (41). Therefore, we hypothesize that menstrual blood immunophenotyping might provide valuable insight prior to conception in those patients (34).

Discussion

"The European League Against Rheumatism (EULAR) recommendations for women's health and pregnancy in patients with SLE or APS" encourage risk stratification before pregnancy considering disease activity, comorbidities, autoantibody profile and medication use as an essential tool to improve pregnancy outcomes in these patients (13). Nevertheless, a high proportion of patients still develop APO. Although different pathologic mechanisms have been associated with APO development, the specific chain of events preceding APO is not fully understood. Moreover, associations between disease activity, and endometrial immune changes are unknown.

A better understanding of pathophysiology, including new valid biomarkers, developing clinical instruments for obstetric risk assessment and new personalized interventions are essential to improve pregnancy outcomes in these patients. Given its association with APO development, endometrial immune profiling might be a new tool for preconceptional assessment in patients with systemic autoimmune diseases such as SLE, APS and SjD.

We hypothesize that non-optimal endometrial immune imbalances might be related to APO in women with autoimmune diseases. In Figure 1, we propose a methodology combining endometrial immune profiling with assessment of clinical data, peripheral blood cells and interferon signature analysis for SLE, APS and SjD patients. We aim for a new approach combining previously described preconception risk factors and immunological changes in the endometrium (5, 7, 42). Briefly, we suggest assessing T cell, uNK, monocyte/macrophages and B cells in peripheral and menstrual blood as the principal immune cells involved in early placentation and widely linked with the pathogenesis of these autoimmune diseases (43, 44).

In conclusion, there is a lack of evidence regarding the endometrial immune environment in women with systemic autoimmune diseases in relation to APO. Due to the systemic pro-inflammatory state in these women, we expect an altered endometrial immune environment that can influence implantation and decidualization processes. The clinical value of endometrial immune profiling, in combination with previously used parameters for obstetric risk assessment, should be

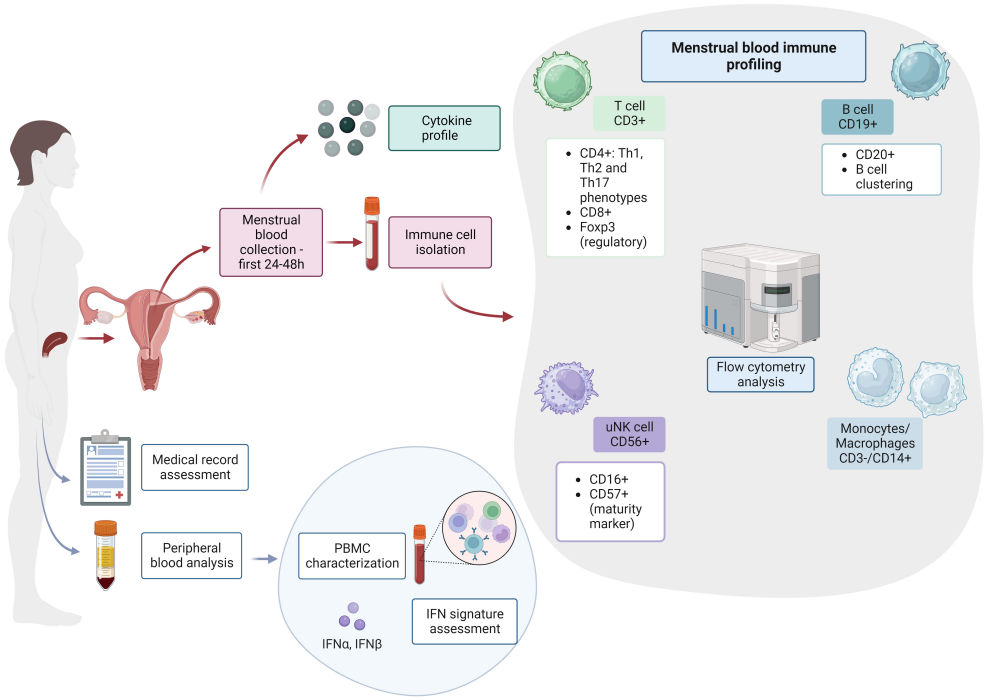


FIGURE 1
Endometrial immune profiling in patients with systemic autoimmune diseases. PBMC, peripheral blood mononuclear cell; IFN, interferon; T cell, T lymphocytes; B cell, B lymphocytes; uNK, uterine natural killer cells. This figure was created with BioRender.com.

investigated in future studies. In particular, the use of menstrual blood seems to be a promising new and non-invasive technique, given the close resemblance with the endometrium biopsies.

Author contributions

JF: Conceptualization, Methodology, Writing – original draft. JP: Writing – review & editing. SH: Writing – review & editing. HB: Writing – review & editing. JW: Writing – review & editing. KdL: Conceptualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. JF is a recipient of a doctoral scholarship from Ministerio de Ciencia, Tecnología e Innovación (MinCiencias, Colombia), (906-2021).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Dorner T, Furie R. Novel paradigms in systemic lupus erythematosus. *Lancet* (2019) 393:2344–58. doi: 10.1016/S0140-6736(19)30546-X

2. Barbhaiya M, Zuily S, Naden R, Hendry A, Manneville F, Amigo M-C, et al. 2023 ACR/EULAR antiphospholipid syndrome classification criteria. *Arthritis Rheumatol* (2023) 82(10):1258–70. doi: 10.1002/art.42624

3. Chan T-M, Wu C-E, Yu H-H, Hsiao C-Y, Su T-H, Chen C-B, et al. Fetal-neonatal and maternal outcomes in women with Sjögren syndrome: a population-based registry linkage study. *Rheumatology* (2023) 62:2820–8. doi: 10.1093/rheumatology/keac711

4. Bundhun PK, Soogund MZ, Huang F. Impact of systemic lupus erythematosus on maternal and fetal outcomes following pregnancy: A meta-analysis of studies published

between years 2001-2016. *J Autoimmun* (2017) 79:17–27. doi: 10.1016/j.jaut.2017.02.009

5. Andrade D, Kim M, Blanco LP, Karumanchi SA, Koo GC, Redecha P, et al. Interferon-α and angiogenic dysregulation in pregnant lupus patients who develop preeclampsia. *Arthritis Rheumatol* (2015) 67:977–87. doi: 10.1002/art.39029

6. Xourgia E, Tektonidou MG. Type I interferon gene expression in antiphospholipid syndrome: Pathogenetic, clinical and therapeutic implications. *J Autoimmun* (2019) 104:102311. doi: 10.1016/j.jaut.2019.102311

7. Fierro JJ, Prins JR, Verstappen GM, Bootsma H, Westra J, de Leeuw K. Preconception clinical factors related to adverse pregnancy outcomes in patients with systemic lupus erythematosus or primary Sjögren's syndrome: a retrospective cohort study. *RMD Open* (2023) 9(3):e003439. doi: 10.1136/rmdopen-2023-003439

8. Oertelt-Prigione S. Immunology and the menstrual cycle. *Autoimmun Rev* (2012) 11:A486–92. doi: 10.1016/j.autrev.2011.11.023

9. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. *J Reprod Immunol* (2017) 124:44–53. doi: 10.1016/j.jri.2017.10.045

10. Altmäe S, Koel M, Võsa U, Adler P, Suhorutšenko M, Laisk-Podar T, et al. Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers. *Sci Rep* (2017) 7:10077. doi: 10.1038/s41598-017-10098-3

11. Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, et al. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. *J Reprod Immunol* (2010) 85:121–9. doi: 10.1016/j.jri.2010.02.006

12. Ashary N, Tiwari A, Modi D. Embryo implantation: war in times of love. *Endocrinology* (2018) 159:1188–98. doi: 10.1210/en.2017-03082

13. Andreoli L, Bertsias GK, Agmon-Levin N, Brown S, Cervera R, Costedoat-Chalumeau N, et al. EULAR recommendations for women's health and the management of family planning, assisted reproduction, pregnancy and menopause in patients with systemic lupus erythematosus and/or antiphospholipid syndrome. *Ann Rheum Dis* (2017) 76:476–485. doi: 10.1136/annrheumdis-2016-209770

14. Lédée N, Petitbarat M, Prat-Ellenberg L, Dray G, Cassuto GN, Chevrier L, et al. Endometrial immune profiling: A method to design personalized care in assisted reproductive medicine. *Front Immunol* (2020) 11:1032. doi: 10.3389/fimmu.2020.01032

15. Lédée N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The uterine immune profile may help women with repeated unexplained embryo implantation failure after *in vitro* fertilization. *Am J Reprod Immunol* (2016) 75:388–401. doi: 10.1111/aji.12483

16. Ng S-W, Norwitz GA, Pavlicev M, Tilburgs T, Simón C, Norwitz ER. Endometrial decidualization: the primary driver of pregnancy health. *Int J Mol Sci* (2020) 21(11):4092. doi: 10.3390/ijms21114092

17. Xie M, Li Y, Meng Y-Z, Xu P, Yang Y-G, Dong S, et al. Uterine natural killer cells: A rising star in human pregnancy regulation. *Front Immunol* (2022) 13:918550. doi: 10.3389/fimmu.2022.918550

18. Rabaglino MB, Post Uiterweer ED, Jeyabalan A, Hogge WA, Conrad KP. Bioinformatics approach reveals evidence for impaired endometrial maturation before and during early pregnancy in women who developed preeclampsia. *Hypertens* (2015) 65:421–9. doi: 10.1161/HYPERTENSIONAHA.114.04481

19. Zhang J, Dunk CE, Shynlova O, Caniggia I, Lye SJ. TGFb1 suppresses the activation of distinct dNK subpopulations in preeclampsia. *EBioMedicine* (2019) 39:531–9. doi: 10.1016/j.ebiom.2018.12.015

20. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. *J Immunol* (2007) 178:2572–8. doi: 10.4049/jimmunol.178.4.2572

21. Krop J, Heidt S, Claas FHJ, Eikmans M. Regulatory T cells in pregnancy: it is not all about foxP3. *Front Immunol* (2020) 11:1182. doi: 10.3389/fimmu.2020.01182

22. Robertson SA, Green ES, Care AS, Moldenhauer LM, Prins JR, Hull ML, et al. Therapeutic potential of regulatory T cells in preeclampsia-opportunities and challenges. *Front Immunol* (2019) 10:478. doi: 10.3389/fimmu.2019.00478

23. Mjösberg J, Berg G, Jenmalm MC, Ernerudh J. FOXP3+ regulatory T cells and T helper 1, T helper 2, and T helper 17 cells in human early pregnancy decidua. *Biol Reprod* (2010) 82:698–705. doi: 10.1095/biolreprod.109.081208

24. Colamatteo A, Fusco C, Micillo T, D'Hooghe T, de Candia P, Alviggi C, et al. Immunobiology of pregnancy: from basic science to translational medicine. *Trends Mol Med* (2023) 29:711–25. doi: 10.1016/j.molmed.2023.05.009

25. Ng SC, Gilman-Sachs A, Thaker P, Beaman KD, Beer AE, Kwak-Kim J. Expression of intracellular Th1 and Th2 cytokines in women with recurrent spontaneous abortion, implantation failures after IVF/ET or normal pregnancy. *Am J Reprod Immunol* (2002) 48:77–86. doi: 10.1034/j.1600-0897.2002.01105.x

26. Ribeiro VR, Romao-Veiga M, Romagnoli GG, Matias ML, Nunes PR, Borges VTM, et al. Association between cytokine profile and transcription factors produced by T-cell subsets in early- and late-onset pre-eclampsia. *Immunology* (2017) 152:163–73. doi: 10.1111/imm.12757

27. Wang S-C, Li Y-H, Piao H-L, Hong X-W, Zhang D, Xu Y-Y, et al. PD-1 and Tim-3 pathways are associated with regulatory CD8+ T-cell function in decidua and maintenance of normal pregnancy. *Cell Death Dis* (2015) 6:e1738. doi: 10.1038/cddis.2015.112

28. Wang S, Sun F, Li M, Qian J, Chen C, Wang M, et al. The appropriate frequency and function of decidual Tim-3(+)CTLA-4(+)CD8(+) T cells are important in maintaining normal pregnancy. *Cell Death Dis* (2019) 10:407. doi: 10.1038/s41419-019-1642-x

29. Kavvadas D, Karachrysafi S, Anastasiadou P, Kavvada A, Fotiadou S, Papachristodoulou A, et al. Immunohistochemical evaluation of CD3, CD4, CD8, and CD20 in decidual and trophoblastic tissue specimens of patients with recurrent pregnancy loss. *Clin Pract* (2022) 12:177–93. doi: 10.3390/clinpract12020022

30. Ma H, Cai S, Yang L, Wang L, Ding J, Li L, et al. How do pre-pregnancy endometrial macrophages contribute to pregnancy? *J Reprod Immunol* (2022) 154:103736. doi: 10.1016/j.jri.2022.103736

31. Diao L, Cai S, Huang C, Li L, Yu S, Wang L, et al. New endometrial immune cell-based score (EI-score) for the prediction of implantation success for patients undergoing IVF/ICSI. *Placenta* (2020) 99:180–8. doi: 10.1016/j.placenta.2020.07.025

32. Lédée N, Prat-Ellenberg L, Chevrier L, Balet R, Simon C, Lenoble C, et al. Uterine immune profiling for increasing live birth rate: A one-to-one matched cohort study. *J Reprod Immunol* (2017) 119:23–30. doi: 10.1016/j.jri.2016.11.007

33. Lédée N, Petitbarat M, Prat-Ellenberg L, Dray G, Cassuto GN, Chevrier L, et al. The uterine immune profile: A method for individualizing the management of women who have failed to implant an embryo after IVF/ICSI. *J Reprod Immunol* (2020) 142:103207. doi: 10.1016/j.jri.2020.103207

34. Marron K, Harrity C. Endometrial lymphocyte concentrations in adverse reproductive outcome populations. *J Assist Reprod Genet* (2019) 36:837–46. doi: 10.1007/s10815-019-01427-8

35. Kliman HJ, Frankfurter D. Clinical approach to recurrent implantation failure: evidence-based evaluation of the endometrium. *Fertil Steril* (2019) 111:618–28. doi: 10.1016/j.fertnstert.2019.02.011

36. Almquist LD, Likes CE, Stone B, Brown KR, Savaris R, Forstein DA, et al. Endometrial BCL6 testing for the prediction of in *vitro* fertilization outcomes: a cohort study. *Fertil Steril* (2017) 108:1063–9. doi: 10.1016/j.fertnstert.2017.09.017

37. Freitag N, Baston-Buest DM, Kruessel J-S, Markert UR, Fehm TN, Bielfeld AP. Eutopic endometrial immune profile of infertility-patients with and without endometriosis. *J Reprod Immunol* (2022) 150:103489. doi: 10.1016/j.jri.2022.103489

38. Van Der Molen RG, Schutten JHF, Van Cranenbroek B, Ter Meer M, Donckers J, Scholten RR, et al. Menstrual blood closely resembles the uterine immune micro-environment and is clearly distinct from peripheral blood. *Hum Reprod* (2014) 29:303–14. doi: 10.1093/humrep/det398

39. Vomstein K, Egerup P, Kolte AM, Behrendt-Møller I, Boje AD, Bertelsen M-L, et al. Biopsy-free profiling of the uterine immune system in patients with recurrent pregnancy loss and unexplained infertility. *Reprod BioMed Online* (2023) 47:103207. doi: 10.1016/j.rbmo.2023.03.018

40. Marron K, Harrity C. Potential utility of a non-invasive menstrual blood immunophenotype analysis in reproductive medicine. *Reprod Fertil* (2022) 3:255–61. doi: 10.1530/RAF-22-0047

41. Crona Guterstam Y, Strunz B, Ivarsson MA, Zimmer C, Melin A-S, Jonasson AF, et al. The cytokine profile of menstrual blood. *Acta Obstet Gynecol Scand* (2021) 100:339–46. doi: 10.1111/aogs.13990

42. Walter IJ, Klein Haneveld MJ, Lely AT, Bloemenkamp KWM, Limper M, Kooiman J. Pregnancy outcome predictors in antiphospholipid syndrome: A systematic review and meta-analysis. *Autoimmun Rev* (2021) 20:102901. doi: 10.1016/j.autrev.2021.102901

43. Vrzić Petronijević S, Vilotić A, Bojić-Trbojević Ž, Kostić S, Petronijević M, Vićovac L, et al. Trophoblast cell function in the antiphospholipid syndrome. *Biomedicines* (2023) 11(10):2681. doi: 10.3390/biomedicines11102681

44. Zhou H-Y, Luo Q, Sui H, Du X-N, Zhao Y-J, Liu L, et al. Recent advances in the involvement of epigenetics in the pathogenesis of systemic lupus erythematosus. *Clin Immunol* (2023) 258:109857. doi: 10.1016/j.clim.2023.109857

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 01 February 2024
DOI 10.3389/fimmu.2024.1339304

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Andrea Ivonne Loewendorf,
ImmunoVation, United States
Margaret A. Jordan,
James Cook University, Australia

*CORRESPONDENCE
Tyana T. Joseph
tjoseph@msm.edu

RECEIVED 16 November 2023
ACCEPTED 15 January 2024
PUBLISHED 01 February 2024

CITATION
Joseph TT, Schuch V, Hossack DJ, Chakraborty R and Johnson EL (2024) Melatonin: the placental antioxidant and anti-inflammatory.
Front. Immunol. 15:1339304.
doi: 10.3389/fimmu.2024.1339304

COPYRIGHT
© 2024 Joseph, Schuch, Hossack, Chakraborty and Johnson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Melatonin: the placental antioxidant and anti-inflammatory

Tyana T. Joseph[1]*, Viviane Schuch[1], Daniel J. Hossack[1], Rana Chakraborty[2] and Erica L. Johnson[1]

[1]Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA, United States, [2]Department of Pediatric and Adolescent Medicine, Mayo Clinic College of Medicine and Science, Rochester, MN, United States

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine hormone with many physiological and biological roles. Melatonin is an antioxidant, anti-inflammatory, free radical scavenger, circadian rhythm regulator, and sleep hormone. However, its most popular role is the ability to regulate sleep through the circadian rhythm. Interestingly, recent studies have shown that melatonin is an important and essential hormone during pregnancy, specifically in the placenta. This is primarily due to the placenta's ability to synthesize its own melatonin rather than depending on the pineal gland. During pregnancy, melatonin acts as an antioxidant and anti-inflammatory, which is necessary to ensure a stable environment for both the mother and the fetus. It is an essential antioxidant in the placenta because it reduces oxidative stress by constantly scavenging for free radicals, i.e., maintain the placenta's integrity. In a healthy pregnancy, the maternal immune system is constantly altered to accommodate the needs of the growing fetus, and melatonin acts as a key anti-inflammatory by regulating immune homeostasis during early and late gestation. This literature review aims to identify and summarize melatonin's role as a powerful antioxidant and anti-inflammatory that reduces oxidative stress and inflammation to maintain a favorable homeostatic environment in the placenta throughout gestation.

KEYWORDS

melatonin, antioxidant, anti-inflammatory, placenta, pregnancy, HCMV, oxidative stress, NLRP3 inflammasome

1 Introduction

Throughout gestation, melatonin (N-acetyl-5-methoxytryptamine) is a powerful hormone with many physiological and biological roles that ensure a stable environment for both the mother and the fetus. Melatonin is an antioxidant, anti-inflammatory, free radical scavenger, circadian rhythm regulator, and sleep hormone (1, 2). A homeostatic balance between reactive oxygen species (ROS) and antioxidants is needed during

pregnancy to maintain a stable and healthy placenta. Without this balance, oxidative stress can occur in the placenta, allowing adverse conditions like pre-eclampsia, preterm birth, and intrauterine growth restriction (IUGR) to occur (3–5). Melatonin is an essential antioxidant in the placenta that reduces oxidative stress during gestation (6, 7). Melatonin is a lipophilic, hydrophilic indolamine that can rapidly cross the placenta and diffuse into cells (1, 8). In addition, this hormone is endogenously produced in both the ovaries and the placenta, which results in increased levels of systemic melatonin in pregnant women compared to non-pregnant women (9, 10). However, melatonin's mechanistic role and signaling pathways during pregnancy are largely unknown (1, 11–13). In this review, we will provide in-depth insight into melatonin's role in the placenta as an anti-inflammatory and antioxidant during inflammation, oxidative stress, and viral infection.

2 The placenta

2.1 Overview

The placenta is a multifaceted, temporary organ with numerous biological functions largely considered endocrinologic and immunologic (14). The critical roles of the placenta include facilitating embryonic implantation into the uterine wall, promoting fetal growth, and maintaining maternal–fetal tolerance (15). In addition, this organ removes harmful waste products and carbon dioxide from the fetal circulation, provides nutrients and oxygen, and envelopes the fetus in a protective immunologic membrane throughout gestation (15–17). The umbilical cord transfers blood through the placenta to supply the fetus with adequate oxygen and nutrients for survival throughout pregnancy (18). This exchange occurs without the fetal blood and the maternal blood intermingling (17, 18). Hormones produced by the placenta include estrogen and progesterone, which promote the expansion of the uterus to accommodate the growing fetus and placenta (19). Other key immunoprotective roles include facilitating the passive transfer of IgG from the maternal to the fetal circulation and affording protection against invasive pathogens in intrauterine and postnatal life (20, 21).

2.2 Development

The formation of the placenta begins after the fertilized egg is implanted into the uterus, which occurs approximately 8 to 10 days after conception (15, 17). The placenta will gradually grow during the first 3 months of pregnancy and then increase in size corresponding to the uterus after 4 months (15, 17). There are several layers and sublayers of tissue that constitute the multifunctional placenta. This review will primarily focus on a sublayer in the chorion layer, namely, the trophoblast, and the decidual layer of the placenta.

The placenta comprises two distinct sides, the maternal and the fetal sides (Figure 1). The chorion is a highly vascular outer embryonic membrane layer surrounding the fetus (14, 22). The chorion has two sublayers: the trophoblast (cytotrophoblast and syncytiotrophoblast) and the extraembryonic mesoderm (Figure 1) (15, 20). The trophoblast layer is positioned on the fetal side of the placenta. Trophoblast cells are essential for implantation because they interact with the maternal uterine endometrium, which promotes syncytiotrophoblast and cytotrophoblast development (20). Trophoblast differentiation and implantation ensure adequate blood supply and limit fetal immune rejection (16, 20). Cytotrophoblast cells are progenitor stem cells of the syncytiotrophoblast that can differentiate into syncytiotrophoblast cells as finger-like projections and ensure that the fetus receives an adequate blood supply with nutrients and oxygen (15, 20, 23). Syncytiotrophoblast cells transport nutrients to the fetus and remove waste products (16). This requires the syncytiotrophoblast to have ample blood supply and blood flow. In addition, these cells must undergo apoptosis continuously throughout gestation for the appropriate channeling of blood (24, 25). Because the syncytiotrophoblast cells have this specialized function and undergo apoptosis, these cells must be regenerated regularly to ensure adequate and continuous blood transport between the maternal and fetal circulations (26). A defect in trophoblast differentiation may compromise the integrity of the placenta, resulting in pregnancy complications, such as preterm birth, preeclampsia, and IUGR (27).

On the maternal side, the primary tissue that comprises this side of the placenta is the decidua, which originates from the endometrium (14). The decidua develops after the blastocyst attaches to the uterine wall, which involves tissue remodeling that supports both residential and immune cells (28). It includes terminally differentiated endometrial stromal cells, maternal blood, and maternal vascular cells (14, 16, 28). There are three distinct sections of decidua, which are named relative to the embryo: the decidua capsularis that covers the implanted embryo, the decidua basalis which is the region between the embryo and the myometrium, and the decidua parietalis, which lines the fetal membrane and the remaining endometrium of the placenta (14, 28, 29). Anchoring villi hold the chorion and the decidua together by securing the decidua basalis to the cytotrophoblast layer (14, 30). Like the trophoblast, the decidua promotes immune tolerance of the semi-allogenic (half of genes from the mother and half of genes from the father) fetus by limiting recognition from maternal immune cells. The decidua also provides nutritional support before placenta formation (28). Defects in the development of the decidua or decidualization may result in implantation failure, pregnancy loss, or pregnancy complications later in gestation (28).

2.3 Immune tolerance

The human placenta is complex and unique, allowing for an intimate contact between maternal and fetal cells throughout gestation (31). The developing fetus has both maternal and paternal antigens, to which the mother's immune system recognizes the paternal antigens as foreign and leads to the activation of the maternal immune system (31). Therefore, a highly regulated immune system is needed between mother and child to create a beneficial immunological environment that protects the

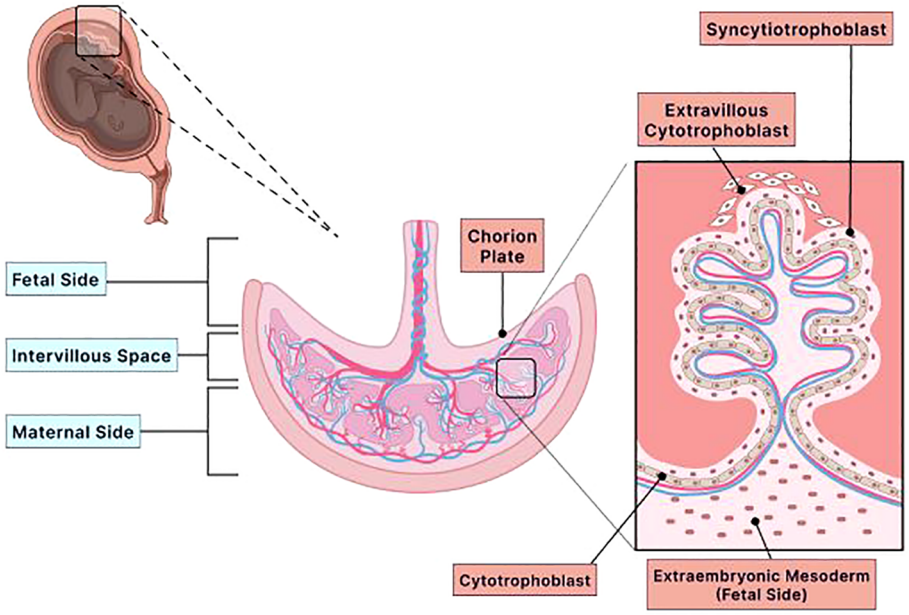


FIGURE 1
Structure of the placenta. The placenta is a multifaceted organ with many biological functions that are endocrinologic and immunologic. This temporary organ provides nutrients and oxygen, removes harmful waste products and carbon dioxide, and envelops the fetus in a protective immunologic membrane throughout gestation. Furthermore, the placenta has two distinct sides, fetal and maternal, and several layers and sublayers. The chorion layer is an immensely vascular membrane layer that surrounds the fetus. The chorion has two sublayers: the extraembryonic mesoderm and the trophoblast (cytotrophoblast and syncytiotrophoblast). Both layers are located on the fetal side of the placenta. The extraembryonic mesoderm is a tissue that contributes to the epithelium of the yolk sac, amnion, and chorionic villi (chorion plate). The trophoblasts are specialized cells crucial for embryo implantation because they interact with the maternal uterine environment to limit fetal immune rejection and ensure an adequate blood supply. The two types of trophoblast cells are cytotrophoblast and syncytiotrophoblast. Both cells cover the outer surface of the chorionic villus. The cytotrophoblast is the innermost lining of the chorionic villus, while the syncytiotrophoblast is the outermost lining of the chorionic villus that is bathed in maternal blood. Because the syncytiotrophoblast is bathed in maternal blood, it has the vital role of transporting nutrients to the fetus and removing waste. Therefore, these cells must have ample blood supply and blood flow and undergo apoptosis regularly to maintain adequate blood circulation. The cytotrophoblast cells are progenitor stem cells of the syncytiotrophoblast. These cells differentiate into the syncytiotrophoblast cells. Therefore, the cytotrophoblast has the specialized role of ensuring that the placenta has ample blood supply with nutrients and oxygen. Adequate blood supply and blood flow by the cytotrophoblast and syncytiotrophoblast are essential in maintaining the integrity of the placenta. If the integrity of the placenta becomes compromised, then adverse pregnancy complications, such as pre-eclampsia, preterm birth, and intrauterine growth restriction, will occur. Created with Biorender.com.

growing fetus from maternal–fetal tolerance, inflammation, and invading pathogens, such as viral infections (32, 33). The placenta is composed of various immune cells, such as natural killer T (NKT) cells, decidual natural killer (dNK) cells, T cells, dendritic cells, B cells, and macrophages (Hofbauer cells and decidual) (31–34). An immense network of cellular connections is formed in the placenta via the interaction of these immune cells, trophoblast cells, and decidual stromal cells to form the immune system in the placenta. An imbalance in this network allows pathogens to infect the placenta and cross the placental barrier to infect the fetus, along with pregnancy complications, such as preterm birth, preeclampsia, spontaneous abortion, and IUGR (33, 35).

3 Oxidative stress and ROS in the placenta

Oxygen is an essential element needed to sustain life. However, the presence of excessive oxygen or limited oxygen can lead to fatal toxicity of cells (36). The formation of ROS occurs as a natural byproduct of cellular oxidative metabolism resulting from the reduction of molecular oxygen generated by the mitochondria during oxidative phosphorylation (37). During mitochondrial oxidative phosphorylation, electrons transfer across respiratory chain enzymes and leak molecular oxygen (38). These electrons can leak prematurely and react with oxygen, producing ROS (38). ROS holds an oxygen atom with an unpaired electron in its outer shell. ROS are essential in regulating cell differentiation, cell signaling, cell differentiation, and inflammation-related factor production (39). However, considerable amounts of ROS at the molecular level can cause cellular and tissue damage, damaging nucleic acids, proteins, organelles, and membranes and inducing cell death or apoptosis (40, 41). Molecules and enzymes that are beneficial in reducing ROS and reducing the effects of ROS include antioxidants. Antioxidants inhibit oxidation and prevent or delay cell damage (42). The generation of ROS and antioxidants should be balanced to achieve homeostasis at the cellular and molecular levels. An imbalance between the formation of ROS and antioxidants leads to oxidative stress (42). This imbalance should be avoided for cellular processes to remain regulated.

Initially, at 0 to 9 weeks of pregnancy, the placenta develops in a state of low oxygen with an ambient pO_2 <20 mmHg due to

the blockage of maternal blood flow to the placenta by endovascular plugs of extravillous trophoblast (36, 43, 44). Previous studies have confirmed that pregnancies that are less than 10 weeks of gestation had no blood flow into the intervillous space, with *in vivo* measurements that indicate pO_2 <20 mmHg (36, 43, 45). Therefore, a premature increase of oxygen tension within the first 10 weeks of gestation can lead to an increased risk of pregnancy loss, primarily due to the detrimental effects of ROS (46, 47). At 10 to 12 weeks gestation, the endovascular trophoblast plugs are lost, which allows maternal blood to perfuse in the intervillous space, thus increasing oxygen tension (36). Throughout pregnancy, the placenta adapts to the changing oxygen levels to support normal placental function by increasing the antioxidant defense within cells (48).

The placenta should remain homeostatic and balanced across gestation with no evidence of oxidative stress (5, 49, 50). Because the syncytiotrophoblast forms by fusion and differentiation of the cytotrophoblast, it must continuously undergo apoptosis to maintain homeostasis (15). Studies have shown that placental oxidative stress is a potent inducer of increased syncytiotrophoblast apoptosis through the mitochondrial oxidative pathway (51, 52). Increased programmed cell death of the syncytiotrophoblast disrupts the layer, resulting in homeostatic imbalance, which causes placental-derived material to be released into the maternal circulation, including tumor necrosis factor (TNF-alpha) and syncytiotrophoblast microparticles (SBTM) (51). Placental trophoblast and endothelial cells form the barrier separating the maternal and fetal circulations (53). Oxidative stress in these cells can lead to the rupture of this barrier and reduce placenta oxygenation or the intermixing of the maternal–fetal circulations (16, 54). Therefore, low levels of ROS are necessary for syncytiotrophoblast formation. In contrast, an imbalance between the ratio of ROS and antioxidants adversely downregulates the syncytiotrophoblast due to increased apoptosis (4, 37, 52).

4 Melatonin in the placenta

4.1 Melatonin and the circadian rhythm in the placenta

Every living organism and nearly every organ has a circadian clock that governs the daily rhythmicity of several physiological and biological processes (55). Core clock genes control the circadian rhythm in the suprachiasmatic nucleus (SCN), the master central pacemaker in the hypothalamus (34, 56). The SCN regulates the photoperiodic programming of the daily circadian clock and coordinates the clock machinery in peripheral tissues (57, 58). The cellular clock oscillates through core clock genes depending on the time of day. Through a transcriptional/translational feedback loop, the heterodimer *BMAL1/CLOCK* activates the heterodimer *Per/Cry* expression that suppresses the transcription of *BMAL1* and *CLOCK* (59–61). Another short feedback loop involves the participation of *BMAL1/CLOCK* to activate the expression of *REV-ERBα* and *RORα* through binding to the nuclear orphan receptor (*ROR*) at the promoter site (34, 62).

The uterus and the placenta utilize circadian rhythm to carry out certain physiological functions, e.g., hormone release, parturition, and immune function, and entrain the fetus' circadian rhythm (2, 63–66). Studies have shown that melatonin can synchronize the clock machinery in healthy and damaged cells to upregulate or downregulate specific clock genes to sustain optimal physiology in cells (34, 67, 68). Moreover, the placenta employs a circadian rhythm for the rhythmic release of melatonin. Melatonin is a ubiquitous molecule that conducts many functions. It is mainly known for its role as one of the circadian rhythm regulators, primarily synthesized in the pineal gland by pinealocytes under dark conditions (34, 69). During darkness, melatonin is rhythmically produced by the pineal gland—concentrations peak between the hours of 2 a.m. and 4 a.m (8).

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized from serotonin (5-hydroxytryptamine) through a two-step reaction (Figure 2). In the first step, serotonin becomes acetylated by arylalkylamine N-acetyltransferase (AANAT) to become N-acetyl serotonin, the rate-limiting step. AANAT is the rate-limiting enzyme of melatonin because it controls the circadian rhythm of melatonin production via the pineal gland. The second step involves N-acetyl serotonin becoming methylated by acetyl serotonin o-methyltransferase (ASMT) to become melatonin (70–72). Given that serum melatonin concentrations are higher in pregnant than in non-pregnant women, studies have indicated that the primary source of this melatonin is the placenta (8–10). How the placenta produces melatonin without the need for the pineal gland or whether the circadian rhythm plays a role in this production has to be studied. The need for melatonin in the placenta is crucial for both the mother and the fetus because it depends on placental melatonin and maternal serum melatonin to provide photoperiodic information to control the internal rhythms of the fetus (34, 64). The placenta exposes maternal melatonin to the fetus at daily rhythmic intervals, with low concentrations during the day and high concentrations at night (8). Disrupting this rhythm during pregnancy may lead to detrimental outcomes for the mother and the growing fetus *in utero* and in adult life (8, 56, 61, 73). The development of the embryo, uterine implantation, placentation, and delivery may be regulated by the clock molecular machinery in the circadian rhythm (73). Circadian disruption or chronodisruption in pregnant women can lead to adverse outcomes in the offspring (61). Maternal chronodisruption is caused by mistimed eating, shift work, traveling across time zones, and immoderate artificial light exposure at nighttime (61). The impairment of the circadian rhythm during pregnancy compromises melatonin production, inhibiting the rhythm of melatonin release (74, 75). Chronodisruption may promote chronic illnesses in postnatal life, including diabetes, obesity, and cardiovascular disease (61).

4.2 Melatonin receptors in the placenta

Melatonin's effects are mediated by two receptors, MT1 (Mel1a) and MT2 (Mel1b) (Figure 3) (76, 77). The MT1 receptor is approximately 350 amino acids long, while the MT2 receptor is 362 amino acids long (78). The molecular weight of both receptors

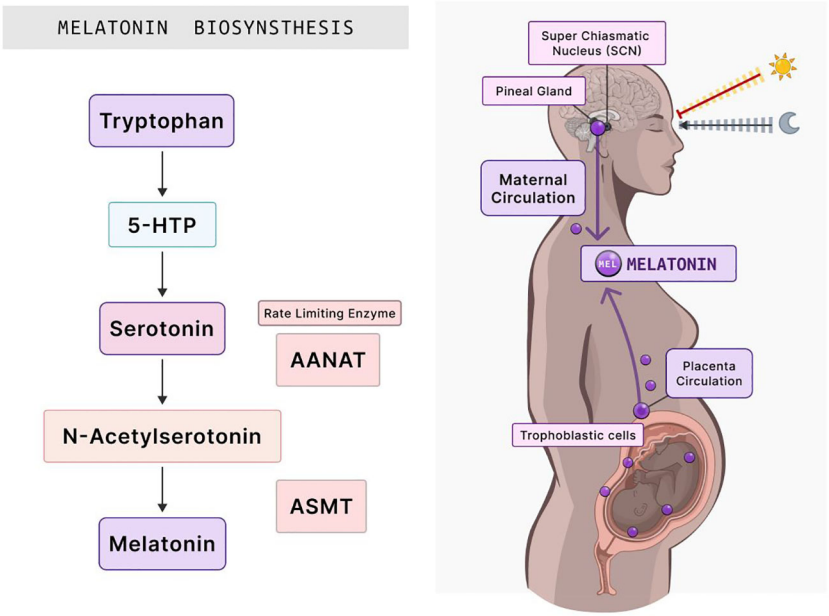


FIGURE 2
Melatonin biosynthesis and its distribution in the human body during pregnancy. The synthesis of melatonin begins with the amino acid tryptophan, which subsequently transforms into 5-HTP. This intermediate is then converted into serotonin. The rate-limiting enzyme, AANAT, acts on serotonin, culminating in N-acetylserotonin production. ASMT then catalyzes the final step, yielding melatonin. Once synthesized, the SCN in the brain, which regulates circadian rhythms, signals the pineal gland to release melatonin into the maternal circulation. Notably, melatonin is also synthesized within the placenta by trophoblastic cells. This melatonin subsequently influences various processes in placental function, essential for fetal growth and development. 5-HTP, 5-hydroxytryptophan; AANAT, arylalkylamine N-acetyltransferase; ASMT, acetylserotonin O-methyltransferase; SCN, suprachiasmatic nucleus. Created with Biorender.com.

is about 39–40 kDa (78). MT1 and MT2 are G-protein coupled receptors. G-protein coupled receptors are essential membrane proteins that form the fourth-largest superfamily in the human genome (79). The G-protein receptor has three subunits: α, β, and γ. When a ligand binds to the extracellular receptor, this initiates a signal transduction cascade that induces a conformational change, promoting the activation of the heterotrimeric GTP-binding protein (G-protein) (79). As the G-protein coupled receptor becomes activated, the α subunit disassociates from the β and γ subunits and releases guanine diphosphate (GDP). It binds to guanine triphosphate (GTP), which results in the conformational changes that later trigger the signal transduction of different G-proteins: inhibitory G-protein (G_i), stimulatory G-protein (G_s), and guanine-nucleotide binding protein (G_q) (79). Each of these proteins has different functions that activate distinct signaling pathways. The G_s protein stimulates the increase of cyclic AMP (cAMP) levels by activating adenylyl cyclase, while G_i inhibits cAMP levels (79). Moreover, G_q induces the expression of phospholipase C, which stimulates protein kinase C (PKC) and calcium (79). Consequently, the MT1 receptor homodimer and MT2 homodimer bind and activate the Gi–G protein and inhibits adenyl cyclase pathway signaling, decreasing forskolin-stimulated cAMP and protein kinase A signaling (60, 77, 80, 81). The MT1 and MT2 receptor heterodimer is associated with G_q, which enables the production of protein kinase C (PKC) and the increase of calcium associated with IP_3 (60, 77, 80, 81). MT1 and MT2 are widely distributed throughout the body and are present in almost all human cells (72, 82, 83). The primary responsibility of the MT1 receptor involves regulating the circadian cycle, while MT2 receptors control the body temperature through the peripheral tissue. In both the cytotrophoblast and the syncytiotrophoblast, studies have detected the presence of melatonin receptors in both primary isolated cells of the placenta and placental trophoblast cell lines (JEG-3 and BeWo) (34, 72, 82). The placenta constantly synthesizes melatonin receptors to promote the survival of the cytotrophoblast by decreasing free radicals and angiogenesis (34). Moreover, when disease (preeclampsia and preterm birth) is present in the placenta, melatonin receptor activity and melatonin levels are both decreased (34, 84). The binding of melatonin on MT1 homodimers and MT2 homodimers and the activation of the receptors indicate autocrine and paracrine activity needed for placental homeostasis and fetal development (34).

4.3 Melatonin in the immune microenvironment of the placenta

Pregnancy is associated with immunological changes that result in the fetus avoiding immune rejection by the mother. Increasing evidence associates immunological changes in pregnancy with melatonin and the circadian clock and synchronous programming of the immune system (34, 85). Melatonin possesses immunomodulatory properties (85) that use circadian regulation to stimulate cytokine production and lymphocyte proliferation and enhance phagocytosis (85–90). In both the luteal phase and early gestation, uterine NK cells represent most of the lymphocyte population. The decidual NK cells

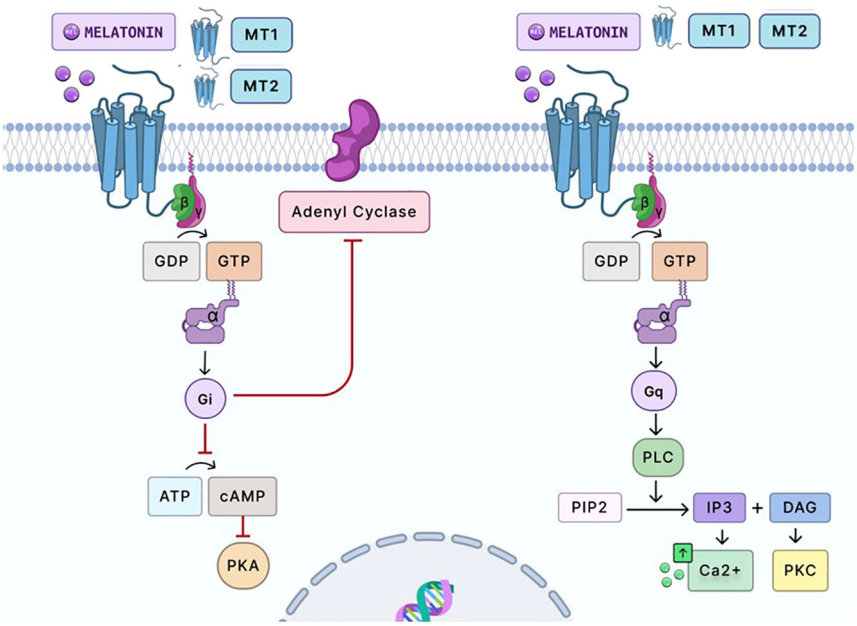


FIGURE 3
Signaling pathway of melatonin via G-protein-coupled receptors. Melatonin primarily signals through two G-protein-coupled receptors, MT1 and MT2. Upon melatonin binding, the MT1 homodimer receptor and MT2 homodimer receptor couples with Gi proteins, inhibiting adenylate cyclase. This results in decreased cAMP production and subsequent reduced activation of PKA. MT1 and MT2 heterodimer activation can also couple with Gq/11 proteins to stimulate PLC. This enzyme catalyzes the conversion of PIP2 into IP3 and DAG. IP3 subsequently promotes calcium release from intracellular stores, elevating intracellular calcium levels. Simultaneously, DAG and calcium can activate PKC, which influences a broad spectrum of cellular activities. PKC modulates numerous cellular processes by phosphorylating target proteins. The heterodimerization of MT1 and MT2 in the placenta has yet to be studied. However, the binding of melatonin on the MT1 homodimer and MT2 homodimer has been shown to elicit antioxidant and anti-inflammatory responses in placental cells by reducing oxidative stress and inflammation. Moreover, activating these receptors in these cells indicates autocrine and paracrine activity, imperative to placental homeostasis and fetal development. Gi and Gq, G-proteins; GDP and GTP, guanosine diphosphate and guanosine triphosphate, respectively; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; Ca^{2+}, calcium ions; PKC, protein kinase C. Created with Biorender.com.

$CD56^{dim}$ and $CD56^{bright}$ comprise most of the leukocyte population in the decidua (91). Decidual cells activate thymocytes, macrophages, neutrophils, and NK cells (34, 92). At the same time, prostaglandins aid in uterine contractions and amplify TNF-α and IL-6 release (93). Alterations in NK cells, T regulatory cells (Tregs), Th17, and Th1/Th2 ratio may be associated with pregnancy-related complications, pregnancy loss, implantation failure, and preeclampsia (92). Melatonin affects the T cell subpopulation in gestation by increasing the differentiation of Th17 and Tregs (94, 95). Melatonin rhythm may be synchronized with Th1/Th2 cell populations in protecting and maintaining fetal survival (96).

Melatonin levels have a significant role in immunity during gestation by enhancing innate and cellular immunity. In the first trimester of pregnancy, macrophages are the second most abundant leukocyte population in the decidual layer of the placenta (33). They have the prominent role of sensing pathogens and immune effector cells, which suggests a central role in the inflammatory response of both placental and decidual infections (97). Melatonin activates the progenitor cell production of macrophages, NK cells, and granulocytes (98). Furthermore, melatonin promotes immune homeostasis during late and early pregnancy (34, 97). It can modify many transcription factors in macrophages, including hypoxia-inducible factor (HIF-1 α), nuclear factor-k-B binding (NF-kB), and interferon regulatory factors (IRFs) (99). In addition, melatonin inhibits cytokine expression in cells treated with lipopolysaccharide (LPS) (100).

4.4 Role of melatonin as an antioxidant and anti-inflammatory in the placenta during oxidative stress

Melatonin has a wide array of functions due to its ubiquitous nature. In reproductive physiology, melatonin exerts endocrine, autocrine, intracrine, and paracrine effects in the placenta (1, 8, 101). Melatonin can pass through the placental barrier and enter the fetal circulation to promote fetal growth and entrain the circadian rhythm of the fetus (34, 69). During pregnancy, melatonin levels increase throughout gestation and peak at term, promoting placental trophoblast cell survival and homeostasis and regulating hormonal production (102). Besides being a circadian-regulating hormone, melatonin is a powerful antioxidant and anti-inflammatory that can offset the effects of free radicals and scavenge ROS (103, 104). Melatonin can act directly on the free radicals or indirectly by stimulating antioxidant enzymes through its receptors (103, 105). Melatonin is both hydrophilic and lipophilic, which means that it can

maneuver between cellular compartments easily and has access to ROS produced by the mitochondria (106, 107). In the presence of oxidative stress, melatonin can protect placental trophoblast cells against damage (71, 108). Melatonin decreases ROS levels by neutralizing and increasing the antioxidant enzymes' (superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase) expression and activity (104, 109, 110). Moreover, melatonin can protect trophoblasts from apoptosis when oxidative stress occurs (108, 111), preventing pregnancy complications. In immunocompromised pregnancies that have rendered pregnancy complications, a supraphysiological dosage of melatonin has been shown to reverse the adverse effects of these complications for both the mother and the fetus (2, 34, 86).

Dysfunction of the placenta and oxidative stress contribute to unfavorable conditions in pregnancy, such as pre-eclampsia and preterm birth (2, 4, 24, 50, 112). Preterm birth is characterized as parturition that occurs before 37 weeks of gestation and is commonly associated with intrauterine infection and inflammation (2, 3, 113). A recent study found that melatonin treatment offsets placental inflammation in offspring exposed to LPS-induced maternal inflammation (84). Preeclampsia occurs after 20 weeks of pregnancy and is associated with hypertension and proteinuria, which induces oxidative stress in placental cells (3, 5, 114, 115). A study noted reduced serum melatonin levels and MT1 receptors in preeclamptic women (116). This reduction may have been due to the decreased placental secretion of melatonin induced by preeclampsia (116). Moreover, another study by Sagrillo-Fagundes and his group found that melatonin significantly regulated autophagy and inflammation in primary trophoblast cells that were exposed to a hypoxic/reoxygenation environment, mimicking preeclamptic pregnancy, thus reducing apoptosis in these cells. This study suggests that the placenta's endogenous melatonin production is inhibited during preeclampsia, thus limiting melatonin in maternal blood and the placenta (111). Additionally, melatonin has been shown to reverse other complications during pregnancy and maintain a healthy pregnancy. In a study in mice, melatonin improved the efficiency of the placenta by increasing the expression of antioxidant enzymes in undernourished pregnancies (117). In a similar study, gestating sows were supplemented with melatonin, which indicated that melatonin could improve the redox status of the mother, fetus, and placenta and amplify the placental growth and function. This may be primarily due to melatonin's ability to enhance the placenta's antioxidant status and inflammatory response and activation (118). In all of these studies, melatonin had the potential benefit of acting as a preventative therapeutic agent for preterm birth, preeclampsia, and other pregnancy complications (84, 101, 116). In future studies, it will be essential to understand how melatonin uses its anti-inflammatory and antioxidant properties to target inflammation and oxidative stress in the placenta.

The induction of oxidative stress and inflammation at the maternal–fetal interface leads to activation of the NLRP3 inflammasome (119–121). The NLRP3 inflammasome complex is an intermediary in innate immune signaling that contributes to the pathogenesis of several diseases (122). Therefore, inhibition of the NLRP3 inflammasome pathway is a potential therapeutic target for inflammatory-related disorders (120, 122). The formation of the NLRP3 inflammasome is triggered by both internal and external factors, such as pathogen-associated molecular patterns (PAMPs), danger/damage-associated molecular patterns (DAMPs), and ROS (121, 123). An inducer of NLRP3 activation is the disassociation of thioredoxin-interacting protein (TXNIP), an intracellular redox regulator that inhibits antioxidants, from TRX (thioredoxin), a redox protein, via ROS or oxidative stress (122, 124). TXNIP and TRX form a complex with a redox relationship, which neutralizes the inhibitory properties of TXNIP, such as over-accumulation of ROS. After TXNIP disassociates and binds to NLRP3, it activates inflammasome (122, 124). The activation of the inflammasome complex entails two consecutive signals, namely (1): the priming signal is established by the activation of TLR receptors (PAMPs) through (for example) LPS, which leads to the secretion of inflammatory cytokines by the nuclear translocation of NF-kb (122, 123, 125, 126) and (2) the activation signal occurs due to the stimulation and binding of several factors that the complex recognizes, such as ROS and TXNIP/TRX disassociation, leading to the generation of caspase-1 (122, 123, 125, 126). Melatonin induces the inhibitory function of the NLRP3 inflammasome by either inhibiting or activating several different proteins and pathways (122). One of the several proteins and pathways melatonin uses to inhibit NLRP3 activation is NF-kb signaling, a master regulator of the priming phase of NLRP3 inflammasome activation (34). Melatonin also reduces TXNIP, which leads to the suppression of ROS and NLRP3 (122). Lastly, melatonin upregulates s Nrf2, an antioxidant protein that promotes ROS scavenging and elimination to facilitate protection against NLRP3 inflammasome activity through ROS scavenging and elimination mediated by Nrf2 (102).

5 Viral infections during pregnancy

Inflammation at the maternal–fetal interface puts the developing fetus at risk for IUGR (127). Placental inflammation can induce oxidative stress, resulting in an overproduction of free radicals/ROS and reduced antioxidant ability (Figure 4) (113). Furthermore, when a mother either contracts a viral infection during pregnancy or acquires a viral infection before pregnancy, this can cause an inflammatory cascade at the maternal–fetal interface (115, 127–129). Some viral infections can cross the placental barrier and influence viral-mediated inflammatory oxidative stress on placental trophoblast cells (127, 130). These viruses include human cytomegalovirus, HIV, herpes simplex virus, and Zika virus (115, 127, 130). These viruses can transmit vertically to the fetus (131, 132). Viral activation in pregnancy is associated with releasing inflammatory chemokines and cytokines in the placenta, enhancing ROS generation in immune and trophoblast cells and resulting in oxidative stress (Figure 4). Viral activation can also cause the syncytiotrophoblast cells to be more susceptible to apoptosis at a much faster rate (26).

The circadian rhythm and CLOCK genes are essential in regulating the reproductive system (73, 133) and parturition (56). Misalignment of this rhythm (chronodisruption) can adversely affect reproductive function and birthing outcomes (73). A potential link between disruption of the circadian rhythm and

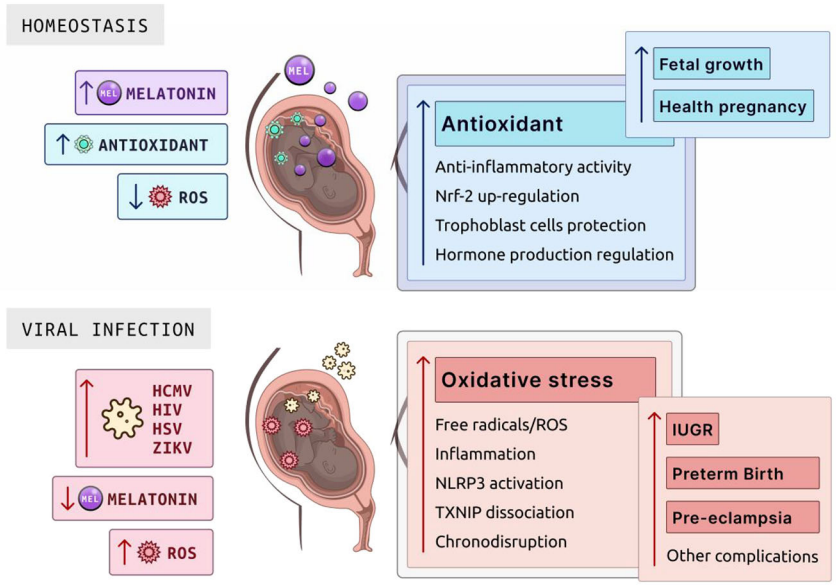


FIGURE 4
Role of melatonin in homeostasis and viral infection during pregnancy. Melatonin is pivotal in maintaining cellular homeostasis due to its antioxidant properties, which neutralize ROS. In the placenta, melatonin supports a healthy pregnancy through its antioxidant activity, anti-inflammatory actions, Nrf-2 upregulation to enhance cellular defense mechanisms, trophoblast cell protection essential for placental and fetal development, and hormone production regulation to balance the hormonal environment for fetal growth. In the context of viral infections such as HCMV, HIV, HSV, and ZIKV, melatonin levels drop significantly, leading to an increase in ROS. This surge in oxidative stress can cause complications, including free radicals/ROS production, NLRP3 activation, inflammation, TXNIP dissociation affecting cellular stress responses, and chronodisruption, which together elevate the risks of IUGR, preterm birth, pre-eclampsia, and other potential complications. ROS, reactive oxygen species; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; ZIKV, Zika virus; IUGR, intrauterine growth restriction; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; TXNIP, thioredoxin-interacting protein. Created with Biorender.com.

viral infections suppressing the immune system has been suggested by several investigators (113, 115, 134, 135). Viruses can rework the biological processes of the infected host to accelerate replication in the body. An interchange between host immunity, virus, and biological clock may influence disease outcomes (134)—the mechanism of this in the placenta has yet to be widely studied. However, evidence demonstrates how viral-induced inflammation can incite chronodisruption to inhibit melatonin and induce oxidative stress and inflammation in the placenta (2, 51, 71, 113, 115, 128, 136–138). A therapeutic strategy with melatonin during pregnancy may be plausible to increase physiological melatonin depletion due to chronodisruption and viral infection.

Melatonin's pro-apoptotic, antioxidant, and anti-inflammatory properties can alleviate inflammatory-mediated oxidative stress by neutralizing and scavenging ROS. However, the clinical capabilities of melatonin to relieve inflammatory oxidative stress caused by a viral infection in the placenta have yet to be elucidated as a therapeutic. In a healthy pregnancy, there is a high concentration of serum melatonin in maternal blood and the placenta (9). Melatonin can alleviate oxidative stress by keeping a homeostatic balance of ROS and antioxidants at the maternal–fetal dyad (Figure 4) (9, 51, 138). The placenta is a crucial source of oxidative stress, and this homeostatic imbalance can lead to inflammatory disorders like preterm birth and preeclampsia (3, 49–51). Furthermore, melatonin can use its anti-inflammatory properties to directly target inflammatory pathways within the innate immune system to reduce inflammation and oxidative stress. Examples of inflammatory pathways include the TLR/NLRP3 pathway, where melatonin upregulates Nrf2 to inhibit NF-kb signaling, inhibiting NLRP3 and cytokine release (139, 140).

6 Conclusion

This article has reviewed melatonin's beneficial properties as an antioxidant and anti-inflammatory in oxidative stress and inflammation in the placenta. Melatonin can be a potential novel therapeutic for oxidative stress-induced pregnancy complications like preeclampsia and preterm birth. More clinical studies are needed to analyze and observe melatonin in pregnant women and characterize its impact on placental cells in potentially reducing inflammatory-mediated oxidative stress.

6.1 Future directions

Although there is no strong evidence currently to corroborate melatonin as a powerful antioxidant and anti-inflammatory in the placenta during oxidative stress, inflammation, and viral infection, future work should address whether melatonin's antioxidant properties in the placenta are due to direct scavenging of ROS (supraphysiological) or receptor-mediated (physiological). Moreover, future work should address whether melatonin increases Nrf2 to deactivate the NLRP3 inflammasome during a viral infection

at the maternal–fetal interface. Lastly, potential studies should determine safe and effective dosages that can be given to pregnant women during adverse pregnancy events like preeclampsia.

Author contributions

TJ: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization. VS: Visualization, Writing – review & editing. DH: Writing – review & editing. RC: Writing – review & editing. EJ: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by R01HD97843 and R01MD017690.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Chen YC, Sheen JM, Tiao MM, Tain YL, Huang LT. Roles of melatonin in fetal programming in compromised pregnancies. *Int J Mol Sci* (2013) 14(3):5380–401. doi: 10.3390/ijms14035380

2. Mark PJ, Crew RC, Wharfe MD, Waddell BJ. Rhythmic three-part harmony: the complex interaction of maternal, placental and fetal circadian systems. *J Biol Rhythms* (2017) 32(6):534–49. doi: 10.1177/0748730417728671

3. Moore TA, Ahmad IM, Zimmerman MC. Oxidative stress and preterm birth: an integrative review. *Biol Res Nurs* (2018) 20(5):497–512. doi: 10.1177/1099800418791028

4. Myatt L, Cui X. Oxidative stress in the placenta. *Histochem Cell Biol* (2004) 122 (4):369–82. doi: 10.1007/s00418-004-0677-x

5. Hansson SR, Naav A, Erlandsson L. Oxidative stress in preeclampsia and the role of free fetal hemoglobin. *Front Physiol* (2014) 5:516. doi: 10.3390/ijms19051496

6. Gonzalez-Candia A, Veliz M, Carrasco-Pozo C, Castillo RL, Cardenas JC, Ebensperger G, et al. Antenatal melatonin modulates an enhanced antioxidant/pro-oxidant ratio in pulmonary hypertensive newborn sheep. *Redox Biol* (2019) 22:101128. doi: 10.1016/j.redox.2019.101128

7. Langston-Cox A, Marshall SA, Lu D, Palmer KR, Wallace EM. Melatonin for the management of preeclampsia: A review. *Antioxidants (Basel)* (2021) 10(3):376. doi: 10.3390/antiox10030376

8. Voiculescu SE, Zygouropoulos N, Zahiu CD, Zagrean AM. Role of melatonin in embryo fetal development. *J Med Life* (2014) 7(4):488–92.

9. Ejaz H, Figaro JK, Woolner AMF, Thottakam BMV, Galley HF. Maternal serum melatonin increases during pregnancy and falls immediately after delivery implicating the placenta as a major source of melatonin. *Front Endocrinol (Lausanne)* (2020) 11:623038. doi: 10.3389/fendo.2020.623038

10. Kivela A. Serum melatonin during human pregnancy. *Acta Endocrinol (Copenh)* (1991) 124(3):233–7. doi: 10.1530/acta.0.1240233

11. Aversa S, Pellegrino S, Barberi I, Reiter RJ, Gitto E. Potential utility of melatonin as an antioxidant during pregnancy and in the perinatal period. *J Matern Fetal Neonatal Med* (2012) 25(3):207–21. doi: 10.3109/14767058.2011.573827

12. Reiter RJ, Tan DX, Manchester LC, Paredes SD, Mayo JC, Sainz RM. Melatonin and reproduction revisited. *Biol Reprod* (2009) 81(3):445–56. doi: 10.1095/biolreprod.108.075655

13. Tamura H, Nakamura Y, Terron MP, Flores LJ, Manchester LC, Tan DX, et al. Melatonin and pregnancy in the human. *Reprod Toxicol* (2008) 25(3):291–303. doi: 10.1016/j.reprotox.2008.03.005

14. Herrick EJ, Bordoni B. *Embryology, placenta*. Treasure Island (FL: StatPearls (2023).

15. Turco MY, Moffett A. Development of the human placenta. *Development* (2019) 146(22). doi: 10.1242/dev.163428

16. John R, Hemberger M. A placenta for life. *Reprod BioMed Online* (2012) 25 (1):5–11. doi: 10.1016/j.rbmo.2012.03.018

17. Herrick EJ, Bordoni B. *Embryology, placenta*. Treasure Island (FL: StatPearls (2022).

18. Remien K, Majmundar SH. *Physiology, fetal circulation*. Treasure Island (FL: StatPearls (2023).

19. Tal R, Taylor H, Feingold KR, Anawalt B, Blackman MR, Bryce A. Endocrinology of pregnancy. In: Feingold KR, Anawalt B, Blackman MR, Boyce A, Chrousos G, Corpas E, editors. *Endotext*. South Dartmouth (MA), MDText.com (2000).

20. Ortega MA, Fraile-Martinez O, Garcia-Montero C, Saez MA, Alvarez-Mon MA, Torres-Carranza D, et al. The pivotal role of the placenta in normal and pathological pregnancies: A focus on preeclampsia, fetal growth restriction, and maternal chronic venous disease. *Cells* (2022) 11(3):568. doi: 10.3390/cells11030568

21. Mor G, Cardenas I. The immune system in pregnancy: a unique complexity. *Am J Reprod Immunol* (2010) 63(6):425–33. doi: 10.1111/j.1600-0897.2010.00836.x

22. Kapila V, Chaudhry K. *Physiology, placenta*. Treasure Island (FL: StatPearls (2023).

23. Gauster M, Moser G, Wernitznig S, Kupper N, Huppertz B. Early human trophoblast development: from morphology to function. *Cell Mol Life Sci* (2022) 79 (6):345. doi: 10.1007/s00018-022-04377-0

24. Sharp AN, Heazell AE, Crocker IP, Mor G. Placental apoptosis in health and disease. *Am J Reprod Immunol* (2010) 64(3):159–69. doi: 10.1111/j.1600-0897.2010.00837.x

25. Sagrillo-Fagundes L, Clabault H, Laurent L, Hudon-Thibeault AA, Salustiano EM, Fortier M, et al. Human primary trophoblast cell culture model to study the protective effects of melatonin against hypoxia/reoxygenation-induced disruption. *J Vis Exp* (2016) 113:54228. doi: 10.3791/54228

26. Straszewski-Chavez SL, Abrahams VM, Mor G. The role of apoptosis in the regulation of trophoblast survival and differentiation during pregnancy. *Endocr Rev* (2005) 26(7):877–97. doi: 10.1210/er.2005-0003

27. Woods L, Perez-Garcia V, Hemberger M. Regulation of placental development and its impact on fetal growth-new insights from mouse models. *Front Endocrinol (Lausanne)* (2018) 9:570. doi: 10.3389/fendo.2018.00570

28. Mori M, Bogdan A, Balassa T, Csabai T, Szekeres-Bartho J. The decidua-the maternal bed embracing the embryo-maintains the pregnancy. *Semin Immunopathol* (2016) 38(6):635–49. doi: 10.1007/s00281-016-0574-0

29. Solders M, Gorchs L, Gidlof S, Tiblad E, Lundell AC, Kaipe H. Maternal adaptive immune cells in decidua parietalis display a more activated and coinhibitory phenotype compared to decidua basalis. *Stem Cells Int* (2017) 2017:8010961. doi: 10.1155/2017/8010961

30. Holder B, Aplin JD, Gomez-Lopez N, Heazell AEP, James JL, Jones CJP, et al. 'Fetal side' of the placenta: anatomical mis-annotation of carbon particle 'transfer' across the human placenta. *Nat Commun* (2021) 12(1):7049. doi: 10.1038/s41467-021-26437-y

31. Than NG, Hahn S, Rossi SW, Szekeres-Bartho J. Editorial: fetal-maternal immune interactions in pregnancy. *Front Immunol* (2019) 10:2729. doi: 10.3389/fimmu.2019.02729

32. Balasundaram P, Farhana A. *Immunology at the maternal-fetal interface*. Treasure Island (FL: StatPearls (2023).

33. Yang F, Zheng Q, Jin L. Dynamic function and composition changes of immune cells during normal and pathological pregnancy at the maternal-fetal interface. *Front Immunol* (2019) 10:2317. doi: 10.3389/fimmu.2019.02317

34. Chuffa LGA, Lupi LA, Cucielo MS, Silveira HS, Reiter RJ, Seiva FRF. Melatonin promotes uterine and placental health: potential molecular mechanisms. *Int J Mol Sci* (2019) 21(1):300. doi: 10.3390/ijms21010300

35. Faas MM, De Vos P. Innate immune cells in the placental bed in healthy pregnancy and preeclampsia. *Placenta* (2018) 69:125–33. doi: 10.1016/j.placenta.2018.04.012

36. Tuuli MG, Longtine MS, Nelson DM. Review: Oxygen and trophoblast biology–a source of controversy. *Placenta* (2011) 32 Suppl 2(0 2):S109–18. doi: 10.1016/j.placenta.2010.12.013

37. Schoots MH, Gordijn SJ, Scherjon SA, van Goor H, Hillebrands JL. Oxidative stress in placental pathology. *Placenta* (2018) 69:153–61. doi: 10.1016/j.placenta.2018.03.003

38. Nolfi-Donegan D, Braganza A, Shiva S. Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox Biol* (2020) 37:101674. doi: 10.1016/j.redox.2020.101674

39. Abdal Dayem A, Hossain MK, Lee SB, Kim K, Saha SK, Yang GM, et al. The role of reactive oxygen species (ROS) in the biological activities of metallic nanoparticles. *Int J Mol Sci* (2017) 18(1):120. doi: 10.3390/ijms18010120

40. Su LJ, Zhang JH, Gomez H, Murugan R, Hong X, Xu D, et al. Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. *Oxid Med Cell Longev* (2019) 2019:5080843. doi: 10.1155/2019/5080843

41. Juan CA, Perez de la Lastra JM, Plou FJ, Perez-Lebena E. The chemistry of reactive oxygen species (ROS) revisited: outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *Int J Mol Sci* (2021) 22(9):4642. doi: 10.3390/ijms22094642

42. Burton GJ, Jauniaux E. Oxidative stress. *Best Pract Res Clin Obstet Gynaecol* (2011) 25(3):287–99. doi: 10.1016/j.bpobgyn.2010.10.016

43. Burton GJ, Jauniaux E, Watson AL. Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. *Am J Obstet Gynecol* (1999) 181(3):718–24. doi: 10.1016/S0002-9378(99)70518-1

44. Jauniaux E, Jurkovic D, Campbell S. *In vivo* investigations of the anatomy and the physiology of early human placental circulations. *Ultrasound Obstet Gynecol* (1991) 1(6):435–45. doi: 10.1046/j.1469-0705.1991.01060435.x

45. Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* (1992) 80(2):283–5.

46. Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *Am J Pathol* (2000) 157(6):2111–22. doi: 10.1016/S0002-9440(10)64849-3

47. Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. *J Soc Gynecol Investig* (2004) 11(6):342–52. doi: 10.1016/j.jsgi.2004.03.003

48. Carter AM. Placental oxygen consumption. Part I: in *vivo* studies–a review. *Placenta* (2000) 21 Suppl A:S31–7. doi: 10.1053/plac.1999.0513

49. Marseglia L, D'Angelo G, Manti S, Arrigo T, Barberi I, Reiter RJ, et al. Oxidative stress-mediated aging during the fetal and perinatal periods. *Oxid Med Cell Longev* (2014) 2014:358375. doi: 10.1155/2014/358375

50. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* (2005) 3:28. doi: 10.1186/1477-7827-3-28

51. Lanoix D, Lacasse AA, Reiter RJ, Vaillancourt C. Melatonin: the watchdog of villous trophoblast homeostasis against hypoxia/reoxygenation-induced oxidative stress and apoptosis. *Mol Cell Endocrinol* (2013) 381(1-2):35–45. doi: 10.1016/j.mce.2013.07.010

52. Wu F, Tian FJ, Lin Y. Oxidative stress in placenta: health and diseases. *BioMed Res Int* (2015) 2015:293271. doi: 10.1155/2015/293271

53. Burton GJ, Fowden AL. The placenta: a multifaceted, transient organ. *Philos Trans R Soc Lond B Biol Sci* (2015) 370(1663):20140066. doi: 10.1098/rstb.2014.0066

54. Wu F, Tian FJ, Lin Y, Xu WM. Oxidative stress: placenta function and dysfunction. *Am J Reprod Immunol* (2016) 76(4):258–71. doi: 10.1111/aji.12454

55. Schibler U. The daily rhythms of genes, cells and organs. Biological clocks and circadian timing in cells. *EMBO Rep* (2005) 6 Spec No(Suppl 1):S9–13. doi: 10.1038/sj.embor.7400424

56. Reschke L, McCarthy R, Herzog ED, Fay JC, Jungheim ES, England SK. Chronodisruption: An untimely cause of preterm birth? *Best Pract Res Clin Obstet Gynaecol* (2018) 52:60–7. doi: 10.1016/j.bpobgyn.2018.08.001

57. Albrecht U. The mammalian circadian clock: a network of gene expression. *Front Biosci* (2004) 9:48–55. doi: 10.2741/1196

58. Hastings MH, Reddy AB, Garabette M, King VM, Chahad-Ehlers S, O'Brien J, et al. Expression of clock gene products in the suprachiasmatic nucleus in relation to circadian behaviour. *Novartis Found Symp* (2003) 253:203–17. doi: 10.1002/0470090839.ch15

59. Bates K, Herzog ED. Maternal-fetal circadian communication during pregnancy. *Front Endocrinol (Lausanne)* (2020) 11:198. doi: 10.3389/fendo.2020.00198

60. Valenzuela FJ, Vera J, Venegas C, Pino F, Lagunas C. Circadian system and melatonin hormone: risk factors for complications during pregnancy. *Obstet Gynecol Int* (2015) 2015:825802. doi: 10.1155/2015/825802

61. Hsu CN, Tain YL. Light and circadian signaling pathway in pregnancy: programming of adult health and disease. *Int J Mol Sci* (2020) 21(6):2232. doi: 10.3390/ijms21062232

62. Guillaumond F, Dardente H, Giguere V, Cermakian N. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* (2005) 20(5):391–403. doi: 10.1177/0748730405277232

63. Akiyama S, Ohta H, Watanabe S, Moriya T, Hariu A, Nakahata N, et al. The uterus sustains stable biological clock during pregnancy. *Tohoku J Exp Med* (2010) 221(4):287–98. doi: 10.1620/tjem.221.287

64. Seron-Ferre M, Valenzuela GJ, Torres-Farfan C. Circadian clocks during embryonic and fetal development. *Birth Defects Res C Embryo Today* (2007) 81(3):204–14. doi: 10.1002/bdrc.20101

65. Reiter RJ, Tan DX, Korkmaz A, Rosales-Corral SA. Melatonin and stable circadian rhythms optimize maternal, placental and fetal physiology. *Hum Reprod Update* (2014) 20(2):293–307. doi: 10.1093/humupd/dmt054

66. Tarocco A, Caroccia N, Morciano G, Wieckowski MR, Ancora G, Garani G, et al. Melatonin as a master regulator of cell death and inflammation: molecular mechanisms and clinical implications for newborn care. *Cell Death Dis* (2019) 10(4):317. doi: 10.1038/s41419-019-1556-7

67. Vriend J, Reiter RJ. Melatonin feedback on clock genes: a theory involving the proteasome. *J Pineal Res* (2015) 58(1):1–11. doi: 10.1111/jpi.12189

68. Jung-Hynes B, Reiter RJ, Ahmad N. Sirtuins, melatonin and circadian rhythms: building a bridge between aging and cancer. *J Pineal Res* (2010) 48(1):9–19. doi: 10.1111/j.1600-079X.2009.00729.x

69. Brzezinski A, Rai S, Purohit A, Pandi-Perumal SR. Melatonin, clock genes, and mammalian reproduction: what is the link? *Int J Mol Sci* (2021) 22(24):13240. doi: 10.3390/ijms222413240

70. Sagrillo-Fagundes L, Soliman A, Vaillancourt C. Maternal and placental melatonin: actions and implication for successful pregnancies. *Minerva Ginecol* (2014) 66(3):251–66.

71. Soliman A, Lacasse AA, Lanoix D, Sagrillo-Fagundes L, Boulard V, Vaillancourt C. Placental melatonin system is present throughout pregnancy and regulates villous trophoblast differentiation. *J Pineal Res* (2015) 59(1):38–46. doi: 10.1111/jpi.12236

72. Lanoix D, Beghdadi H, Lafond J, Vaillancourt C. Human placental trophoblasts synthesize melatonin and express its receptors. *J Pineal Res* (2008) 45(1):50–60. doi: 10.1111/j.1600-079X.2008.00555.x

73. Sati L. Chronodisruption: effects on reproduction, transgenerational health of offspring and epigenome. *Reproduction* (2020) 160(5):R79–94. doi: 10.1530/REP-20-0298

74. Reiter RJ, Tan DX, Korkmaz A, Erren TC, Piekarski C, Tamura H, et al. Light at night, chronodisruption, melatonin suppression, and cancer risk: a review. *Crit Rev Oncog.* (2007) 13(4):303–28. doi: 10.1615/CritRevOncog.v13.i4.30

75. Reiter RJ, Rosales-Corral S, Coto-Montes A, Boga JA, Tan DX, Davis JM, et al. The photoperiod, circadian regulation and chronodisruption: the requisite interplay between the suprachiasmatic nuclei and the pineal and gut melatonin. *J Physiol Pharmacol* (2011) 62(3):269–74.

76. Li DY, Smith DG, Hardeland R, Yang MY, Xu HL, Zhang L, et al. Melatonin receptor genes in vertebrates. *Int J Mol Sci* (2013) 14(6):11208–23. doi: 10.3390/ijms140611208

77. Liu J, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. MT1 and MT2 melatonin receptors: A therapeutic perspective. *Annu Rev Pharmacol Toxicol* (2016) 56:361–83. doi: 10.1146/annurev-pharmtox-010814-124742

78. Reppert SM, Weaver DR, Godson C. Melatonin receptors step into the light: cloning and classification of subtypes. *Trends Pharmacol Sci* (1996) 17(3):100–2. doi: 10.1016/0165-6147(96)10005-5

79. Rehman S, Rahimi N, Dimri M. *Biochemistry, G Protein Coupled Receptors* Treasure Island (FL: StatPearls (2023).

80. Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. *Pharmacol Rev* (2010) 62(3):343–80. doi: 10.1124/pr.110.002832

81. Ekmekcioglu C. Melatonin receptors in humans: biological role and clinical relevance. *BioMed Pharmacother* (2006) 60(3):97–108. doi: 10.1016/j.biopha.2006.01.002

82. Lanoix D, Guerin P, Vaillancourt C. Placental melatonin production and melatonin receptor expression are altered in preeclampsia: new insights into the role of this hormone in pregnancy. *J Pineal Res* (2012) 53(4):417–25. doi: 10.1111/j.1600-079X.2012.01012.x

83. Lanoix D, Ouellette R, Vaillancourt C. Expression of melatoninergic receptors in human placental choriocarcinoma cell lines. *Hum Reprod* (2006) 21(8):1981–9. doi: 10.1093/humrep/del120

84. Lee JY, Song H, Dash O, Park M, Shin NE, McLane MW, et al. Administration of melatonin for prevention of preterm birth and fetal brain injury associated with premature birth in a mouse model. *Am J Reprod Immunol* (2019) 82(3):e13151. doi: 10.1111/aji.13151

85. Uzun M, Gencer M, Turkon H, Oztopuz RO, Demir U, Ovali MA. Effects of melatonin on blood pressure, oxidative stress and placental expressions of TNFalpha, IL-6, VEGF and sFlt-1 in RUPP rat model of preeclampsia. *Arch Med Res* (2017) 48 (7):592–8. doi: 10.1016/j.arcmed.2017.08.007

86. Hardeland R. Melatonin and inflammation-Story of a double-edged blade. *J Pineal Res* (2018) 65(4):e12525. doi: 10.1111/jpi.12525

87. Miller SC, Pandi-Perumal SR, Esquifino AI, Cardinali DP, Maestroni GJ. The role of melatonin in immuno-enhancement: potential application in cancer. *Int J Exp Pathol* (2006) 87(2):81–7. doi: 10.1111/j.0959-9673.2006.00474.x

88. Kuhlwein E, Irwin M. Melatonin modulation of lymphocyte proliferation and Th1/Th2 cytokine expression. *J Neuroimmunol* (2001) 117(1-2):51–7. doi: 10.1016/S0165-5728(01)00325-3

89. Paredes SD, Sanchez S, Parvez H, Rodriguez AB, Barriga C. Altered circadian rhythms of corticosterone, melatonin, and phagocytic activity in response to stress in rats. *Neuro Endocrinol Lett* (2007) 28(4):489–95.

90. Alvarez-Garcia V, Gonzalez A, Alonso-Gonzalez C, Martinez-Campa C, Cos S. Melatonin interferes in the desmoplastic reaction in breast cancer by regulating cytokine production. *J Pineal Res* (2012) 52(3):282–90. doi: 10.1111/j.1600-079X.2011.00940.x

91. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. *Tissue Antigens* (2004) 63(1):1–12. doi: 10.1111/j.1399-0039.2004.00170.x

92. Man GCW, Zhang T, Chen X, Wang J, Wu F, Liu Y, et al. The regulations and role of circadian clock and melatonin in uterine receptivity and pregnancy-An immunological perspective. *Am J Reprod Immunol* (2017) 78(2):10.1111/aji.12715. doi: 10.1111/aji.12715

93. Galazka K, Wicherek L, Pitynski K, Kijowski J, Zajac K, Bednarek W, et al. Changes in the subpopulation of CD25+ CD4+ and FOXP3+ regulatory T cells in decidua with respect to the progression of labor at term and the lack of analogical changes in the subpopulation of suppressive B7-H4 macrophages–a preliminary report. *Am J Reprod Immunol* (2009) 61(2):136–46. doi: 10.1111/j.1600-0897.2008.00674.x

94. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol* (2010) 63(6):601–10. doi: 10.1111/j.1600-0897.2010.00852.x

95. Glebezdina NS, Nekrasova IV, Olina AA, Sadykova GK, Kuklina EM. Differentiation of T cells producing interleukin-17 (Th17) against the background of exogenous melatonin during pregnancy. *J Pineal Res* (2023) 75(4):e12904. doi: 10.1111/jpi.12904

96. Szereday L, Varga P, Szekeres-Bartho J. Cytokine production by lymphocytes in pregnancy. *Am J Reprod Immunol* (1997) 38(6):418–22. doi: 10.1111/j.1600-0897.1997.tb00321.x

97. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol* (2013) 31:387–411. doi: 10.1146/annurev-immunol-032712-100003

98. Srinivasan V, Maestroni GJ, Cardinali DP, Esquifino AI, Perumal SR, Miller SC. Melatonin, immune function and aging. *Immun Ageing* (2005) 2:17. doi: 10.1186/1742-4933-2-17

99. Xia Y, Chen S, Zeng S, Zhao Y, Zhu C, Deng B, et al. Melatonin in macrophage biology: Current understanding and future perspectives. *J Pineal Res* (2019) 66(2): e12547. doi: 10.1111/jpi.12547

100. Kadena M, Kumagai Y, Vandenbon A, Matsushima H, Fukamachi H, Maruta N, et al. Microarray and gene co-expression analysis reveals that melatonin attenuates immune responses and modulates actin rearrangement in macrophages. *Biochem Biophys Res Commun* (2017) 485(2):414–20. doi: 10.1016/j.bbrc.2017.02.063

101. Dominguez Rubio AP, Sordelli MS, Salazar AI, Aisemberg J, Bariani MV, Cella M, et al. Melatonin prevents experimental preterm labor and increases offspring survival. *J Pineal Res* (2014) 56(2):154–62. doi: 10.1111/jpi.12108

102. Sagrillo-Fagundes L, Bienvenue-Pariseault J, Vaillancourt C. Melatonin: The smart molecule that differentially modulates autophagy in tumor and normal placental cells. *PloS One* (2019) 14(1):e0202458. doi: 10.1371/journal.pone.0202458

103. Chitimus DM, Popescu MR, Voiculescu SE, Panaitescu AM, Pavel B, Zagrean L, et al. Melatonin's impact on antioxidative and anti-inflammatory reprogramming in homeostasis and disease. *Biomolecules* (2020) 10(9):1211. doi: 10.3390/biom10091211

104. Galano A, Tan DX, Reiter RJ. Melatonin as a natural ally against oxidative stress: a physicochemical examination. *J Pineal Res* (2011) 51(1):1–16. doi: 10.1111/j.1600-079X.2011.00916.x

105. Reiter RJ. Melatonin: lowering the high price of free radicals. *News Physiol Sci* (2000) 15:246–50. doi: 10.1152/physiologyonline.2000.15.5.246

106. Tan DX, Manchester LC, Qin L, Reiter RJ. Melatonin: A mitochondrial targeting molecule involving mitochondrial protection and dynamics. *Int J Mol Sci* (2016) 17(12):2124. doi: 10.3390/ijms17122124

107. Loh D, Reiter RJ. Melatonin: regulation of biomolecular condensates in neurodegenerative disorders. *Antioxidants (Basel)* (2021) 10(9):1483. doi: 10.3390/antiox10091483

108. Hannan NJ, Binder NK, Beard S, Nguyen TV, Kaitu'u-Lino TJ, Tong S. Melatonin enhances antioxidant molecules in the placenta, reduces secretion of soluble fms-like tyrosine kinase 1 (sFLT) from primary trophoblast but does not rescue endothelial dysfunction: An evaluation of its potential to treat preeclampsia. *PloS One* (2018) 13(4):e0187082. doi: 10.1371/journal.pone.0187082

109. Vazquez J, Grillitsch K, Daum G, Mas A, Torija MJ, Beltran G. Melatonin minimizes the impact of oxidative stress induced by hydrogen peroxide in saccharomyces and non-conventional yeast. *Front Microbiol* (2018) 9:1933. doi: 10.3389/fmicb.2018.01933

110. Bantounou M, Plascevic J, Galley HF. Melatonin and related compounds: antioxidant and anti-inflammatory actions. *Antioxidants (Basel)* (2022) 11(3):532. doi::10.3390/antiox11030532

111. Sagrillo-Fagundes L, Assuncao Salustiano EM, Ruano R, Markus RP, Vaillancourt C. Melatonin modulates autophagy and inflammation protecting human placental trophoblast from hypoxia/reoxygenation. *J Pineal Res* (2018) 65(4): e12520. doi: 10.1111/jpi.12520

112. Aouache R, Biquard L, Vaiman D, Miralles F. Oxidative stress in preeclampsia and placental diseases. *Int J Mol Sci* (2018) 19(5):1496. doi: 10.3390/ijms19051496

113. Cappelletti M, Della Bella S, Ferrazzi E, Mavilio D, Divanovic S. Inflammation and preterm birth. *J Leukoc Biol* (2016) 99(1):67–78. doi: 10.1189/jlb.3MR0615-272RR

114. Park B, Khanam R, Vinayachandran V, Baqui AH, London SJ, Biswal S. Epigenetic biomarkers and preterm birth. *Environ Epigenet* (2020) 6(1):dvaa005. doi: 10.1093/eep/dvaa005

115. Giakoumelou S, Wheelhouse N, Cuschieri K, Entrican G, Howie SE, Horne AW. The role of infection in miscarriage. *Hum Reprod Update* (2016) 22(1):116–33. doi: 10.1093/humupd/dmv041

116. Zeng K, Gao Y, Wan J, Tong M, Lee AC, Zhao M, et al. The reduction in circulating levels of melatonin may be associated with the development of preeclampsia. *J Hum Hypertens* (2016) 30(11):666–71. doi: 10.1038/jhh.2016.37

117. Richter HG, Hansell JA, Raut S, Giussani DA. Melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy. *J Pineal Res* (2009) 46(4):357–64. doi: 10.1111/j.1600-079X.2009.00671.x

118. Peng X, Cai X, Li J, Huang Y, Liu H, He J, et al. Effects of melatonin supplementation during pregnancy on reproductive performance, maternal-placental-fetal redox status, and placental mitochondrial function in a sow model. *Antioxidants (Basel)* (2021) 10(12):1867. doi: 10.3390/antiox10121867

119. Alfian I, Chakraborty A, Yong HEJ, Saini S, Lau RWK, Kalionis B, et al. The placental NLRP3 inflammasome and its downstream targets, caspase-1 and interleukin-6, are increased in human fetal growth restriction: implications for aberrant inflammation-induced trophoblast dysfunction. *Cells* (2022) 11(9):1413. doi: 10.3390/cells11091413

120. Shirasuna K, Karasawa T, Takahashi M. Role of the NLRP3 inflammasome in preeclampsia. *Front Endocrinol (Lausanne)* (2020) 11:80. doi: 10.3389/fendo.2020.00080

121. Socha MW, Malinowski B, Puk O, Dubiel M, Wicinski M. The NLRP3 inflammasome role in the pathogenesis of pregnancy induced hypertension and preeclampsia. *Cells* (2020) 9(7):1642. doi: 10.3390/cells9071642

122. Arioz BI, Tarakcioglu E, Olcum M, Genc S. The role of melatonin on NLRP3 inflammasome activation in diseases. *Antioxidants (Basel)* (2021) 10(7):1020. doi: 10.3390/antiox10071020

123. Zhao C, Zhao W. NLRP3 inflammasome-A key player in antiviral responses. *Front Immunol* (2020) 11:211. doi: 10.3389/fimmu.2020.00211

124. Spindel ON, World C, Berk BC. Thioredoxin interacting protein: redox dependent and independent regulatory mechanisms. *Antioxid Redox Signal* (2012) 16(6):587–96. doi: 10.1089/ars.2011.4137

125. Xu J, Nunez G. The NLRP3 inflammasome: activation and regulation. *Trends Biochem Sci* (2022) 48(4):331–344. doi: 10.1016/j.tibs.2022.10.002

126. Nunes PR, Mattioli SV, Sandrim VC. NLRP3 activation and its relationship to endothelial dysfunction and oxidative stress: implications for preeclampsia and pharmacological interventions. *Cells* (2021) 10(11):2828. doi: 10.3390/cells10112828

127. Leon-Juarez M, Martinez-Castillo M, Gonzalez-Garcia LD, Helguera-Repetto AC, Zaga-Clavellina V, Garcia-Cordero J, et al. Cellular and molecular mechanisms of viral infection in the human placenta. *Pathog Dis* (2017) 75(7):ftx093. doi: 10.1093/femspd/ftx093

128. Zaga-Clavellina V, Diaz L, Olmos-Ortiz A, Godinez-Rubi M, Rojas-Mayorquin AE, Ortuno-Sahagun D. Central role of the placenta during viral infection: Immuno-competences and miRNA defensive responses. *Biochim Biophys Acta Mol Basis Dis* (2021) 1867(10):166182. doi: 10.1016/j.bbadis.2021.166182

129. Srinivas SK, Ma Y, Sammel MD, Chou D, McGrath C, Parry S, et al. Placental inflammation and viral infection are implicated in second trimester pregnancy loss. *Am J Obstet Gynecol* (2006) 195(3):797–802. doi: 10.1016/j.ajog.2006.05.049

130. Adams Waldorf KM, McAdams RM. Influence of infection during pregnancy on fetal development. *Reproduction* (2013) 146(5):R151–62. doi: 10.1530/REP-13-0232

131. Arora N, Sadovsky Y, Dermody TS, Coyne CB. Microbial vertical transmission during human pregnancy. *Cell Host Microbe* (2017) 21(5):561–7. doi: 10.1016/j.chom.2017.04.007

132. Auriti C, De Rose DU, Santisi A, Martini L, Piersigilli F, Bersani I, et al. Pregnancy and viral infections: Mechanisms of fetal damage, diagnosis and prevention of neonatal adverse outcomes from cytomegalovirus to SARS-CoV-2 and Zika virus.

Biochim Biophys Acta Mol Basis Dis (2021) 1867(10):166198. doi: 10.1016/j.bbadis.2021.166198

133. Sen A, Hoffmann HM. Role of core circadian clock genes in hormone release and target tissue sensitivity in the reproductive axis. *Mol Cell Endocrinol* (2020) 501:110655. doi: 10.1016/j.mce.2019.110655

134. Mazzoccoli G, Vinciguerra M, Carbone A, Relogio A. The circadian clock, the immune system, and viral infections: the intricate relationship between biological time and host-virus interaction. *Pathogens* (2020) 9(2):83. doi: 10.3390/pathogens9020083

135. Zhuang X, Rambhatla SB, Lai AG, McKeating JA. Interplay between circadian clock and viral infection. *J Mol Med (Berl)* (2017) 95(12):1283–9. doi: 10.1007/s00109-017-1592-7

136. Borrmann H, McKeating JA, Zhuang X. The circadian clock and viral infections. *J Biol Rhythms* (2021) 36(1):9–22. doi: 10.1177/0748730420967768

137. Rijo-Ferreira F, Takahashi JS. Circadian rhythms in infectious diseases and symbiosis. *Semin Cell Dev Biol* (2022) 126:37–44. doi: 10.1016/j.semcdb.2021.09.004

138. Morvaridzadeh M, Sadeghi E, Agah S, Nachvak SM, Fazelian S, Moradi F, et al. Effect of melatonin supplementation on oxidative stress parameters: A systematic review and meta-analysis. *Pharmacol Res* (2020) 161:105210. doi: 10.1016/j.phrs.2020.105210

139. Ahmadi Z, Ashrafizadeh M. Melatonin as a potential modulator of Nrf2. *Fundam Clin Pharmacol* (2020) 34(1):11–9. doi: 10.1111/fcp.12498

140. Bona S, Fernandes SA, Moreira ACJ, Rodrigues G, Schemitt EG, Di Naso FC, et al. Melatonin restores zinc levels, activates the Keap1/Nrf2 pathway, and modulates endoplasmic reticular stress and HSP in rats with chronic hepatotoxicity. *World J Gastrointest Pharmacol Ther* (2022) 13(2):11–22. doi: 10.4292/wjgpt.v13.i2.11

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 23 February 2024
DOI 10.3389/fimmu.2024.1351898

Check for updates

OPEN ACCESS

EDITED BY
Mahmood Jeddi-Tehrani,
Culture and Research, Iran

REVIEWED BY
Nathalie Ledee,
Hôpital Cochin, France
A. Inkeri Lokki,
University of Helsinki, Finland

*CORRESPONDENCE
Uday Kishore
ukishore@hotmail.com;
uday.kishore@uaeu.ac.ae
Roberta Bulla
rbulla@units.it

†These authors have contributed equally to this work and share first authorship

RECEIVED 07 December 2023
ACCEPTED 30 January 2024
PUBLISHED 23 February 2024

CITATION
Yasmin H, Agostinis C, Toffoli M, Roy T, Pegoraro S, Balduit A, Zito G, Di Simone N, Ricci G, Madan T, Kishore U and Bulla R (2024) Protective role of complement factor H against the development of preeclampsia. *Front. Immunol.* 15:1351898. doi: 10.3389/fimmu.2024.1351898

COPYRIGHT
© 2024 Yasmin, Agostinis, Toffoli, Roy, Pegoraro, Balduit, Zito, Di Simone, Ricci, Madan, Kishore and Bulla. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Protective role of complement factor H against the development of preeclampsia

Hadida Yasmin[1†], Chiara Agostinis[2†], Miriam Toffoli[3], Tamali Roy[1], Silvia Pegoraro[2], Andrea Balduit[2], Gabriella Zito[2], Nicoletta Di Simone[4,5], Giuseppe Ricci[2,3], Taruna Madan[6], Uday Kishore[7,8*] and Roberta Bulla[9*]

[1]Immunology and Cell Biology Laboratory, Department of Zoology, Cooch Behar Panchanan Barma University, Cooch Behar, West Bengal, India, [2]Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy, [3]Department of Medical, Surgical and Health Science, University of Trieste, Trieste, Italy, [4]Department of Biomedical Sciences, Humanitas University, Milan, Italy, [5]Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Humanitas Research Hospital, Milan, Italy, [6]Department of Innate Immunity, ICMR-National Institute for Research in Reproductive and Child Health (NIRRCH), Mumbai, India, [7]Department of Veterinary Medicine, U.A.E. University, Al Ain, United Arab Emirates, [8]Zayed Centre for Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates, [9]Department of Life Sciences, University of Trieste, Trieste, Italy

Pregnancy is an immunologically regulated, complex process. A tightly controlled complement system plays a crucial role in the successful establishment of pregnancy and parturition. Complement inhibitors at the feto-maternal interface are likely to prevent inappropriate complement activation to protect the fetus. In the present study, we aimed to understand the role of Factor H (FH), a negative regulator of complement activation, in normal pregnancy and in a model of pathological pregnancy, *i.e.* preeclampsia (PE). The distribution and expression of FH was investigated in placental tissues, various placental cells, and in the sera of healthy (CTRL) or PE pregnant women *via* immunohistochemistry, RT-qPCR, ELISA, and Western blot. Our results showed a differential expression of FH among the placental cell types, decidual stromal cells (DSCs), decidual endothelial cells (DECs), and extravillous trophoblasts (EVTs). Interestingly, FH was found to be considerably less expressed in the placental tissues of PE patients compared to normal placental tissue both at mRNA and protein levels. Similar results were obtained by measuring circulating FH levels in the sera of third trimester CTRL and PE mothers. Syncytiotrophoblast microvesicles, isolated from the placental tissues of PE and CTRL women, downregulated FH expression by DECs. The present study appears to suggest that FH is ubiquitously present in the normal placenta and plays a homeostatic role during pregnancy.

KEYWORDS

factor H, complement system, pregnancy, placenta, preeclampsia, microvesicles

1 Introduction

Pregnancy is a delicate biological process where the maternal immune system undergoes several immunomodulatory changes involving both innate and adaptive immunity to ensure tolerance at the feto-maternal interface. Complement is a crucial player in the tightly regulated immunological mechanisms for successful implantation, placental development, and parturition (1–3), displaying also non-canonical functions. Several years after its discovery, the contribution of the complement system to a successful pregnancy has increasingly been recognised (4).

Complement is a pivotal component of humoral innate immunity, which is made up of over 50 fluid-phase, membrane-bound and intracellular proteins that participate in three independent but collaborative activation pathways: classical, lectin and alternative. These three pathways converge on the activation of common component C3, and on the generation of anaphylatoxins such as C3a, C4a, C5a, and C5a-desArg, opsonins such as, C3b, iC3b, C3d, C4b, and cytolytic membrane attack complex (MAC) (5). Complement activation must be tightly regulated since complement dysregulation or over-activation can turn it from a homeostatic to a pathological effector driving several inflammatory conditions.

Several studies have demonstrated that dysregulation of complement activation predisposes to the development of pregnancy-related complications, such as pre-term birth, spontaneous abortion, recurrent miscarriage, and preeclampsia (PE) (6–9). PE is a common pregnancy disorder characterized by high blood pressure with proteinuria or end-organ injury. It is characterized by abnormal placentation, which may be related to defective decidualization, implantation, and angiogenesis (10–12). Besides shallow trophoblast invasion and inadequate remodeling of uterine arteries, dysregulation of immune and non-immune system is also a focal point in the development of PE. Excessive increase in the apoptosis of villous trophoblast can also cause over-activation of complement, enhancing pro-inflammatory response at the feto-maternal interface as observed in the case of PE (13). Moreover, increased levels of complement components and their activation-dependent cleaved products, including C3a, C5a, and C5b-9 complex, have been reported in the circulation of PE women (14), even though their exact contribution to PE pathophysiology is not fully understood.

Complement activation is tightly controlled through several complement regulatory proteins, Factor H (FH) being one of them in pregnancy. FH is 155 kDa soluble protein. Its gene is located on human chromosome 1 at the RAC (regulators of complement activation) gene cluster (15–17). Under physiological conditions, FH levels in the plasma range widely from 116 to 562 µg/ml, depending on genetic and environmental factors (18). FH is the main negative regulator of the complement alternative pathway in solution phase, but it is also involved in the inhibition of complement amplification on target cells and in the extracellular matrix of host tissues. It is a versatile innate immune molecule that protects host tissues by attenuating C3b attached to self surfaces and by restricting the formation of MAC. FH regulates enzymatic cleavage of C3 via its decay accelerating activity as well as cofactor activity for factor I–mediated C3b cleavage (19–22). Thus, by acting as a central negative regulator of the alternative pathway, FH limits the complement-mediated tissue damage leading to several diseases (23–25). FH can bind to molecules/ligands exposed on apoptotic and necrotic cells, including DNA, histones, and annexin II (and possibly the altered glycocalyx), in order to minimize the downstream pro-inflammatory effects of complement activation and the release of autoantigens that would potentially trigger autoimmunity (26–28).

Recent reports suggest that the FH level rises in maternal circulation during the first trimester of normal pregnancy (7), whereas it appears to decrease in severe PE (29). The presence of FH in placental tissue is largely attributed to the circulating blood in the placental vessels; whether it is synthesized locally by the major cell types constituting the placenta has not been investigated. The current study investigates the expression of FH in serum, placental tissues as well as various placental cells in normal pregnancy. FH levels were also measured in PE women to establish its contribution to the mechanisms underlying complement-dependent PE pathophysiology.

2 Materials and methods

2.1 Human subjects and samples

For this study, multiple cohorts of pregnant women at different stages of pregnancy were included (Supplementary Figure 1). For each cohort, PE was defined as the new onset of hypertension (systolic blood pressure ≥ 140 mmHg or diastolic ≥ 90 mmHg) on at least two occasions within 24 hours, along with the new onset of proteinuria (≥ 300 mg in a 24-hour urine collection, 50 mg/mmol protein/creatinine ratio, or at least 2+ on dipstick testing following two consecutive measurements).

A first cohort of patients was enrolled at the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" (Trieste, Italy) between October 2007 and April 2009, as described by Di Lorenzo and colleagues (30). From this serum bank, we selected first trimester sera of women who subsequently developed PE (n = 20) and sera of healthy pregnant women (n = 20) matched for maternal age (32 ± 4).

The third-trimester sera cohort was collected at the IRCCS "Policlinico Agostino Gemelli" (Rome, Italy). In particular, PE patients (n = 31) and CTRL (n = 26) had singleton pregnancies with undetected fetal abnormality and were matched for gestation week (32.7 ± 3.7 vs. 32.9 ± 4.5) (Table 1). A random selection from this cohort of samples was made to obtain pooled sera for cell stimulation. Blood samples were centrifuged at 500 *xg* for 7 min and the serum was immediately stored at -80°C.

Tissue samples of first-trimester placenta and decidua were obtained from healthy women undergoing elective termination of pregnancy at 8-12 weeks of gestation at the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" (Trieste, Italy). Within the same institute, term placenta samples were collected from both PE patients (n = 7) and healthy women (n = 8). A portion of these samples was fixed in 10%v/v buffered formalin for histological investigations, while another portion was shredded, added to 5

TABLE 1 Characteristics of the PE patients' cohort enrolled at Institute "Policlinico Agostino Gemelli" (Rome, Italy).

	PE n=31	CTRL n=26
Gestation weeks	32.7 (± 3.7)	32.9 (± 4.5)
Newborn weight (g)	1732 (± 860)	n.d.
Hypertension	75%	0%
Proteinuria (g/L)	2.7 (± 4)	0%
IUGR	35%	0%
Delivery		
Cesarean	80%	n.d.
Vaginal	20%	n.d.
Pre-existing diseases		
Hypertension	10%	0%
APS	15%	0%
Others	10%	0%

Data are expressed as mean ± standard deviation and as a percentage. n.d., not defined, IUGR, intra uterine growth restriction; APS, antiphospholipid syndrome.

mL TRIzol (ThermoFisher), and stored at -80°C for total mRNA isolation.

The study was reviewed and approved by the Regional Ethical Committee of FVG (CEUR-2020-Os-156; Prot. 0022668/P/GEN/ARCS and CEUR-2019-Sper-17) and adhered to the principles of the Declaration of Helsinki. The written consent was obtained from all the participants.

Syncytiotrophoblast derived microvesicles (STBMs), prepared via dual-placental perfusion of PE and healthy placentae, were obtained from the Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital University of Oxford, United Kingdom, as previously described (31).

2.2 Cell isolation and culture

Endothelial and stromal cells were isolated from first trimester decidual biopsy specimens, as previously described, with minor modifications (32, 33). Briefly, decidual tissues were digested with 0.25% trypsin (Sigma-Aldrich) and 50 µg/mL DNase I (Roche, Milan, Italy) in PBS, overnight at 4°C, and then treated with 3 mg/mL collagenase type I (Worthington Biochemical, DBA, Milano, Italy), for 30 min at 37°C. Following Ficoll-Paque Plus density gradient (GE Healthcare, Euroclone, Milan, Italy) centrifugation, decidual human endothelial cells (DECs) were isolated by positive selection using Dynabeads M-450 (Dynal, Oslo, Norway) coated with lectin Ulex europaeus 1 (Sigma-Aldrich). DECs were cultured in a flask pre-coated with 5 µg/cm^2 fibronectin (Roche) using Human Endothelial Serum Free Medium (HESFM; Gibco, Life Technologies, Milan, Italy), supplemented with 20 ng/mL basic Fibroblast Growth Factor (bFGF; Immunological Sciences), 10 ng/mL Epidermal Growth Factor (EGF; Immunological Sciences), and penicillin (50 U/mL)/streptomycin (50 µg/mL) (PS; Sigma-Aldrich).

Decidual stromal cells (DSCs) were obtained by culturing the endothelial-negative cell fraction in RPMI plus 10% fetal bovine serum (FBS), without growth factors. Non-adherent cells were removed *via* extensive washing; adherent cells were used only when the resulting cell population was negative for CD14, CD45, Cytokeratin 8/18, von Willebrand Factor (vWF), or CD31. The purity of DECs and DSCs isolated from first trimester decidua was routinely assessed by cytofluorimetric and qPCR analysis, as previously reported (34).

Extravillous trophoblasts (EVTs) were purified from placental tissues incubated with Hanks' Balanced Salt Solution (HBSS) containing 0.25% trypsin and 0.2 mg/mL DNase I (Roche) for 20 min at 37°C, following the published procedure (35). Cells were seeded in 25 cm^2-flask pre-coated with 5 µg/cm^2 fibronectin (Roche) in RPMI (Gibco, Life Technologies) supplemented with 10% FBS to remove non-adherent leucocytes and syncytiotrophoblasts, and used within 12 h. The purity of EVTs isolated from first trimester placenta was routinely assessed by immunofluorescence and qPCR analysis, as previously reported (36).

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords of healthy placentae, following a published protocol (37), and cultured in HESFM supplemented with 20 ng/mL EGF, 10 ng/mL bFGF, 1% PS, and 10% FBS. Immortalized human hepatocytes (IHH, kindly provided by Dr. Cristina Bellarosa, Italian Liver Foundation, Trieste, Italy) were cultured in Dulbecco's modified Eagle medium (Gibco), supplemented with non-essential amino acids, 2mM L-glutamine (Gibco). 100 µg/mL of penicillin/streptomycin (Gibco), and 10% FBS at 37°C and 5% CO_2.

2.3 Immunohistochemical analysis

Term placental tissues from PE and healthy women were fixed in 10% *v/v* buffered formalin, paraffin-embedded and stored at 4°C. Tissue sections of 3-4 µm were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol (100%, 95%, 70%) and dH_2O. The antigen unmasking technique was performed using citrate buffer (pH 6), for 20 min at 98°C in thermostatic bath. After neutralization of the endogenous peroxidase activity with 3% *v/v* H_2O_2 for 5 min, sections were incubated with PBS + 2% bovine serum albumin (BSA) for 30 min to block non-specific binding. Next, rabbit anti-human FH polyclonal antibody (1:100, #PA5-83957, Invitrogen) was incubated at 4°C. Staining was revealed *via* anti-rabbit horseradish peroxidase (HRP)-conjugate (1:500, Sigma), incubated for 30 min at room temperature, and 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Sections were counterstained with Mayer Haematoxylin (DiaPath, Italy) and examined under a Leica DM 2000 optical microscope. Kidney was stained as positive control tissue. Images were collected using a Leica DFC 7000 T digital camera (Leica Microsystems, Wetzlar, Germany). To quantify the staining, we utilized an immunoreactive score (38), which is commonly used for immunohistochemical evaluation. For each slide, we blindly analyzed three different visual fields of the microscope, attributing the following scores:

0 = no color reaction, 1 = mild reaction, 2 = moderate reaction, 3 = intense reaction.

2.4 Real time quantitative PCR

Total RNA was extracted from placental tissues of PE and healthy (CTRL) women as well as from isolated placental cells. The isolation of RNA from tissue samples was carried out using Phenol-Chloroform extraction method. Placental cells (EVTs, DECs, and DSCs) and IHH were stimulated for 24 h with 10% sera of PE or CTRL women (5 pooled sera), 100 ng/mL TNF-α, 10 ng/mL IL-1β or 50 µg/mL STBMs derived from PE or CTRL women. After incubation, cells were lysed and the total RNA was isolated. For each placental cell type (EVTs, DECs or DSCs), the experiments were repeated using at least four different cell populations. Total RNA was isolated using an RNA purification kit (Norgen Biotek Company, Thorold, Canada). RNA quantification was performed *via* NanoDrop® spectrometer (NanoDrop). RNA was converted to cDNA using SensiFAST™ cDNA Synthesis Kit (Meridian Bioscience, Memphis, TN, USA).

For Real-Time quantitative PCR (RT-qPCR), SYBR Select Master Mix (Applied Biosystems, ThermoFisher) was used. The reaction was performed by the Rotor-Gene 6000 (Corbett, Explera), *via* 45 cycles of denaturation (10 sec at 95°C), and annealing (45 sec at 60°C, melting temperature of the primers). Following primers were used: *CFH* (forward 5'- ACC GTG AAT GTG ACA CAG ATG G-3'; reverse 5'- ATT CCC GAT CTG GTT CCA TTG C-3'), *GAPDH* (forward 5'-GAT CAT CAG CAA TGC CTC CT-3'; reverse 5'- GT GGT CAT GAG TCC TTC CA-3'). The mRNA expression level of FH was evaluated with a comparative quantification provided with Rotor Gene 1.7 Software (Corbett Research) and normalized with the expression of a housekeeping gene (*GAPDH*).

2.5 Western blot analysis

Placental STBMs, isolated from CTRL ($n = 4$) or PE patients ($n = 4$), were lysed. After boiling, 20 µg of STBMs, diluted in Laemmli buffer, were subjected to a 10% *v/v* SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane using the wet system of Mini Blot Module (Thermo Fisher Scientific), at a constant voltage for 60 min. An hour of blocking step with 5% skimmed milk in Tris-Buffered Saline + Tween 20 (TBST; 10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) was followed by an overnight incubation at 4°C with primary antibodies: anti- FH (1:1000; #PA5-83957, Invitrogen) and anti-actin (1:1000; sc-8432, Santa Cruz). The following day, the membrane was incubated with anti-rabbit LI-COR IRDye 800CW and anti-mouse LI-COR IRDye 680CW (1:10,000; LI-COR Biosciences, Lincoln, NE, USA), for 1 h at room temperature. The fluorescence intensity was acquired in the Odyssey® CLx near-infrared scanner (LI-COR Biosciences, Lincoln, NE, USA) and normalized for anti-actin. Image acquisition, processing and data analysis were performed via Image Studio Ver 5.2 (LI-COR Biosciences).

2.6 Enzyme-linked immunosorbent assay

Human Complement FH DuoSet ELISA kit (#DY4779; R&D Systems, Inc., Minneapolis, Canada) was used to quantify FH in serum samples, following the manufacturer's instructions. The plate was read by the PowerWave X Microplate Reader (Bio-Tek Instruments) spectrophotometer at 450 nm.

2.7 Statistical analysis

Data obtained from *in vitro* cell stimulation assays were analyzed by unpaired non-parametric test (Wilcoxon) comparing resting and treated conditions, while data obtained from patient samples were analyzed by unpaired t-test comparing PE *vs* controls healthy. STBM analysis was statistically evaluated using Mann-Whitney test. Results were expressed as mean ± standard deviations. *p*-values (p) < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism software 9.0 (GraphPad Software Inc., La Jolla, CA, USA).

3 Results

3.1 FH expression in placental tissues and cells in normal pregnancy

To investigate the physiological role of FH in placenta, we first assessed the basal expression of FH in placental tissues and cells. We analyzed FH tissue distribution by immunohistochemistry (IHC) using first trimester and term placental sections with rabbit anti-human FH antibodies. As shown in Figures 1A, B, the positivity for FH staining in first trimester placentae was particularly intense in decidual spiral arteries and in the syncytiotrophoblast monolayer, whereas in third trimester placenta, FH was present in the inner of villi vessels and at the level of intervillous spaces (Figures 1C, D). In order to confirm the local synthesis of FH, we performed RT-qPCR using placental tissue mRNA isolated from first and third trimester normal placenta. In placental tissues, FH expression was observed both in the first ($n = 4$) as well as third ($n = 4$) trimester samples derived from healthy pregnancies, where both the decidua and the fetal villi expressed FH (Figure 1E). The expression levels were widely variable among samples. No significant difference could be observed between first and third trimester.

To identify cells that were responsible for the local production of FH, we performed RT-qPCR on isolated first trimester placental cells (EVTs, DECs, and DSCs). FH gene expression was analyzed using HUVECs as a calibrator, since they showed the highest FH mRNA expression. FH was expressed at similar levels by all the three placental cell investigated (DSCs, DECs, and EVTs) (Figure 1F).

10.3389/fimmu.2024.1351898

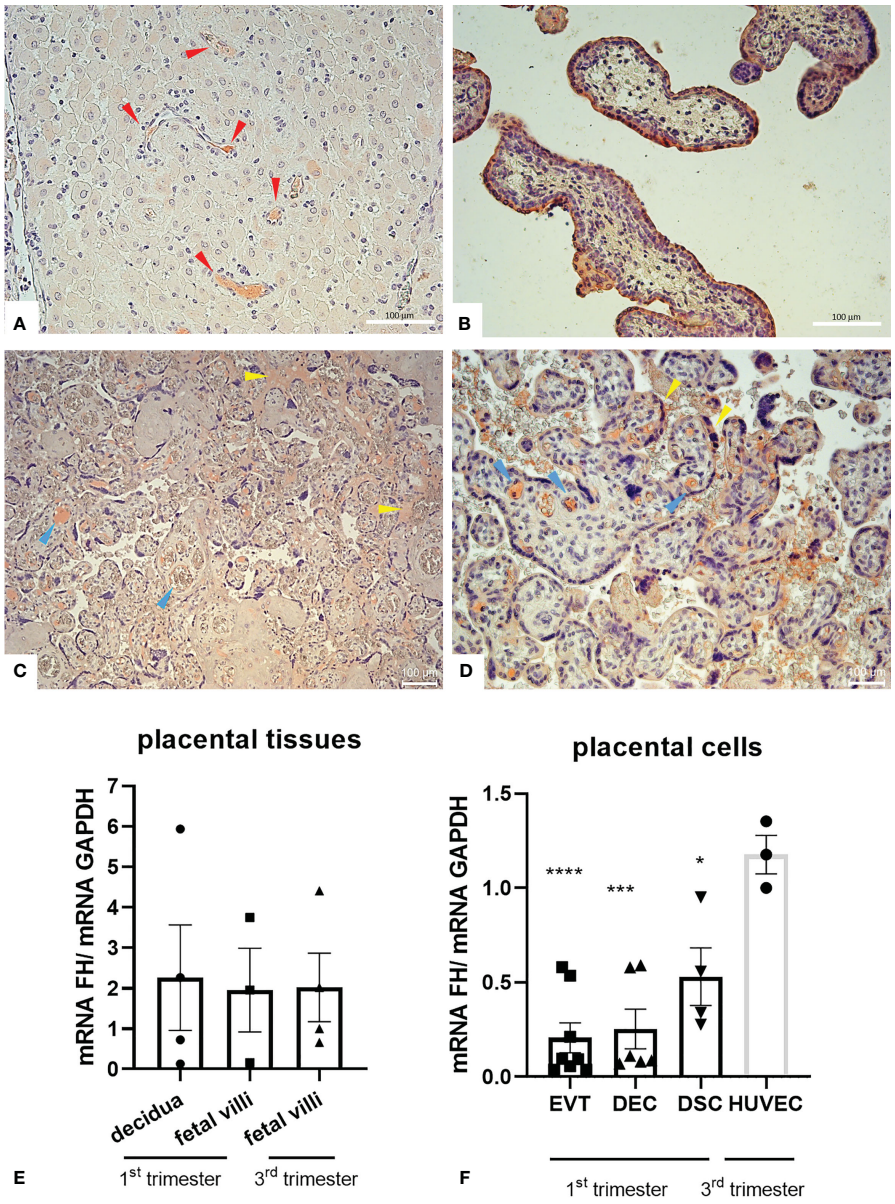


FIGURE 1
(A–D) Immunohistochemical analysis for the distribution of Factor H (FH) in placental tissues. First trimester [**(A, B)** *n* = 3] and term human placental [**(C, D)** *n* = 8] sections were stained with anti-FH polyclonal antibody. Staining was detected *via* 3-amino-9-ethylcarbazole (AEC) substrate chromogen. Nuclei were stained with Mayer's Hematoxylin. The positivity for FH staining in first trimester placentae was particularly intense in decidual vessels (red arrows) and in the syncytiotrophoblast monolayer, whereas in villi vessels (yellow arrows) and in intervillous spaces and syncythium surface (blue arrows) for term placentae. Magnification, **(A, B, D)** 200x, **(C)** 200x. Scale bar, 100 μm. **(E, F)** mRNA expression levels of FH were measured by RT-qPCR in placental tissues **(E)** and placental cells **(F)** isolated from normal pregnancies. FH was expressed in all placental tissues (*n* = 4 first trimester decidua samples, *n* = 3 first trimester villous samples and *n* = 4 term villous samples) and placental cells examined, *i.e.*, extravillous trophoblast (EVT, *n* = 8), decidual endothelial cells (DECs, *n* = 6), and decidual stromal cells (DSCs, *n* = 4). FH gene expression was analyzed using HUVECs as a calibrator, since they showed the highest FH expression. *GAPDH* was used as a housekeeping gene to normalize gene expression results. Data are expressed as mean ± SD of two independent experiments performed in triplicate. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, as compared to HUVECs (Mann-Whitney test).

3.2 Higher levels of FH detected in normal placentae compared to PE placentae

To better elucidate the role of FH in pregnancy, we first investigated its expression levels in a pathological condition that affects placental development, such as PE. Thus, we compared the local distribution of FH in CTRL and PE placental tissues by IHC. Placentae from CTRL women (*n* = 8) had higher FH expression than PE pregnancies (*n* = 7) (Figure 2, Supplementary Figure 2). FH was almost undetectable in PE placentae (Figures 2C, D), making it difficult to define its possible localization, even though villi vessels were slightly positive; FH in CTRL placentae was mainly localized in

10.3389/fimmu.2024.1351898

the fetal villous endothelium (blue arrows) and intervillous spaces (red arrows) (Figures 2A, B). The IHC quantitation demonstrated a significantly lower level of FH in PE placentae (Figure 2E). Our results were partially confirmed by RT-qPCR analysis of placental tissue. Although no statistical significance was reached, the results showed lower FH gene expression in PE placental tissue compared to CTRL placenta (Figure 2F). When dividing patients into late-onset (LO) and early-onset EOPE, we found that EOPE patients had the lowest FH levels (Figure 2F), suggesting the involvement of FH in PE-associated pathologic mechanisms.

3.3 Modulation of FH gene expression by sera of CTRL and PE women

Based on previous observations, we hypothesized that microenvironmental factors, including pro-inflammatory cytokines present in the PE placentae, could be responsible for the decreased FH expression by placental cells. Thus, DECs and DSCs isolated from first trimester placentae were stimulated with a pool of 5 different CTRL or PE sera, and analyzed for FH mRNA expression levels. As shown in Figures 3A, B, FH expression was significantly reduced in

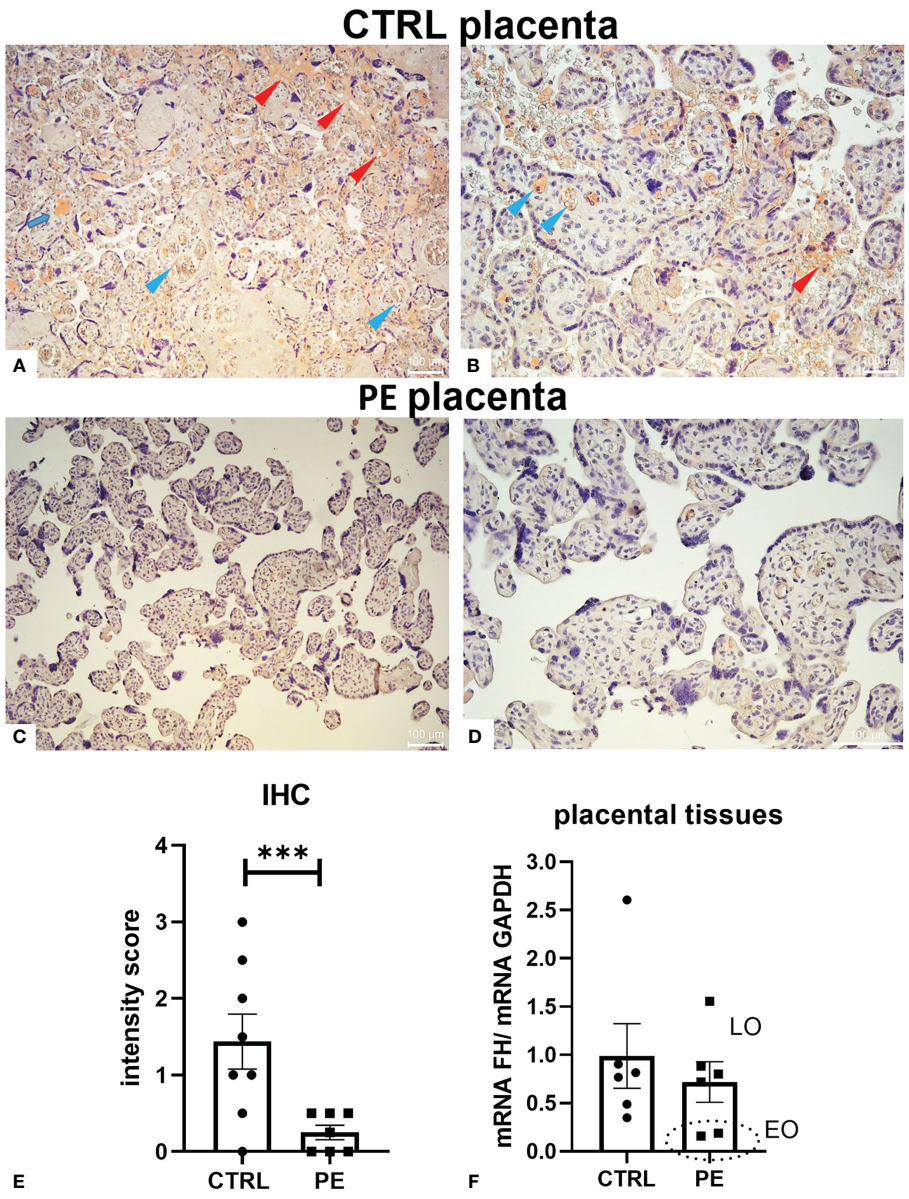


FIGURE 2
Localization of Factor H (FH) in placental tissues from normal (CTRL) and preeclamptic (PE) tissue. **(A–D)** The samples representative of CTRL [**(A)** original magnification 10 X; **(B)** original magnification 20X] or PE [**(C)** original magnification 10X; **(D)** original magnification 20 X] pregnancies were stained with anti-FH antibody. FH staining was detected in all the normal placental tissues, being mainly localized in fetal villi endothelium (blue arrows) and intervillous spaces (red arrows), whereas it was almost undetectable in PE placentae **(C, D)**. Staining was detected *via* 3-amino-9-ethylcarbazole (AEC) substrate chromogen. Nuclei were stained with Mayer's Hematoxylin. Scale bars, 100 µm. **(E)** Quantitation of IHC staining using an immunoreactive score (PE n = 7, CTRL n = 8). For each slide, three different visual fields of the microscope, were analyzed giving a score as described in Materials and Methods section. ***$p < 0.001$ (t-test). **(F)** FH mRNA expression levels in term placental tissues of CTRL (n = 6) and PE [further divided into early onset, (EO, n = 2), and late onset, (LO, n = 4)] pregnancies were assessed by RT-qPCR. FH expression was lower in PE placentae compared to CTRL placentae; interestingly, EOPE samples exhibited minimal expression (marked with a circle). *GAPDH* was used as a housekeeping gene to normalize gene expression levels.

DECs and DSCs following incubation with PE sera compared to cells incubated with matched CTRL sera. We also investigated the ability of PE sera to modulate FH expression using an immortalized hepatocyte cell line (IHH), but we did not observe any significant modulation of FH transcript levels (Figure 3C).

To determine which humoral factors in PE sera might be responsible for modulating FH expression, placental cells and IHH were stimulated with key pro-inflammatory cytokines known to be elevated in PE sera, such as TNF-α (39) and IL-1β (40). Surprisingly, none of these cytokines were able to induce a decrease in FH expression by the placental cells, but rather we observed their upregulation (Figures 3D, E). In contrast, IHH showed a significant downregulation of FH expression, especially after stimulation with TNF-α (Figure 3F).

Out of a range of immunomodulatory properties exerted by the placenta, the shedding of STBMs may contribute significantly to pregnancy success (41, 42). Interestingly, STBM levels are elevated in women with PE, possibly contributing to vascular endothelial dysfunction (43). Therefore, DECs were incubated for 24 h with STBMs isolated from the placenta of PE and CTRL women. Consistent with the earlier study, STBMs isolated from PE placentae were able to downregulate FH expression (Supplementary Figure 3); however, we were unable to obtain a statistically significant result.

3.4 Circulating levels of FH in healthy pregnant women are higher than in PE patients

To examine whether the observations gathered at the placental level might also reflect at the systemic level, circulating FH levels were measured by ELISA in the sera of PE women collected at the time of diagnosis and in the sera of matched CTRL women.

In agreement with the results we obtained using placenta, we found lower circulating FH levels in the sera of PE women compared to CTRL (healthy) women (Figure 4). Therefore, we investigated whether the differences in FH levels between PE patients and CTRL women could be used to define FH as an early marker for PE. Thus, we quantified FH in serum samples collected between 11 and 13 weeks of gestation from pregnant women who later developed PE and in sera of matched CTRL women. Compared to PE, FH concentrations were significantly higher in the sera of CTRL women during the third trimester of pregnancy (Figure 4A, CTRL: 556.95 μg/mL ± SD 191.52 *vs* PE: 324.21 μg/mL ± SD 131.04), and, to a lesser extent, in the first trimester (Figure 4B CTRL: 624.06 μg/mL ± SD 273.05 *vs* PE: 450.97 μg/mL ± SD 226.16). Since a significant difference in FH levels was observed between PE and CTRL sera, we hypothesized that FH may bind to apoptotic bodies of the placenta. Therefore, we investigated the presence of FH deposition on STBMs, a method previously described by Tannetta et al. (44). We performed Western blot analysis using solubilized STBMs from PE (n = 4) and CTRL placentas (n = 4), and measured FH protein expression levels. As shown in Figures 4C, D, FH was present in STBMs from both PE placenta and CTRL, with significantly higher levels in PE.

4 Discussion

Pregnancy is a complex biological process in which the maternal immune system undergoes several changes that are necessary for the protection of the mother and the fetus from pathogenic challenges and for a successful delivery of the fetus (4).

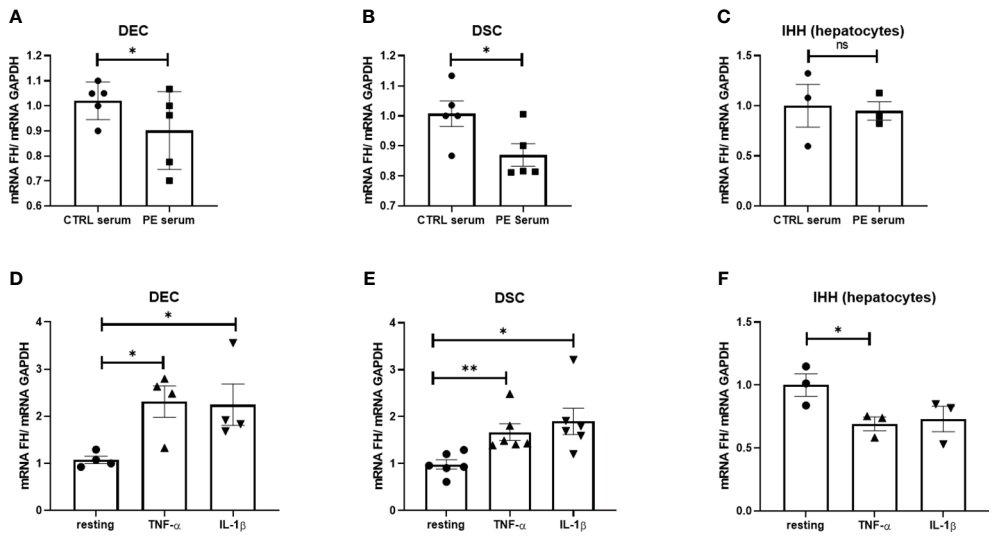


FIGURE 3
(A–C) Levels of Factor H (FH) mRNA expression in DECs **(A)**, DSCs **(B)** and IHH **(D)** following *in vitro* stimulation with serum from normal (CTRL) and PE women for 24 h. Pooled PE (n = 5) or CTRL (n = 5) serum samples were used. **(D–F)** Levels of FH mRNA expression in DECs **(D)**, DSCs **(E)** or IHH **(F)** stimulated with TNF-α or IL-1β for 24h. FH mRNA levels were significantly increased in DECs and DSCs after stimulation with TNF-α and IL-1β, whereas in IHH, FH transcripts were down-regulated with only TNF-α. Data are expressed as mean ± SD of two independent experiments performed in triplicate. *p < 0.05, **p < 0.01, as compared to resting (Mann-Whitney test). ns, non significant.

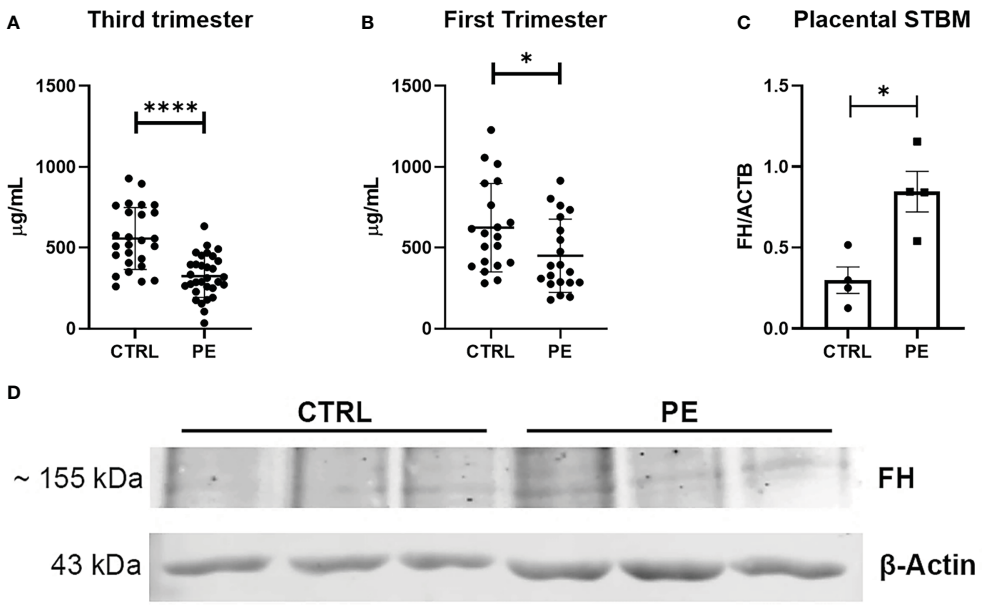


FIGURE 4
(A, B) Measurement of circulating Factor H (FH) levels in sera of normal (CTRL) and preeclamptic (PE) women, collected during third **(A)** or first **(B)** trimester, by ELISA. During both the first trimester and the third trimester lower levels of FH were observed in PE pregnancies compared to CTRL, showing a highly significant difference (n = 26 PE vs n =31 CTRL sera were compared for third trimester of pregnancy; n = 20 PE vs n = 20 CTRL for first trimester of pregnancy). *p < 0.05, ****p < 0.0001 (t-test). **(C, D)** Evaluation of FH deposition on placental STBMs via Western blot. STBMs derived from PE (n = 4) and CTRL (n = 4) were solubilized and separated by SDS-PAGE under reducing conditions. After transfer, the membrane was probed with anti-FH antibody and IRDye 800CW secondary antibody. Signal intensity was detected using an Odyssey CLx near-infrared scanner (LI-COR Biosciences, Lincoln, NE, USA). Image acquisition, processing and data analysis were performed with Image Studio 5.2 (LI-COR Biosciences). Histograms represent the mean ± SD of two independent experiments performed in duplicate. Beta-actin (ACTB) was used to normalize the results. *p < 0.05 (Mann-Whitney test).

The complement system is critical for a successful pregnancy (1, 2), thus dysregulation along the complement cascade may play a major role in pregnancy-associated complications, including PE. In particular, complement regulators impose strict control on complement activation. FH, a negative regulator of complement alternative pathway, is a very multifaceted molecule which is able to bind a wide range of target ligands and fulfil different functions.

Aiming to investigate the role of FH in pregnancy, we first explored its expression pattern during different trimesters of the pregnancy. Our results confirmed a local production of FH at the feto-maternal interface, both in the decidua and in the villi, but showed no differences in FH transcript levels between the first and third trimester of pregnancy. IHC staining pattern was very different between the first trimester and term placenta: in first-trimester double-layered syncytium, FH staining was widespread; conversely, in term placenta monolayered villi, FH was present in maternal intervillous space and into the fetal villous vessels. Placental tissue may present intravillous fibrin deposits, which are expected to stain for FH (45). This is likely to represent the maternal FH, while the expected endothelial staining of villous vessels is the fetal FH; thus, the two display the expression of FH molecules of different origins.

To determine which placental cells were responsible for the local production of FH, we analyzed the cells isolated from first trimester placental tissue by RT-qPCR. All the placental cell types (DSCs, DECs, and EVTs) expressed FH to a similar degree, although the highest FH expression was observed in HUVECs, which we used as a positive control based on an earlier study by Rayes et al. (46).

Compared to PE, placentae from CTRL women had higher FH staining, which allowed us to conclude that FH was mainly localized in the fetal villous endothelium and intervillous spaces. Conversely, FH in PE placenta was almost undetectable. The presence of FH in healthy placenta has been previously reported with a significantly variable staining pattern, being uniformly localized in the syncytiotrophoblast layer in 70% of the samples, but not in the villous vessels (45). However, in agreement with our results, most of PE placentae remained negative for FH staining (45). Lokki and Heikkinen-Eloranta demonstrated intracellular localization of FH in the placental syncytiotrophoblast of PE placenta, and suggested that FH may exacerbate thrombotic microangiopathy in PE independent of complement activation (47). However, the existence of an association between FH and PE should be further confirmed by analyzing FH localization and synthesis *via* IHC and RT-qPCR using a greater number of cases, via an accurate clustering of the patients based on severity and pathogenesis of the disease. The results of Belmonte et al. demonstrated the involvement of the lectin pathway in complement deposition in PE placenta (48). This evidence is in accordance with our results that failed to detect FH deposition in PE placentae. However, we cannot exclude that FH staining was undetectable due to the damage and the dysmorphia typical of the PE villi.

Under physiological conditions, plasma FH levels can vary between 116 and 562 µg/mL depending on genetic and

environmental factors, but they may increase in pregnant women (18). There are several reports about an increase in the circulating FH levels during pregnancy (7, 14, 49). Our study also suggests that soluble FH levels remain high during pregnancy both in the first and the third trimester. Interestingly, we observed a decrease in circulating FH levels when comparing PE with healthy pregnancy, particularly in the third trimester. Our results also highlighted a statistically significant difference between PE and healthy controls from the prospective study cohort. In the first trimester of pregnancy, patients who subsequently (after the 20th week of gestation) developed PE had lower FH levels, indicating FH as a potential predictive marker of PE, although further studies are needed. Previous studies have already reported lower circulating levels of FH in PE patients (29, 50), in manner independent of the levels of anti-FH autoantibodies (50). In a prospective study, serum levels of FH were shown to increase in the middle and late stages of pregnancy, whereas FH was reduced in both EOPE and LOPE compared to control groups (29). However, these differences were not observed in the second and third trimesters of pregnancy. Another study pointed out that FH concentrations in the serum of PE patients were higher during the first trimester than in a normal pregnancy; subsequently, these values were similar to those in a normal pregnancy (7). Ari et al. found that serum C3 and FH levels in patients with HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome were not significantly different from those of PE patients and healthy pregnant women (51).

Insufficient levels of FH can be attributed to decreased FH expression due to mutations. Five rare variants (L3V, R127H, R166Q, C1077S, and N1176K) in the FH gene have been found to predispose to the occurrence of severe PE (52). Moreover, FH reduction can also be due to the presence of anti-FH autoantibodies, consumption of FH, or by the fact that FH is overwhelmed by a massive disease burden (53).

The observation of both local and systemic lower FH levels in PE led us to consider that microenvironmental factors may be responsible for reduced FH production by placental cells. After stimulation with pooled PE or control sera, we thus analyzed FH mRNA expression in DECs and DSCs. Our results showed a significantly reduced FH expression in cells stimulated with PE sera compared to control sera. The downregulation of FH gene may be explained by the particular conditions of PE, where the placenta is characterized by increased oxidative stress and complement activation. Thus, upregulation of the FH gene probably protects the cells by inhibiting complement activation under physiological conditions, while its downregulation can potentially lead to adverse pregnancy outcomes.

To identify the factors present in PE sera that might modulate FH expression, cells were incubated with pro-inflammatory cytokines known to be increased in PE women's blood, such as TNF-α and IL-1β (39, 54–59). Previously, Daha's group demonstrated that stimulation of HUVECs with IL-1 led to a decrease in FH expression (60). Unexpectedly, FH mRNA expression was increased in DECs and DSCs after stimulation with pro-inflammatory cytokines. Due to the multifactorial nature of PE (61), the *in vitro* stimulation with these two cytokines may likely be insufficient to mimic a PE-like scenario in placental cells. Moreover, the results obtained with IHH suggested that the selected amount of TNF-α (100 ng/mL) and IL-1β (10 ng/mL) was higher compared to their levels in PE sera. We can hypothesize differential levels of cytokine sensitivity between placental cells and hepatocytes. Since the potent pro-inflammatory properties of TNF-α and IL-1β can trigger excessive inflammation, and directly or indirectly, activate the complement system, placental cells upregulate FH expression possibly to control complement activation.

STBMs, released into the maternal circulation to ensure feto-maternal communication, are significantly increased in PE and can contribute to the local pro-inflammatory properties by carrying a variety of placental proteins into the maternal circulation (62). Tanetta et al. found 538 unique proteins in STBMs of PE, including several complement proteins and regulators (*e.g.*, C1q, C3, CD55, CD59, and vitronectin) (63). In another study, Baig et al. identified 25 differentially expressed proteins in PE, with CD59 being downregulated (64). Our results showed that STBMs, isolated from PE placentae, downregulated FH gene expression in DECs, suggesting that binding of STBMs to DECs may have an inhibitory effect on FH production. Surprisingly, we also found higher levels of FH in STBMs isolated from PE samples than in control samples. Despite this high proportion of FH in STBMs, complement activation is dysregulated/poorly controlled in PE. One possibility is that FH expressed by/on the placental cells is sequestered into STBMs, which may act as decoy substrates. A previous study in tumour models demonstrated that FH can successfully be carried by extracellular vesicles (65). Thus, FH may not be available to the cells for complement regulation, or alternatively, its ability to inhibit complement activation may be masked. In fact, it is widely recognized that FH must be in soluble form to be biologically effective, which is not the case in PE, consistent with the lower circulating FH levels observed in PE mothers when analyzing serum, tissues, and isolated cells. Therefore, it is important to investigate the immunomodulatory properties of FH-loaded STBMs in PE immunopathology.

5 Conclusions

Under physiological conditions, FH is locally expressed at the feto-maternal interface in all trimesters of pregnancy. However, it can be localized in syncytiotrophoblasts in the first trimester and in villous endothelium in the third trimester placenta. Decidual cells (DECs, EVTs, and DSCs) appear to synthesize FH. PE placentae are almost negative for FH staining. Circulating levels of FH are significantly lower in the PE sera compared to controls. The causes for this reduction could be multiple (*e.g.*, mutations, consumption, less synthesis). PE sera contain humoral factors that can cause downregulation of FH expression by placental cells, possibly due to the presence of STBMs highly loaded with FH in PE. Further studies are required to understand the mechanisms linking FH and PE onset.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was reviewed and approved by the Regional Ethical Committee of FVG (CEUR-2020-Os-156; Prot. 0022668/P/GEN/ARCS and CEUR-2019-Sper-17) and adhered to the principles of the Declaration of Helsinki. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HY: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. CA: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. MT: Investigation, Methodology, Software, Writing – review & editing. TR: Investigation, Methodology, Validation, Writing – review & editing. SP: Data curation, Investigation, Software, Writing – review & editing. AB: Data curation, Methodology, Software, Writing – review & editing. GZ: Formal analysis, Methodology, Software, Writing – review & editing. ND: Data curation, Investigation, Methodology, Writing – review & editing. GR: Formal analysis, Investigation, Methodology, Writing – review & editing. TM: Conceptualization, Investigation, Writing – review & editing. UK: Conceptualization, Formal analysis, Resources, Supervision, Writing – review & editing. RB: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Ministry of Health, Rome -Italy, in collaboration with the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste -Italy (RC24/19 to GR, RC09/21, RC01/23 to CA, and RC03/23 to AB), DST-SERB-TARE project, Government of India (Sanction No. TAR/2022/000626), to HY and UAEU Start Up grant #12F043 and UPAR grant #12F061 to U.K.

Acknowledgments

We thank Prof. Ian Sargent and his group of the Nuffield Department of Obstetrics and Gynaecology, University of Oxford (John Radcliffe Hospital, Oxford, UK) for providing STBM samples, and Dr. Cristina Bellarosa (Liver Foundation, Trieste, Italy) for providing IHH cell line.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1351898/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Graphical description of the multiple cohorts of pregnant women at different stages of pregnancy.

SUPPLEMENTARY FIGURE 2
IHC analysis of FH in placental tissues from three normal (CTRL) and three preeclamptic (PE) tissues. Original magnification 10X. FH staining was detected in all the normal placental tissues (left panels), whereas it was almost undetectable in PE placentae (right panels). Staining was detected *via* 3-amino-9-ethylcarbazole (AEC) substrate chromogen. Nuclei were stained with Mayer's Hematoxylin. Scale bars, 100 µm.

SUPPLEMENTARY FIGURE 3
Levels of FH mRNA expression in DECs stimulated for 24h with 50 µg/mL STBMs of PE or CTRL placentae. Data are expressed as mean ± SD of two independent experiments performed in triplicate.

References

1. Bulla R, Bossi F, Tedesco F. The complement system at the embryo implantation site: friend or foe? *Front Immunol* (2012) 3:55. doi: 10.3389/fimmu.2012.00055

2. Madhukaran SP, Alhamlan FS, Kale K, Vatish M, Madan T, Kishore U. Role of collectins and complement protein C1q in pregnancy and parturition. *Immunobiology* (2016) 221(11):1273–88. doi: 10.1016/j.imbio.2016.06.002

3. Girardi G, Lingo JJ, Fleming SD, Regal JF. Essential role of complement in pregnancy: from implantation to parturition and beyond. *Front Immunol* (2020) 11:1681. doi: 10.3389/fimmu.2020.01681

4. Mor G, Cardenas I. The immune system in pregnancy: a unique complexity. *Am J Reprod Immunol* (2010) 63(6):425–33. doi: 10.1111/j.1600-0897.2010.00836.x

5. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol* (2015) 6:262. doi: 10.3389/fimmu.2015.00262

6. Buurma A, Cohen D, Veraar K, Schonkeren D, Claas FH, Bruijn JA, et al. Preeclampsia is characterized by placental complement dysregulation. *Hypertension* (2012) 60(5):1332–7. doi: 10.1161/HYPERTENSIONAHA.112.194324

7. He YD, Xu BN, Wang ML, Wang YQ, Yu F, Chen Q, et al. Dysregulation of complement system during pregnancy in patients with preeclampsia: A prospective study. *Mol Immunol* (2020) 122:69–79. doi: 10.1016/j.molimm.2020.03.021

8. Regal JF, Gilbert JS, Burwick RM. The complement system and adverse pregnancy outcomes. *Mol Immunol* (2015) 67(1):56–70. doi: 10.1016/j.molimm.2015.02.030

9. Teirilä L, Heikkinen-Eloranta J, Kotimaa J, Meri S, Lokki AI. Regulation of the complement system and immunological tolerance in pregnancy. *Semin Immunol* (2019) 45:101337. doi: 10.1016/j.smim.2019.101337

10. Laresgoiti-Servitje E. A leading role for the immune system in the pathophysiology of preeclampsia. *J Leukoc Biol* (2013) 94(2):247–57. doi: 10.1189/jlb.1112603

11. Jung E, Romero R, Yeo L, Gomez-Lopez N, Chaemsaithong P, Jaovisidha A, et al. The etiology of preeclampsia. *Am J Obstet Gynecol* (2022) 226(2S):S844–66. doi: 10.1016/j.ajog.2021.11.1356

12. Magee LA, Nicolaides KH, von Dadelszen P. Preeclampsia. *N Engl J Med* (2022) 386(19):1817–32. doi: 10.1056/NEJMra2109523

13. Raguema N, Moustadraf S, Bertagnolli M. Immune and apoptosis mechanisms regulating placental development and vascularization in preeclampsia. *Front Physiol* (2020) 11:98. doi: 10.3389/fphys.2020.00098

14. Derzsy Z, Prohászka Z, Rigó J, Füst G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol* (2010) 47 (7-8):1500–6. doi: 10.1016/j.molimm.2010.01.021

15. Sim RB, DiScipio RG. Purification and structural studies on the complement-system control protein beta 1H (Factor H). *Biochem J* (1982) 205(2):285–93. doi: 10.1042/bj2050285

16. Rodriguez de Cordoba S, Lublin DM, Rubinstein P, Atkinson JP. Human genes for three complement components that regulate the activation of C3 are tightly linked. *J Exp Med* (1985) 161(5):1189–95. doi: 10.1084/jem.161.5.1189

17. Rodriguez de Cordoba S, Rubinstein P. Quantitative variations of the C3b/C4b receptor (CR1) in human erythrocytes are controlled by genes within the regulator of complement activation (RCA) gene cluster. *J Exp Med* (1986) 164(4):1274–83. doi: 10.1084/jem.164.4.1274

18. Esparza-Gordillo J, Soria JM, Buil A, Almasy L, Blangero J, Fontcuberta J, et al. Genetic and environmental factors influencing the human factor H plasma levels. *Immunogenetics* (2004) 56(2):77–82. doi: 10.1007/s00251-004-0660-7

19. Whaley K, Ruddy S. Modulation of the alternative complement pathways by beta 1 H globulin. *J Exp Med* (1976) 144(5):1147–63. doi: 10.1084/jem.144.5.1147

20. Weiler JM, Daha MR, Austen KF, Fearon DT. Control of the amplification convertase of complement by the plasma protein beta1H. *Proc Natl Acad Sci U.S.A.* (1976) 73(9):3268–72. doi: 10.1073/pnas.73.9.3268

21. Morgan HP, Schmidt CQ, Guariento M, Blaum BS, Gillespie D, Herbert AP, et al. Structural basis for engagement by complement factor H of C3b on a self surface. *Nat Struct Mol Biol* (2011) 18(4):463–70. doi: 10.1038/nsmb.2018

22. Kouser L, Abdul-Aziz M, Nayak A, Stover CM, Sim RB, Kishore U. Properdin and factor h: opposing players on the alternative complement pathway "see-saw". *Front Immunol* (2013) 4:93. doi: 10.3389/fimmu.2013.00093

23. Ferreira VP, Pangburn MK, Cortés C. Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol* (2010) 47(13):2187–97. doi: 10.1016/j.molimm.2010.05.007

24. Józsi M. Factor H family proteins in complement evasion of microorganisms. *Front Immunol* (2017) 8:571. doi: 10.3389/fimmu.2017.00571

25. Meri S, Haapasalo K. Function and dysfunction of complement factor H during formation of lipid-rich deposits. *Front Immunol* (2020) 11:611830. doi: 10.3389/fimmu.2020.611830

26. Parente R, Clark SJ, Inforzato A, Day AJ. Complement factor H in host defense and immune evasion. *Cell Mol Life Sci* (2017) 74(9):1605–24. doi: 10.1007/s00018-016-2418-4

27. Leffler J, Herbert AP, Norström E, Schmidt CQ, Barlow PN, Blom AM, et al. Annexin-II, DNA, and histones serve as factor H ligands on the surface of apoptotic cells. *J Biol Chem* (2010) 285(6):3766–76. doi: 10.1074/jbc.M109.045427

28. Ferluga J, Kouser L, Murugaiah V, Sim RB, Kishore U. Potential influences of complement factor H in autoimmune inflammatory and thrombotic disorders. *Mol Immunol* (2017) 84:84–106. doi: 10.1016/j.molimm.2017.01.015

29. Jia K, Ma L, Wu S, Yang W. Serum levels of complement factors C1q, Bb, and H in normal pregnancy and severe pre-eclampsia. *Med Sci Monit* (2019) 25:7087–93. doi: 10.12659/MSM.915777

30. Di Lorenzo G, Ceccarello M, Cecotti V, Ronfani L, Monasta L, Vecchi Brumatti L, et al. First trimester maternal serum PIGF, free β-hCG, PAPP-A, PP-13, uterine artery Doppler and maternal history for the prediction of preeclampsia. *Placenta* (2012) 33(6):495–501. doi: 10.1016/j.placenta.2012.03.003

31. Agostinis C, Stampalija T, Tannetta D, Loganes C, Vecchi Brumatti L, De Seta F, et al. Complement component C1q as potential diagnostic but not predictive marker of preeclampsia. *Am J Reprod Immunol* (2016) 76(6):475–81. doi: 10.1111/aji.12586

32. Agostinis C, Rami D, Zacchi P, Bossi F, Stampalija T, Mangogna A, et al. Pre-eclampsia affects procalcitonin production in placental tissue. *Am J Reprod Immunol* (2018) 79(4):e12823. doi: 10.1111/aji.12823

33. Agostinis C, Masat E, Bossi F, Ricci G, Menegazzi R, Lombardelli L, et al. Transcriptomics and immunological analyses reveal a pro-angiogenic and anti-inflammatory phenotype for decidual endothelial cells. *Int J Mol Sci* (2019) 20 (2):1604. doi: 10.3390/ijms20071604

34. Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, et al. Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* (2008) 111(6):3108–15. doi: 10.1182/blood-2007-08-105965

35. Bulla R, Agostinis C, Bossi F, Rizzi L, Debeus A, Tripodo C, et al. Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Mol Immunol* (2008) 45 (9):2629–40. doi: 10.1016/j.molimm.2007.12.025

36. Bulla R, Bossi F, Agostinis C, Radillo O, Colombo F, De Seta F, et al. Complement production by trophoblast cells at the feto-maternal interface. *J Reprod Immunol* (2009) 82(2):119–25. doi: 10.1016/j.jri.2009.06.124

37. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* (1973) 52(11):2745–56. doi: 10.1172/JCI107470

38. Fedchenko N, Reifenrath J. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review. *Diagn Pathol* (2014) 9:221. doi: 10.1186/s13000-014-0221-9

39. Lau SY, Guild SJ, Barrett CJ, Chen Q, McCowan L, Jordan V, et al. Tumor necrosis factor-alpha, interleukin-6, and interleukin-10 levels are altered in preeclampsia: a systematic review and meta-analysis. *Am J Reprod Immunol* (2013) 70(5):412–27. doi: 10.1111/aji.12138

40. Kalinderis M, Papanikolaou A, Kalinderi K, Ioannidou E, Giannoulis C, Karagiannis V, et al. Elevated serum levels of interleukin-6, interleukin-1β and human chorionic gonadotropin in pre-eclampsia. *Am J Reprod Immunol* (2011) 66 (6):468–75. doi: 10.1111/j.1600-0897.2011.01019.x

41. Chaouat G. Placental immunoregulatory factors. *J Reprod Immunol* (1987) 10 (3):179–88. doi: 10.1016/0165-0378(87)90085-4

42. Mincheva-Nilsson L, Baranov V. Placenta-derived exosomes and syncytiotrophoblast microparticles and their role in human reproduction: immune modulation for pregnancy success. *Am J Reprod Immunol* (2014) 72(5):440–57. doi: 10.1111/aji.12311

43. Redman CW, Sargent IL. Immunology of pre-eclampsia. *Am J Reprod Immunol* (2010) 63(6):534–43. doi: 10.1111/j.1600-0897.2010.00831.x

44. Tannetta DS, Dragovic RA, Gardiner C, Redman CW, Sargent IL. Characterisation of syncytiotrophoblast vesicles in normal pregnancy and pre-eclampsia: expression of Flt-1 and endoglin. *PloS One* (2013) 8(2):e56754. doi: 10.1371/journal.pone.0056754

45. Lokki AI, Heikkinen-Eloranta J, Jarva H, Saisto T, Lokki ML, Laivuori H, et al. Complement activation and regulation in preeclamptic placenta. *Front Immunol* (2014) 5:312. doi: 10.3389/fimmu.2014.00312

46. Rayes J, Roumenina LT, Dimitrov JD, Repessé Y, Ing M, Christophe O, et al. The interaction between factor H and VWF increases factor H cofactor activity and regulates VWF prothrombotic status. *Blood* (2014) 123(1):121–5. doi: 10.1182/blood-2013-04-495853

47. Lokki AI, Heikkinen-Eloranta J. Pregnancy induced TMA in severe preeclampsia results from complement-mediated thromboinflammation. *Hum Immunol* (2021) 82 (5):371–8. doi: 10.1016/j.humimm.2021.03.006

48. Belmonte B, Mangogna A, Gulino A, Cancila V, Morello G, Agostinis C, et al. Distinct roles of classical and lectin pathways of complement in preeclamptic placentae. *Front Immunol* (2022) 13:882298. doi: 10.3389/fimmu.2022.882298

49. Savaris RF, Becker C, E. e. P. Guedes Neto: Maternal plasma levels of complement Factor H in miscarriage and in normal pregnancy: A cohort study. *J Reprod Immunol* (2016) 114:1–5. doi: 10.1016/j.jri.2016.01.002

50. Dijkstra DJ, Lokki AI, Gierman LM, Borggreven NV, van der Keur C, Eikmans M, et al. Circulating levels of anti-C1q and anti-factor H autoantibodies and their targets in normal pregnancy and preeclampsia. *Front Immunol* (2022) 13:842451. doi: 10.3389/fimmu.2022.842451

51. Ari E, Yilmaz Y, Gul A, Alahdab YO, Kedrah AE, Macunluoglu B, et al. Human serum complement C3 and factor H in the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Am J Reprod Immunol* (2009) 62(4):238–42. doi: 10.1111/j.1600-0897.2009.00731.x

52. Lokki AI, Ren Z, Triebwasser M, Daly E, Perola M, Auro K, et al. and FINNPEC: Identification of complement factor H variants that predispose to pre-eclampsia: A genetic and functional study. *BJOG* (2023) 130(12):1473-1482. doi: 10.1111/1471-0528.17529

53. Cortes C, Desler C, Mazzoli A, Chen JY, Ferreira VP. The role of properdin and Factor H in disease. *Adv Immunol* (2022) 153:1–90. doi: 10.1016/bs.ai.2021.12.001

54. Cemgil Arikan D, Aral M, Coskun A, Ozer A. Plasma IL-4, IL-8, IL-12, interferon-γ and CRP levels in pregnant women with preeclampsia, and their relation with severity of disease and fetal birth weight. *J Matern Fetal Neonatal Med* (2012) 25(9):1569–73. doi: 10.3109/14767058.2011.648233

55. Spence T, Allsopp PJ, Yeates AJ, Mulhern MS, Strain JJ, McSorley EM. Maternal serum cytokine concentrations in healthy pregnancy and preeclampsia. *J Pregnancy* (2021) 2021:6649608. doi: 10.1155/2021/6649608

56. Trisnawati E, Nontji W, Nurasni S. Tumour necrosis factor-α (TNF-α) serum levels in preeclampsia pregnant women and pregnant women at risk with preeclampsia. *Enfermería Clínica* (2020) 30:27–30. doi: 10.1016/j.enfcli.2019.07.021

57. Mosimann B, Wagner M, Poon LC, Bansal AS, Nicolaides KH. Maternal serum cytokines at 30-33 weeks in the prediction of preeclampsia. *Prenat Diagn* (2013) 33 (9):823–30. doi: 10.1002/pd.4129

58. Szarka A, Rigó J, Lázár L, Beko G, Molvarec A. Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. *BMC Immunol* (2010) 11:59. doi: 10.1186/1471-2172-11-59

59. Sharma D, Singh A, Trivedi SS, Bhattacharjee J. Role of endothelin and inflammatory cytokines in pre-eclampsia - A pilot North Indian study. *Am J Reprod Immunol* (2011) 65(4):428–32. doi: 10.1111/j.1600-0897.2010.00903.x

60. Brooimans RA, van der Ark AA, Buurman WA, van Es LA, Daha MR. Differential regulation of complement factor H and C3 production in human umbilical vein endothelial cells by IFN-gamma and IL-1. *J Immunol* (1990) 144(10):3835–40.

61. Valenzuela FJ, Pérez-Sepúlveda A, Torres MJ, Correa P, Repetto GM, Illanes SE. Pathogenesis of preeclampsia: the genetic component. *J Pregnancy* (2012) 2012:632732. doi: 10.1155/2012/632732

62. Tannetta D, Masliukaite I, Vatish M, Redman C, Sargent I. Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia. *J Reprod Immunol* (2017) 119:98–106. doi: 10.1016/j.jri.2016.08.008

63. Tannetta D, Mackeen M, Kessler B, Sargent I, Redman C. Multi-dimensional protein identification technology analysis of syncytiotrophoblast vesicles released from perfused preeclampsia placentas. *Pregnancy Hypertens* (2012) 2(3):201–2. doi: 10.1016/j.preghy.2012.04.046

64. Baig S, Kothandaraman N, Manikandan J, Rong L, Ee KH, Hill J, et al. Proteomic analysis of human placental syncytiotrophoblast microvesicles in preeclampsia. *Clin Proteomics* (2014) 11(1):40. doi: 10.1186/1559-0275-11-40

65. Mao X, Zhou L, Tey SK, Ma APY, Yeung CLS, Ng TH, et al. Tumour extracellular vesicle-derived Complement Factor H promotes tumorigenesis and metastasis by inhibiting complement-dependent cytotoxicity of tumour cells. *J Extracell Vesicles* (2020) 10(1):e12031. doi: 10.1002/jev2.12031

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 27 March 2024
DOI 10.3389/fimmu.2024.1382424

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Rachel Elaine Hewitt,
University of Cambridge, United Kingdom
Svetlana Dambaeva,
Rosalind Franklin University of Medicine and Science, United States

*CORRESPONDENCE
Jasper Nörenberg
jasper.norenberg@pte.hu
Alíz Barakonyi
barakonyi.aliz@pte.hu

RECEIVED 05 February 2024
ACCEPTED 18 March 2024
PUBLISHED 27 March 2024

CITATION
Nörenberg J, Vida P, Bösmeier I, Forró B, Nörenberg A, Buda Á, Simon D, Erdő-Bonyár S, Jáksó P, Kovács K, Mikó É, Berki T, Mezősi E and Barakonyi A (2024) Decidual γδT cells of early human pregnancy produce angiogenic and immunomodulatory proteins while also possessing cytotoxic potential. *Front. Immunol.* 15:1382424. doi: 10.3389/fimmu.2024.1382424

COPYRIGHT
© 2024 Nörenberg, Vida, Bösmeier, Forró, Nörenberg, Buda, Simon, Erdő-Bonyár, Jáksó, Kovács, Mikó, Berki, Mezősi and Barakonyi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Decidual γδT cells of early human pregnancy produce angiogenic and immunomodulatory proteins while also possessing cytotoxic potential

Jasper Nörenberg[1,2,3]*, Péter Vida[3], Isabell Bösmeier[1], Barbara Forró[4], Anna Nörenberg[2,3,5], Ágnes Buda[3], Diana Simon[2,6], Szabina Erdő-Bonyár[2,6], Pál Jáksó[4], Kálmán Kovács[2,3], Éva Mikó[1,2,5], Tímea Berki[2,6], Emese Mezősi[2,7] and Alíz Barakonyi[1,2,5]*

[1]Department of Medical Microbiology and Immunology, University of Pécs Medical School, Clinical Center, Pécs, Hungary, [2]National Laboratory on Human Reproduction, University of Pécs, Pécs, Hungary, [3]Department of Obstetrics and Gynaecology, University of Pécs Medical School, Clinical Center, Pécs, Hungary, [4]Department of Pathology, University of Pécs Medical School, Clinical Center, Pécs, Hungary, [5]Janos Szentagothai Research Centre, University of Pécs, Pécs, Hungary, [6]Department of Immunology and Biotechnology, University of Pécs Medical School, Clinical Center, Pécs, Hungary, [7]First Department of Internal Medicine, University of Pécs Medical School, Clinical Center, Pécs, Hungary

During pregnancy, the maternal immune system must allow and support the growth of the developing placenta while maintaining the integrity of the mother's body. The trophoblast's unique HLA signature is a key factor in this physiological process. This study focuses on decidual γδT cell populations and examines their expression of receptors that bind to non-classical HLA molecules, HLA-E and HLA-G. We demonstrate that decidual γδT cell subsets, including Vδ1, Vδ2, and double-negative (DN) Vδ1-/Vδ2- cells express HLA-specific regulatory receptors, such as NKG2C, NKG2A, ILT2, and KIR2DL4, each with varying dominance. Furthermore, decidual γδT cells produce cytokines (G-CSF, FGF2) and cytotoxic mediators (Granulysin, IFN-γ), suggesting functions in placental growth and pathogen defense. However, these processes seem to be controlled by factors other than trophoblast-derived non-classical HLA molecules. These findings indicate that decidual γδT cells have the potential to actively contribute to the maintenance of healthy human pregnancy.

KEYWORDS

decidua, γδT cells, HLA-E, HLA-G, NK receptors, cytokines, angiogenic factors, cytotoxic mediators

1 Introduction

During pregnancy, the coexistence of two genetically and immunologically different individuals within one body challenges primary transplantation and tumor physiology concepts. In their context, the maternal immune system's task is to ensure the integrity of the mother's body and remove foreign or dysplastic tissues. However, the maternal immune system does not attack embryonal or fetal tissues but supports implantation, placentation, and fetal growth (1–3).

In human pregnancy, the trophoblast infiltrates deeply into the decidua and spiral arteries, allowing the establishment of a hemochorial placenta. This type of placentation, in which maternal blood is in direct contact with fetal tissues, ensures a sufficient supply of oxygen and nutrients. Insufficient supply, caused by weak trophoblast invasion during the first trimester, may lead to human pregnancy disorders, like fetal growth restriction or preeclampsia (4–6). Furthermore, early pregnancy loss or infertility cases might be connected to even weaker implantation and invasion. Research of the last decades emphasizes the importance of the interaction of trophoblast and decidua for controlling invasion depth and establishing a healthy placenta (7–9). Cases of the placenta accreta spectrum, in which the trophoblast might even invade neighboring organs, highlight the role of the decidua in this process as they commonly occur when the blastocyte implants at the site of a uterine scar, where decidua is absent (9–11).

At the time of receptivity (window of implantation), leukocytes accumulate in the decidua, dominated by a unique CD56^{bright} innate lymphoid cell population, commonly known as uterine/decidual NK (u/dNK) cells (12). Decidual NK cells have an array of activating and inhibiting receptors, which bind specific classical and non-classical HLA class I molecules (13–17). The extravillous cytotrophoblast (EVT), which is in direct contact with the decidua and invades uterine spiral arteries (Figure 1A), is unique in its HLA class I expression pattern: The highly variable HLA-A and -B are not expressed by the EVT. Instead, its cells express HLA-C and the oligomorphic HLA-E and -G on their cell surface. Under physiological circumstances, HLA-G is exclusively known to be expressed by the EVT (18, 19). Next to the membrane-bound form of HLA-E and HLA-G (mHLA-E/-G), soluble forms (sHLA-E/-G) have been found in the sera of pregnant women (20–23).

Next to dNK cells, γδT cells and their potential roles during pregnancy have attracted interest. They are well known to surveil the tissue integrity of frontiers between the organism and the environment (24, 25). Studies reported a higher prevalence of γδT cells among decidual CD3$^+$ cells compared to the peripheral blood (26–28). These decidual γδT cells, like dNK cells, are either clustered proximate to decidual glands or scattered as intraepithelial lymphocytes (26). The association of decidual γδT cells and dNK cells to glands might be connected to the invasion of the EVT, as the EVT penetrates not only spiral arteries but uterine glands (29). A growing body of evidence attributes a central role to

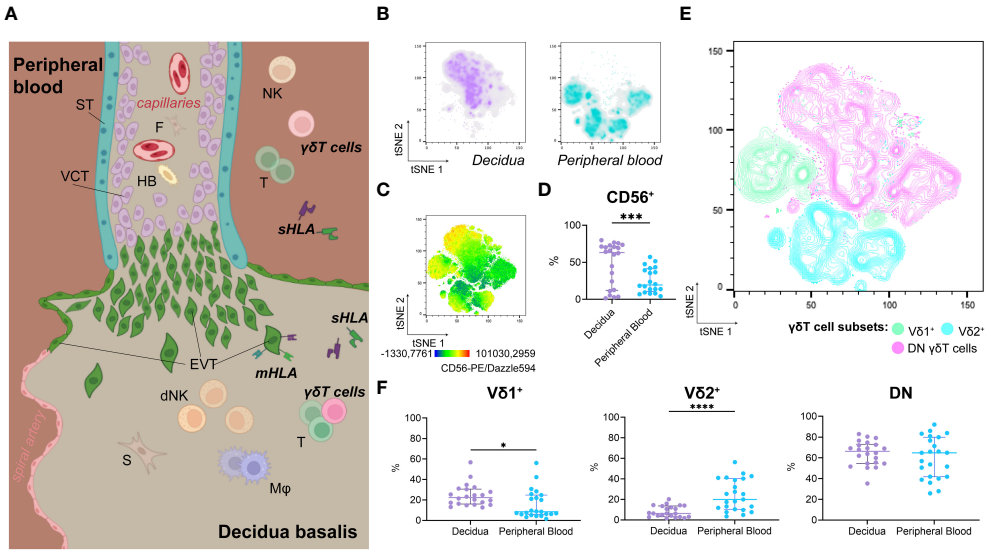


FIGURE 1
(A) Illustration of anatomical structure and spatial relations at the feto-maternal interface. **(B)** Representative isolated depiction of tSNE-clustered decidual (left) and maternal peripheral blood (right) γδT cells as density plots (n=1; Panel A, also see Supplementary Information 1B). **(C)** Representative tSNE-plot depicting fluorescence intensity of CD56-PE/Dazzle594^{TM} on γδT cells from peripheral blood and decidua (n=1; Panel A, also see Supplementary Information 1C). **(D)** Statistical comparison of CD56$^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23) (see also Supplementary Information 1D). **(E)** Representative contour plot overlay of Vδ1$^+$, Vδ2$^+$, and DN γδT cells on clustered γδT cells from peripheral blood and decidua (n=1; Panel A, also see Supplementary Information 1E). **(F)** Statistical comparison of Vδ1$^+$, Vδ2$^+$, and DN γδT cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). Testing for significance was performed with the Wilcoxon test. *: $p \leq 0.05$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$; ST, syncytiotrophoblast; VCT, villous cytotrophoblast; F, fibroblast; HB, Hofbauer cell; (d) NK, (decidual) Natural killer cell; T, T cell; s/mHLA, soluble/membrane-bound Human Leukocyte Antigen class I; EVT, extravillous cytotrophoblast; S, stroma cell; Mφ, Macrophage.

these glands, providing nutrients, growth factors, and cytokines during placentation (29–31).

Innate lymphoid cells, like dNK cells, and γδT are closely related. Although γδT cells have an antigen-recognition receptor, they rely on an arsenal of cytotoxicity receptors for their activity. Furthermore, in contrast to αβT cells, they are not MHC-restricted for their antigen recognition. However, some of these cytotoxicity-related receptors bind HLA class I molecules and transmit activating or inhibiting signaling upon ligation. Consequently, HLA expression is likely to influence γδT cell behavior.

Considering the EVT's unique HLA class I expression profile, three receptor groups come into focus at the maternal-fetal interface. The NKG2 receptor family, with a particular emphasis on activating NKG2C and inhibitory NKG2A, plays a pivotal role in recognizing HLA-E expression (32, 33). HLA-G, conversely, can be bound by KIR2DL4, a receptor from the Killer cell Immunoglobulin-like Receptor (KIR) family (CD158). This family is mainly known for HLA-C binding. However, KIR2DL4 stands out among the KIR receptor family due to its unique ligand preference, location, and function. Unlike other KIR receptors, KIR2DL4 is predominantly located intracellularly and is only expressed on the cell surface during activation states. Although its molecular structure suggests an inhibitory function, KIR2DL4-ligation was shown to trigger cytokine release (34–36). Last, Immunoglobulin-like transcript (ILT2), a member of the leukocyte immunoglobulin-like receptor subfamily B, binds HLA class I molecules, including HLA-G, and transmits an inhibitory signal upon ligation (17, 33).

In addition, the non-classical MHC molecule CD1d, which presents lipid antigens, has the potential to facilitate antigen recognition through the γδTCR (37–39). However, further signals will influence the crosstalk between maternal γδT cells and the fetal EVT in the decidua. Although, investigations in tumor immunology have already demonstrated the expression of different non-classical HLA receptors (40), a detailed expression profile of these receptors on decidual γδT cells has not been published.

Here, we present an expression profile for HLA-E- or HLA-G-binding receptors of decidual γδT cells during early pregnancy. Furthermore, we investigated the potential consequences of respective receptor-ligand interactions. In this context, we focused on γδT cells' secretion of mediators, which may influence vascular transformation or pathogen defense.

2 Results

2.1 Heterogeneity of peripheral and decidual γδT cells during early pregnancy

We found no significant difference between decidual and peripheral blood γδT cells' prevalence among $CD45^+$/live cells (Supplementary Information 1A). To characterize decidual γδT cells and compare them to their circulating counterparts, we utilized the downsampling plugin of FlowJo™ and concatenated previously gated γδT cell populations from decidual mononuclear cells (DMCs) and peripheral blood mononuclear cells (PBMCs). Defined separate clusters with minimal overlap were assigned to decidual or peripheral blood γδT cells, respectively (Figures 1A, B, see also Supplementary Information 1B).

Due to the biological similarities between NK cells and γδT, we investigated the expression of CD56 on γδT cells. While $CD56^{dim}$ expression was detectable in several peripheral blood γδT cell clusters, decidual γδT cells exhibited both $CD56^{dim}$ and $CD56^{bright}$ phenotypes (Figure 1C, see also Supplementary Information 1C). Nevertheless, $CD56^+$ γδT cells are more prevalent in the decidua than in the periphery (Figure 1D, see also Supplementary Information 1D).

Classical γδT cell subsets were associated with distinct clusters. $V\delta1^+$ ($CD45^+TCR\gamma\delta^+V\delta1^+V\delta2^-$) cells were more prevalent in the decidua, while $V\delta2^+$ ($CD45^+TCR\gamma\delta^+V\delta1^-V\delta2^+$) cells were more common among circulating γδT cells. However, double-negative (DN, $CD45^+TCR\gamma\delta^+V\delta1^-V\delta2^-$) γδT cells were the most common in both decidual and peripheral blood (Figures 1E, F, see also Supplementary Information 1F).

2.2 Decidual γδT subsets express receptors that bind to HLA-E or HLA-G molecules

Using two flow cytometric panels (Supplementary Information 6), we investigated the prevalence and expression of HLA-E and HLA-G-binding receptors (NKG2C, NKG2A, and ILT2, KIR2DL4, respectively) on γδT cell subsets in the decidua and the matched peripheral blood (Figure 2A, see also Supplementary Information 1B, E). To estimate the expression intensity, we compared the median fluorescence intensity (normalized to the respective FMO) of all investigated receptors. Peripheral γδT cells show higher expression intensity for KIR2DL4 or ILT2 receptors than that for NKG2C and NKG2A. Within the different decidual γδT cell subpopulations, decidual DN γδT cells exhibited relatively high expression levels for all investigated receptors, while decidual $V\delta1^+$ cells showed a more focused expression of the activating NKG2C and the inhibiting ILT2. In contrast, decidual $V\delta2^+$ cells expressed significantly more NKG2A on their cell surface (Figure 2B, see also Supplementary Information 2A, B).

The prevalence of $NKG2C^+$ cells was generally higher among decidual γδT cells compared to the periphery. However, this difference reached the level of significance only in the $V\delta2^+$ and DN subsets. Furthermore, NKG2C positivity was significantly more common in the $V\delta1^+$ subset compared to the $V\delta2^+$ one (Figure 2C). Likewise, cells expressing the inhibitory counterpart NKG2A were more prevalent in the decidua. While the percentage of $NKG2A^+$ cells among $V\delta2^+$ cells did not differ between decidua and peripheral blood, a significantly higher proportion of DN γδT cells expresses NKG2A and NKG2C in the decidua compared to the periphery (Figure 2D).

The inhibitory HLA-G-binding ILT2 was commonly expressed by γδT cells independently of their origin. Generally, $ILT2^+$ cells were less prevalent in the $V\delta2^+$ subsets than in other γδT cell populations. However, when focusing on the prevalence of $ILT2^+$ cells within each γδT cell subset, significantly fewer decidual DN γδT cells expressed ILT2 than their peripheral blood counterpart (Figure 2E). The HLA-G-binding KIR2DL4 was expressed by the majority of γδT cells (Figure 2F).

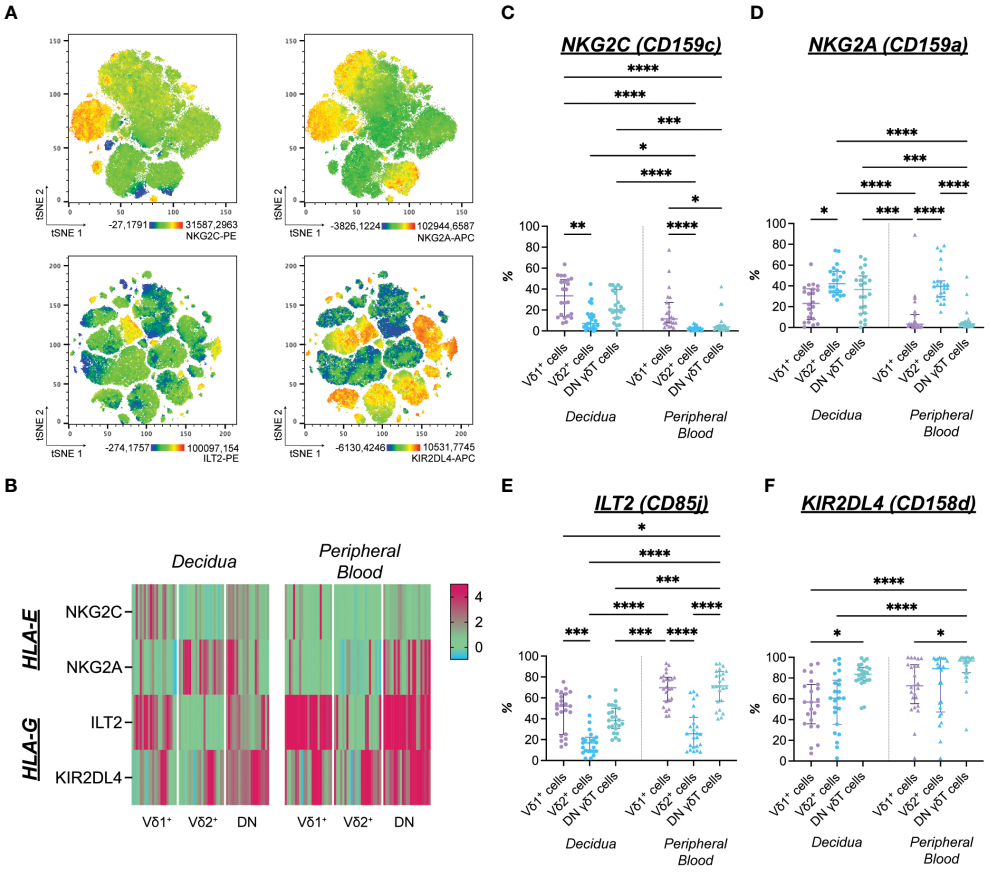


FIGURE 2
(A) Expression distribution of NKG2C-PE (upper left), NKG2A-APC (upper right) of flow cytometric Panel A, ILT2-PE (lower left, see also Supplementary Information A/D), and KIR2DL4-APC (lower right, see also Supplementary Information A/D) of flow cytometric panel B depicted as representative tSNE plots of paired, concatenated decidual and peripheral blood γδT cells (n=1). **(B)** Heatmap of standardized median fluorescence intensity ($[Median_{Subset}\text{-}Median_{FMO}]/rSD_{FMO}$) of NKG2C-PE, NKG2A-APC, ILT2-PE, KIR2DL4-APC on matched decidual and peripheral blood γδT cell subsets (n=22; also see Supplementary Information 2). **(C)** Statistical comparison of $NKG2C^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). **(D)** Statistical comparison of $NKG2A^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). **(E)** Statistical comparison of $ILT2^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). **(F)** Statistical comparison of $KIR2DL4^+$ cells' prevalence among γδT cells from the decidua (n=22) and peripheral blood (n=23). Testing for significance was performed with the Kruskal-Wallis test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$****: $p \leq 0.0001$.

2.3 Decidual γδT cells secrete trophoblastotropic cytokines

To determine the functional consequences of the HLA-E or -G recognition by γδT cells, we incubated purified γδT cells with soluble HLA-E or -G (sHLA-E/-G). Furthermore, we utilized human choriocarcinoma cell lines (JAr) transfected with HLA-E or $HLA\text{-}G_{1m}$ to investigate more complex interactions of membrane-bound HLA-E or -G (mHLA-E/-G) (Figure 3A).

Vascular transformation by the trophoblast and the local immune environment is crucial to establishing a healthy placenta during early pregnancy. Therefore, we analyzed the collected cell co-culture supernatants for potential angiogenic cytokines (Figure 3B). When comparing peripheral blood to decidual γδT cells without HLA molecules ("Control (sHLA)" in Figure 3B), we found significantly higher levels of G-CSF produced by the decidual ones (Figure 3C). Furthermore, decidual γδT cells produced FGF-2, whereas no FGF-2 was detected in the wells of peripheral γδT cells (Figure 3D). On the other hand, peripheral blood γδT cells produce small amounts of EGF, which was not detected in the wells of decidual samples (Figure 3E). While the production of most measured cytokines was not influenced by the presence or absence of HLA-E or -G molecules in our experimental model, incubating mHLA-G with γδT cells, independently from their origin, increased the measured Leptin concentrations (Figure 3F). Additionally, we detected elevated concentrations of Follistatin when incubating peripheral blood γδT cells with mHLA-E. However, compared to all other co-culture wells, the level of significance was not reached (Figure 3G).

2.4 Decidual γδT cells are strong producers of cytotoxic mediators

γδT cells act as first responders in the mucosal defense against pathogens and many frontiers between the body and its environment. Therefore, we also analyzed the intracellular perforin content to determine each γδT cell subset's cytotoxic potential in the decidua.

10.3389/fimmu.2024.1382424

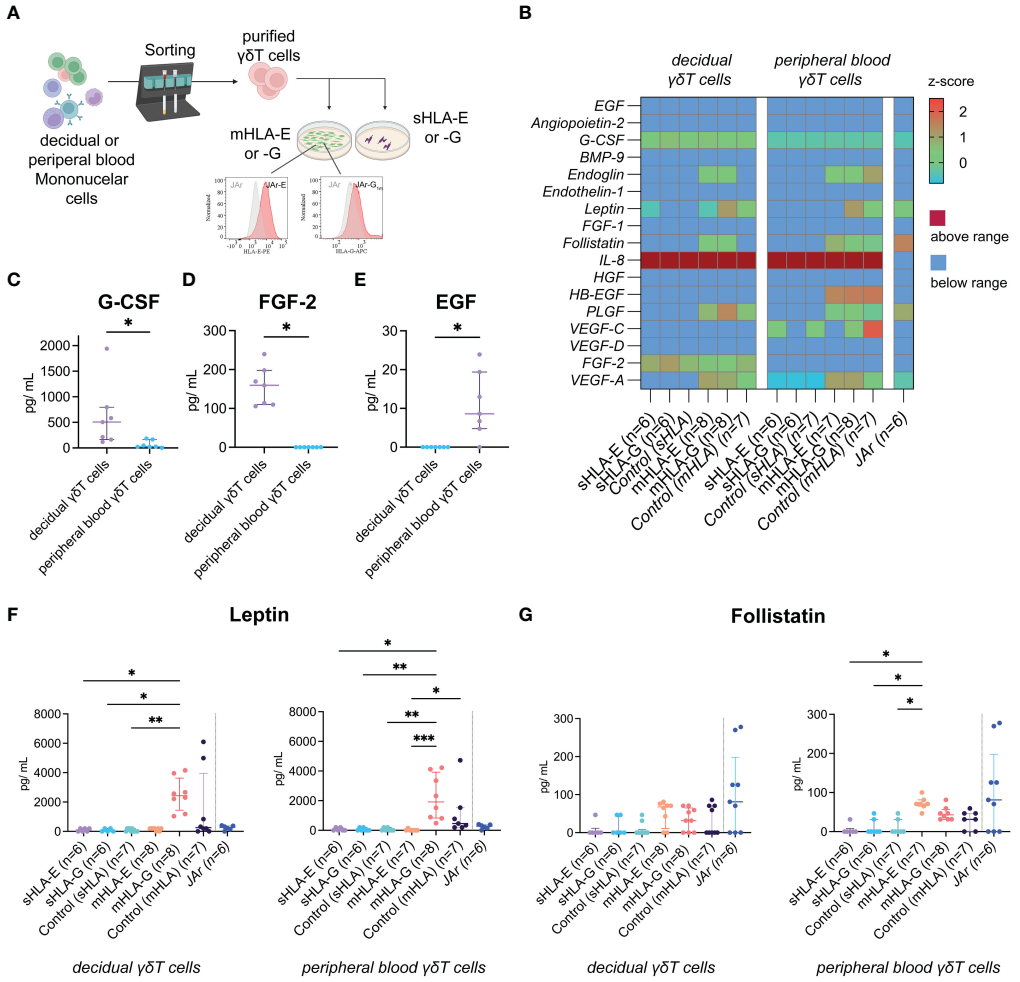


FIGURE 3
(A) Illustration of the experimental setup. **(B)** Heatmap depicting the z-score of the respective angiogenic factor after incubating peripheral blood (left) or decidual (right) γδT cells with soluble (s) or membrane-bound (m)HLA-E or -G. The last column depicts measurements from the human choriocarcinoma cell line JAr without γδT cells as an additional control (also see Supplementary Information 3). **(C)** Statistical comparison of G-CSF concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(D)** Statistical comparison of FGF-2 concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(E)** Statistical comparison of EGF concentrations secreted from decidual (n=7) or peripheral blood (n=7) γδT cells without HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(F)** Statistical comparison of Leptin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. **(G)** Statistical comparison of Follistatin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

Although in the periphery significantly less intracellular Perforin was measured in DN γδT cells compared to the Vδ2 population (Supplementary Information 4A, B), the Perforin content of the different γδT cell subsets did not differ in the decidua. Figure 4A confirms the distinct features of Perforin-positive decidual γδT cell subpopulations, as analyzed in our two flow cytometric antibody panels. (Figure 4A, see also Supplementary Information 4A, B).

Upon interaction with HLA-E or HLA-G, NKG2C, NKG2A, ILT2, and KIR2DL4 are potential regulators of the cytotoxic capability of immune cells. Investigating the perforin content of the different NK receptor-expressing decidual γδT cell populations, we found that the expression of NKG2C and ILT2 was associated with significantly higher levels of intracellular perforin in all decidual γδT cell subsets. The expression of NKG2A, however, correlated only in the $V\delta1^+$ and DN γδT subset with higher levels of intracellular perforin. A similar, significant relation between KIR2DL4 expression and perforin content was only detectable in the DN γδT cell subset (Figure 4B).

To determine if this hypothetical relationship between cytotoxicity and the expression of HLA class I binding receptors has functional consequences, we analyzed the secretion of typical NK cell cytokines and cytotoxicity-related soluble molecules after exposure to sHLA-E/-G or mHLA-E/-G (Figure 4C, see also Supplementary Information 4C-N). However, the measured perforin concentration did not differ significantly (Figure 4D). In addition, we found that decidual γδT cells secrete excessive amounts of granulysin and high levels of interferon-γ (IFN-γ) (Figure 4E).

10.3389/fimmu.2024.1382424

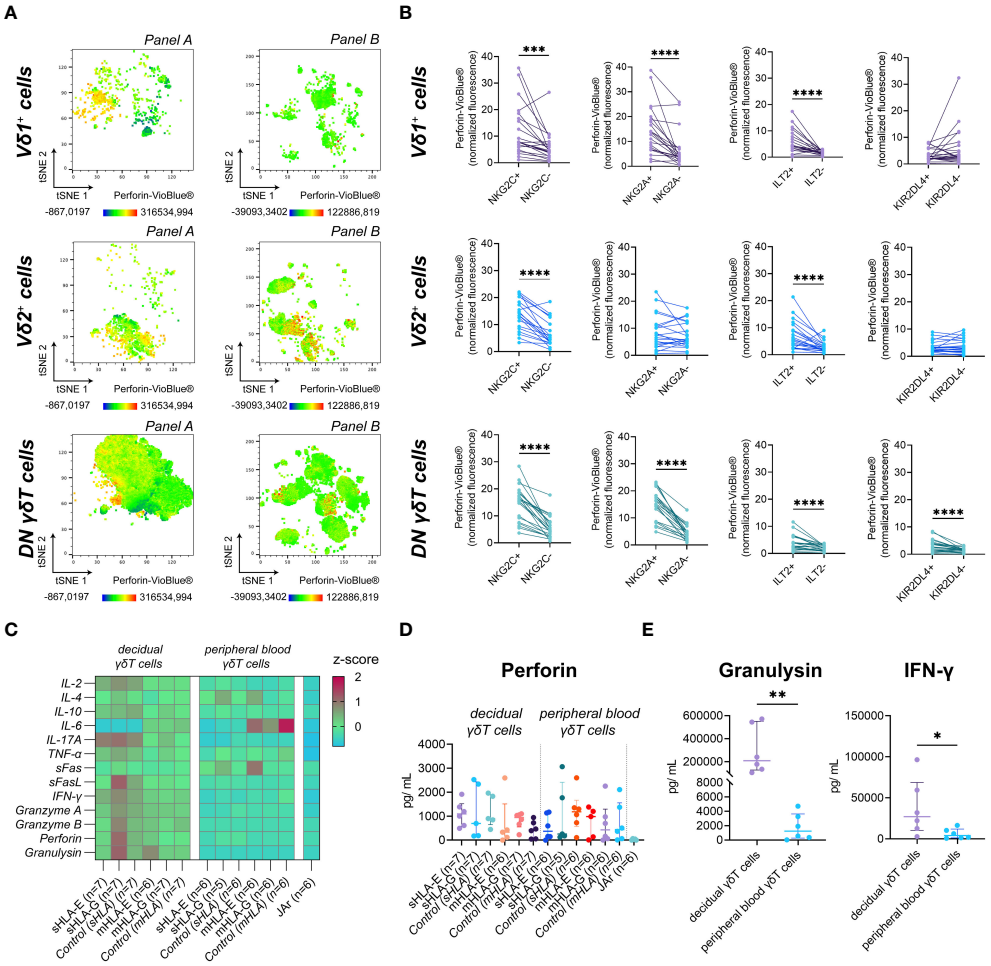


FIGURE 4
(A) Fluorescence intensity of Perforin-VioBlue® on gated γδT cell subsets from the decidua for the two flow cytometric panels depicted as representative tSNE plots (n=1). **(B)** Statistical association of standardized Perforin-VioBlue® median fluorescence intensity ([$Median_{Subset}$-$Median_{FMO}$]/rSD_{FMO}) to the expression of NKG2C, NKG2A, ILT2, and KIR2DL4 on decidual γδT cell subsets (n=22). Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. **(C)** Heatmap depicting the z-score of the respective soluble factor after incubating decidual (left) or peripheral blood (right) γδT cells with soluble (s) or membrane-bound (m) HLA-E or -G. **(D)** Statistical comparison of Perforin concentrations measured after incubating peripheral blood or decidual γδT cells with sHLA or mHLA molecules. Testing for significance was performed with the Kruskal-Wallis test. **(E)** Statistical comparison of Granulysin (left) and INF-γ (right) concentrations secreted from decidual (n=6) or peripheral blood (n=6) γδT cells in the absence of HLA molecules. Testing for significance was performed with the Wilcoxon matched-pairs signed rank test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

3 Discussion

Decidual γδT cells may have several functions in the microenvironment of the maternal-fetal interface between the decidua basalis and the extravillous trophoblast. They could influence the process of implantation and placentation via cytokine secretion or clear out pathogens.

The prevalence of γδT cells among $CD45^+$/live cells was not different between the decidua and the peripheral blood. However, it is important to note that literature data shows that while the prevalence of T cells ($CD3^+$) among all lymphocytes is around 70% in the peripheral blood, it is only around 15% in the early human decidua (41). Furthermore, according to previously published data, the population of γδT cells is markedly expanded in the decidua compared to the periphery (28, 41). Thus, due to the lack of CD3 staining, our prevalence data must be interpreted carefully.

As γδT cells exhibit innate and adaptive immunity aspects, they are often described as a bridge between these two parts of the immune system. Although a more didactic subdivision, γδT cells show quite some plasticity, and some subsets (e.g., Vγ9Vδ2) behave more in an innate manner, while others (e.g., Vδ1) show rather adaptive features (42–49). Therefore, when investigating γδT cells in any context, determining the prevalent subpopulations is a crucial first step before further conclusions.

The increase in the Vδ1 subset is typical for all tissues that frontier the environment and is often understood as a "first line defense" (24, 50). Our data on the prevalence of Vδ1 and Vδ2 subsets are in line with previously published flow cytometric data (51). However, these publications ignored the largest decidual γδT cell population, which expresses neither the Vδ1 nor the Vδ2 chain. Different variants of Vδ-chains exist, which makes DN γδT cells a heterogeneous population. However, a recent study used

sequencing to show that the early human decidua is only inhabited by the Vδ1$^+$, Vδ2$^+$, and Vδ3$^+$ γδT cell subsets (52). Therefore, DN γδT cells of the decidua can be considered as Vδ3$^+$ cells. The Vδ3 subset is generally assumed to induce antigen-presenting cell maturation (53). In the context of pregnancy, the correct presentation of antigens is essential. Further investigations will be necessary to determine if decidual Vδ3 cells could direct antigen-presenting cell maturation toward a tolerance-promoting phenotype. Currently, anti-Vδ3 antibodies are not commercially available. Therefore, we cannot finally confirm that the DN subset represents the Vδ3-one, so henceforward, we refer to this population as DN γδT cells.

Interestingly, the majority of decidual γδT cells are CD56$^+$, suggesting a special characteristic for decidual γδT cells. In addition, all three γδT cell populations contain cell clusters of CD56-expressing cells, which are more prevalent in the decidua. Although these decidual 'NKγδT-like' cells may be associated with dNK cells, as the expression of CD56 on lymphoid cells serves rather as a phenotypical marker, conclusions must be made carefully. To date, investigations indicating physiological similarities are still outstanding.

As mentioned previously, the trophoblast's unique expression pattern of HLA class I molecules is crucial in allowing temporal chimerism during pregnancy. HLA molecules are absent at the maternal-fetal interface between the circulating maternal blood and the fetal syncytiotrophoblast in the intervillous spaces, which may explain why circulating adaptive immune cells do not drive an immune response at this location. However, the decidua directly interacts with the extravillous trophoblast expressing HLA-C, HLA-E, and HLA-G. Under physiological circumstances, HLA-G is uniquely expressed at this interface and is believed to influence the maternal immune system to accept and support viviparity. The expression of HLA-E, on the other hand, is connected to the expression of other HLA class I molecules, as it is loaded with their leader sequence peptides. Therefore, its expression on the cell surface usually is proportional to a given cell's HLA class I expression level (54, 55). In cases of viral infection or malignancies, the expression of HLA class I molecules may alter, which consequently will be reflected in the expression of HLA-E. This can be detected by NK or cytotoxic T cells via the activating receptor NKG2C or the inhibitory receptor NKG2A. However, tumors may utilize upregulated HLA-E expression or secretion as an escape mechanism. Therefore, the extravillous trophoblast's HLA-E positivity might simply result from its HLA-G expression.

Control of γδT cell function is essential, where trophoblast-expressed non-classical HLA molecules could serve as potent mediators. Among all decidual γδT cell subsets, cells expressing receptors for HLA-E or HLA-G are prevalent, suggesting an efficient influence of these non-classical HLA molecules. Especially the decidual Vδ1$^+$ subset shows high expression levels of NKG2C and co-expresses NKG2A with lower intensity. However, considering the higher affinity of NKG2A to HLA-E, this difference in surface expression may be irrelevant (32). Both ILT2 and KIR2DL4 have been shown to bind various ligands, including HLA-G (33, 34). Next to its physiological expression and secretion by the EVT during pregnancy, malignancies are known to express and secret HLA-G. In this context, its ITL2-mediated inhibitory effects on Vγ9Vδ2 cells are well known [reviewed in (56)], and similar effects can be expected in the decidual microenvironment. KIR2DL4 is a unique Killer cell immunoglobulin-like receptor family member, as it is reportedly expressed on the cell surface or intracellularly by activated or non-activated NK cells. According to our findings, both decidual and peripheral γδT cells only express KIR2DL4 intracellularly. Considering its location, interactions with sHLA-G are more likely. However, intercellular HLA-G transfer through mechanisms like trogocytosis, nanotube transfer, or exosome provides an alternative possibility for KIR2DL4-HLA-G interactions (57). Despite its molecular structure suggesting an inhibitory function, it has been shown that the consequence of its ligation depends on the context. Rajagopalan et al., for example, demonstrated the production of angiogenic factors by peripheral blood NK cells due to KIR2DL4 ligation (36, 58).

For deeper investigation of the role of HLA-E and HLA-G in the functional control of different γδT cell subpopulations in the decidua, we analyzed the proportions of HLA class I-binding receptor expressing decidual γδT subsets. Decidual Vδ2 cells showed a reduced potential for activation and an increased potential for inhibition via HLA-E. Therefore, we suppose that HLA-E could be responsible, among others, for the control of Vδ2 cell function in the placenta. Regarding HLA-G, our data suggest that this non-classical HLA molecule preferentially regulates not only decidual γδT cells but also peripheral Vδ1 and DN γδT cells in its soluble form. These effects are mediated through ILT2 and KIR2DL4 receptor functions. In addition, because of the high ratio of KIR2DL4 expressing peripheral Vδ2 cells, soluble HLA-G is potentially able to inhibit Vδ2 cells also. This peripheral inhibition of γδT cell subsets could be part of the known systemic immunological adaptation during pregnancy, which could be measured in the peripheral blood also.

A further interesting issue is whether peripheral and decidual γδT cells differ in their NK cell receptor expression patterns. Regarding the proportion of NK receptor-positive cells, decidual Vδ1 or Vδ2 cells do not differ significantly in their inhibitory NK receptor expression pattern from their peripheral counterpart; however, decidual and peripheral DN γδT cells are phenotypically different and therefore show presumably distinct regulation by the non-classical HLA molecules. Here, in contrast to the peripheral DN γδT cells, the binding of the HLA-E molecule can result in both inhibitory and activating signals in the decidua. Whereas HLA-G primarily inhibits decidual DN γδT cells via KIR2DL4 rather than ILT2.

Although activated decidual γδT cells produce angiogenic factors (G-CSF, FGF-2), the presence of HLA-G did not affect their production *in vitro*. G-CSF is also secreted by decidual NK cells and promotes the disorganization of vascular muscles. This, in turn, aids the invasion of the extravillous trophoblast into the spiral arteries, increasing the blood supply in the placental bed (59, 60). FGF-2, on the other hand, activates MAPK signaling and was assumed to improve endometrial receptivity (61). Furthermore, it was demonstrated that FGF-2 improves proliferation and survival of trophoblast organoid cultures *in vitro* (62). Although the

concentrations of FGF-2 in our experiments were significantly lower than in the the trophoblast culture experiments, it demonstrates that decidual γδT cells contribute to the creation of a nursing environment for the invading trophoblast. Leptin is produced by trophoblast tissue and is known to support trophoblast invasion (63). Administration of leptin increases HLA-G expression on the EVT *in vitro* (64). In this context, we hypothesize that higher leptin concentrations could be connected to the expression of HLA-G itself, indicating an interdependent relationship between HLA-G expression and leptin secretion. In our experiments, leptin concentrations increased only in the presence of decidual but also peripheral blood γδT cells, without a significant difference between the two experimental settings. This generally emphasizes the relevance of immune cells for leptin secretion. Other angiogenic factors like endoglin, placenta growth factor, or vascular endothelial growth factor, which were detected in our experiments, are known to be secreted by trophoblast tissue (65).

Regarding potential defense mechanisms, decidual γδT cells' intracellular perforin level correlates positively with NKG2C and ILT2 expression on all γδT subsets and with NKG2A for Vδ1 and DN subsets. However, our experiments do not show altered secretion of perforin in the presence of HLA-E or -G. While innate lymphoid cells utilize various activating and inhibiting receptors for their activity, γδT cells can use their TCR for antigen recognition. Our results suggest no immediate consequences for the isolated presence of either HLA-E or -G. However, long-term consequences are possible and likely. In peripheral blood NK cells, these receptors are not just associated with immediate cell reaction but also with a process of activation threshold alteration called NK cell education (66). We suggest a similar process might also be possible for γδT cells.

Decidual γδT cells secrete high levels of IFN-γ and impressive levels of granulysin. This emphasizes their importance in pathogen defense, as granulysin allows pathogen eradication from virus-infected trophoblast cells without harming the trophoblast itself (67).

Our findings highlight the multifaceted functions and interactions of γδT cells in decidua during the first trimester, confirming the concept that γδT cells are potential effector immune cells at the feto-maternal interface, contributing to healthy pregnancy. The presented data provide further evidence that decidual γδT lymphocytes significantly differ from peripheral γδT cells - they produce angiogenic and immunomodulatory proteins, have conserved or even increased cytotoxic potential, and they could be controlled by non-classical HLA molecules. Accordingly, HLA-G and HLA-E, expressed by EVT can influence decidual γδT cell function through receptors like NKG2C, NKG2A, ILT2, and KIR2DL4, which interactions may modulate immune responses, adding another layer of complexity to the maternal-fetal interface. However, the detailed consequences of the cross-linking of the non-classical HLA molecules and their receptors on the different γδT cell subpopulations remain to be elucidated in the future. Moreover, our study also reveals the potential research interest of the under-researched DN γδT cell population, which could be a promising target for further investigations in reproductive immunology.

4 Materials and methods

4.1 Human samples

Decidual tissue samples and matched peripheral blood were obtained from healthy pregnant women (n = 27, age *(mean ± SD)* = 25.9 ± 1.4) undertaking an elective pregnancy termination during the first trimester (gestational age *(mean ± SD)*= 9.3 ± 0.3) in the Department of Obstetrics and Gynecology, University of Pécs, Medical School, Hungary.

4.2 Isolation of decidual mononuclear cells

The pregnancy was terminated by vacuum aspiration, and the collected tissue was immediately processed. First, the collected decidual pieces were macroscopically homogenized with scissors (approximately 2 mm^3). Hereafter, the tissue was resuspended with prewarmed (37°C) collagenase type IV (1 mg/mL, Gibco®) and transferred to C-tubes (Miltenyi Biotec). To create a single-cell solution, the samples were then further dissected using a gentleMACS™ dissociator (Miltenyi Biotec) with three fast contrarotating cycles (800 rpm/25 sec/cycle) and slow agitation (40 rpm) for one hour at 37°C. After that, the cells were collected through successive 100 µm, 70 µm, and 40 µm nylon cell strainers (Miltenyi Biotec) and washed in RPMI1640 medium (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza). In the next step, decidual mononuclear cells (DMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. The collected cells were washed and resuspended in RPMI1640 medium (Lonza) containing 20% fetal calf serum (Gibco®) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza). The resuspended cells were distributed onto cell culture dishes and incubated overnight at 37°C and 5% CO_2 to allow the remaining decidual stroma cells to settle and adhere. The next morning, the non-adherent cells were aspirated, washed, controlled for viability with trypan blue, and split for cryopreservation and isolation of γδT cells. Cryopreserved DMCs were used for flow cytometric measurements, while isolated decidual γδT cells were co-cultured with choriocarcinoma cell lines or soluble HLA proteins.

4.3 Isolation of peripheral blood mononuclear cells

Heparinized peripheral blood was diluted with phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ (GE Healthcare) gradient centrifugation. Hereafter, the collected cells were washed and resuspended in RPMI1640 medium (Lonza) containing 20% fetal calf serum (Gibco®) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza), then incubated overnight at 37°C and 5% CO_2. The next morning cells were controlled for viability and split cryopreservation and isolation of γδT cells. Cryopreserved PBMCs were used for flow cytometric

measurements, while isolated peripheral γδT cells were co-cultured with choriocarcinoma cell lines or soluble HLA proteins.

4.4 Cryopreservation

The washed cells were resuspended in heat-inactivated human serum containing 10% dimethyl sulfoxide and frozen at -80°C utilizing MrFrosty™ Freezing Container (Thermo Scientific™) for later analysis.

4.5 Isolation of γδT cells

Decidual and peripheral blood γδT cells were isolated using the 'TCRγ/δ$^+$ T cell Isolation Kit' (Miltenyi Biotech) according to the manufacturer's instructions. The purity of the yielded γδT cells was determined by flow cytometry, and samples showing more than 90% γδT cells (γδTCR$^+$/all living cells) were used for cell co-culture experiments (n=8). Although the precise composition of the antibody cocktail used in this kit is not publicly available, in-house testing confirmed the efficient elimination of CD56$^+$ cells.

4.6 Flow cytometry

Matched decidual and peripheral blood cryopreserved cells of 27 participants were thawed and transferred into prewarmed (37°C) RPMI1640 medium (Lonza) supplemented with 10% fetal calf serum (Gibco®), penicillin (1 x 10^5 U/L) (Lonza) streptomycin (0.05 g/L) (Lonza), and DNase (20µg/mL) (Sigma). Then, the cells were washed at 400x*g* for 7 min, resuspended in protein-free PBS, distributed into round-bottom polystyrene tubes (2 x 10^6/tube), and washed at 400x*g* for 7 min in protein-free PBS. Consecutively, the cells were stained for viability (according to the manufacturer's instructions) and surface antigens (30 min at RT in the dark). Then, the cells were fixed and permeabilized for intracellular target (Perforin, KIR2DL4) staining utilizing the InsideStain Kit (according to the manufacturer's instructions) (Miltenyi Biotec). The fluorochrome-conjugated antibodies used in each panel are summarized in Supplementary Table 1. Finally, the cells were resuspended in PBS with 1% paraformaldehyde and stored in the dark at 4°C until measurement on a Navios™ flow cytometer (Beckman Coulter). Due to low live cell count or poor sample quality, five decidual and four peripheral blood samples were excluded during preanalytical quality control in FlowJo™. Compensation matrices were calculated by FlowJo™ using CompBeads (BD™) and MACS® Comp Bead Kit, anti-REA (Miltenyi Biotec) for fluorochrome-labeled antibodies, and PBMCs for the viability dye. Gamma/delta T cells were defined as lymphocytes → single cells → ZombieNIR$^-$CD45$^+$TCRγδ$^+$ events. Decidual cells were further defined as residency marker (CD69) positive to exclude peripheral blood-derived cells in the decidual sample (68). All gates are based on fluorescence-minus-one controls (FMO; also see Supplementary Information 5). Due to day-to-day variability and the different fluorophores, we standardized fluorescence intensity data to the individual FMO:

$$Standardized\ Fluorescence\ Intensity = \frac{Median_{Population} - Median_{FMO}}{robust\ standard\ deviation_{FMO}}$$

4.7 Cell (co-)culture

Three human choriocarcinoma cell lines (JAr) were used as model tissues: A standard JAr cell line (HLA class I$^-$) and the two JAr lines transfected with either HLA-E or HLA-G$_{1m}$ (JArE and JArG$_{1m}$, respectively).

The cell lines were donated by P. Le Bouteiller (INSERM UMR 1043, Toulouse, France). JArG$_{1m}$ was produced by transfection of the pCDNA3/*HLA-G1m* plasmid, a gift of Dr. M. Lopez-Botet (Department of Immunology, University Hospital la Princesa, Madrid, Spain), in which the HLA-G leader sequence was modified as follows: the methionyl residue at position 2 was mutated to threonine; therefore, it could not provide a functional signal peptide for the expression of HLA-E ensuring the exclusive expression of HLA-G (69). JAR-E was transfected with a cd3.14 cosmid encoding HLA-E, a gift of M. Ulbrecht (Institute of Anthropology and Human Genetics, Munich, Germany) (70), in which the HLA-E leader sequence was replaced by that of HLA-A2, providing stable peptides for the expression of HLA-E, as described by Lee et al. (71). Upon arrival, aliquots of all cell lines were stored in our liquid nitrogen biobank. Low passage-count aliquots were thawed for our experiments. The cell lines were cultured in RPMI1640 medium (Lonza) supplemented with penicillin (1 x 10^5 U/L) (Lonza) and streptomycin (0.05 g/L) (Lonza), pyruvate (100mM) (Gibco®), geneticin (300 mg/mL) (Gibco®) for all transfectants and 10% fetal calf serum (Gibco®) at 37°C and 5% CO_2. The expression of HLA-E or HLA-G was regularly confirmed via flow cytometry (Figure 3).

On the day of sample acquisition, cells of all three cell lines were seeded onto 96-well plates (30,000 cells/well). After that, cells were incubated at 37°C and 5% CO_2 overnight for confluent growth.

On the next day, the old culture medium was carefully aspirated. Then, 100 µL of freshly isolated, matched decidual and peripheral blood γδT cells (10^6 γδT cells/mL), resuspended in cell line culture medium, were pipetted into the wells. In addition to membrane-bound HLA-E and G$_{1m}$, decidual and peripheral blood γδT cells were incubated with soluble HLA-E (0.5 µg/mL) or HLA-G (0.5 µg/mL) (both from OriGene Technologies) in independent wells. All tests were performed as biological duplicates. All wells were activated using ionomycin (1 µg/mL) (Sigma-Aldrich) and phorbol myristate acetate (25 ng/mL) (Sigma-Aldrich) for 18h. Hereafter, the 96-well plates were centrifuged, and aliquots of the supernatants were cryopreserved at -80°C for batched analysis.

4.8 Measurement of cell-(co-) culture supernatants

Diluted (1:10) cell culture supernatants were analyzed for IL-2 (6.5 – 20,000), IL-4 (9.04 – 14,000), IL-10 (5.06 – 14,000), IL-6

(11.58 – 15,000), IL-17A (8.51 – 18,000), TNF-α (12.68 – 12,000), sFas (4.53 – 81,000), sFasL (7.37 – 11,000), IFN-γ (57.13 – 20,000), granzyme A (62.96 – 15,000), granzyme B (24.99 – 52,000), perforin (60.18 – 12,000) and granulysin (175.95 – 57,000) utilizing the Human LegendPlex™ CD8/NK Panel (BioLegend) on a Canto 2 flow cytometer (BD Bioscience) according to the manufacturer's instructions.

Undiluted cell culture supernatants were analyzed for Angiopoietin-2 (13.7 – 10,000), BMP-9 (2.7 – 2,000), EGF (2.7 – 2,000), Endoglin (13.7 – 20,000), Endothelin-1 (2.7 – 2,000), FGF-1 (13.7 – 10,000), FGF-2 (13.7 – 10,000), Follistatin (27.4 – 10,000), G-CSF (13.7 – 10,000), HB-EGF (1.4 – 1,000), HGF (27.4 – 20,000), IL-8 (1.4 – 1,000), Leptin (137.2 – 100,000), PLGF (6.9 – 1,000), VEGF-A (13.7 – 10,000), VEGF-C (6.9 – 5,000) and VEGF-D (6.9 – 5,000) utilizing the MILLIPLEX® Human Angiogenesis/Growth Factor Magnetic Bead Panel - Cancer Multiplex Assay Enzyme-linked Immunosorbent Assay (Millipore) according to the manufacturer's instructions. The parentheses' numbers indicate each cytokine's detection range (pg/mL).

4.9 Statistics and data presentation

All statistical tests were performed in GraphPad Prism 9. Datasets were checked for Gaussian distribution by the D'Agostino-Pearson omnibus normality test. The test used in each comparison is indicated in the respective figure legend. Generally, p-values ≤ 0.05 were considered significant. Illustrations were produced using BioRender and Adobe Illustrator 23.0.4. Plots of flow cytometric data were exported from FlowJo™. Diagrams and Heatmaps were created using GraphPad Prism 9.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by University of Pecs - Medical School, Ethics Committee (5643-PTE 2019, 5643-PTE 2023). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. PV: Data curation, Investigation, Project administration, Writing – review & editing. IB: Data curation, Investigation, Methodology, Writing – review & editing. BF: Data curation, Formal analysis, Methodology, Validation, Writing – review & editing. AN: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. ÁB: Data curation, Investigation, Validation, Writing – review & editing. DS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. SE-B: Data curation, Investigation, Methodology, Writing – review & editing. PJ: Data curation, Methodology, Resources, Supervision, Validation, Writing – review & editing. KK: Investigation, Resources, Supervision, Writing – review & editing. ÉM: Writing – review & editing. TB: Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. EM: Validation, Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision. AB: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. PTE ÁOK-KA grants funded this research (No: KA-2019-28, KA-2019-37), the Thematic Excellence Program 2020 - Institutional Excellence Sub-program of the Ministry for Innovation and Technology in Hungary, within the framework of the second thematic program of the University of Pécs and the European Union and co-financed by the European Social Fund: Comprehensive Development for Implementing Smart Specialization Strategies at the University of Pécs (EFOP-3.6.1.-16-2016-00004). This work was supported by the National Research, Development and Innovation Fund of Hungary, financed under the TKP-2021-EGA-10 funding scheme. Further support was granted by the "National Laboratory on Human Reproduction", project no. RRF-2.3.1-21-2022-00012, titled National Laboratory on Human Reproduction has been implemented with the support provided by the Recovery and Resilience Facility of the European Union within the framework of Programme Széchenyi Plan Plus.

Acknowledgments

We are grateful to our participants for their sample donations. Furthermore, we thank Philippe Le Bouteiller for providing the JAr cell line and transfectants. The research was performed in collaboration with the Flow Cytometry Core Facility at the Szentágothai Research Centre of the University of Pécs.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1382424/full#supplementary-material

References

1. Chavan AR, Griffith OW, Wagner GP. The inflammation paradox in the evolution of mammalian pregnancy: turning a foe into a friend. *Curr Opin Genet Dev*. (2017) 47:24–32. doi: 10.1016/J.GDE.2017.08.004

2. Kieckbusch J, Gaynor LM, Moffett A, Colucci F. MHC-dependent inhibition of uterine NK cells impedes fetal growth and decidual vascular remodelling. *Nat Commun*. (2014) 5:3359. doi: 10.1038/ncomms4359

3. Xiong S, Sharkey AM, Kennedy PR, Gardner L, Farrell LE, Chazara O, et al. Maternal uterine NK cell–activating receptor KIR2DS1 enhances placentation. *J Clin Invest*. (2013) 123:4264–72. doi: 10.1172/JCI68991

4. Redman CWG, Sargent IL. The pathogenesis of pre-eclampsia. *Gynecol Obstet Fertil*. (2001) 29:518–22. doi: 10.1016/S1297-9589(01)00180-1

5. Redman CWG, Sargent IL. Immunology of pre-eclampsia. *Am J Reprod Immunol*. (2010) 63:534–43. doi: 10.1111/j.1600-0897.2010.00831.x

6. Sargent IL, Borzychowski AM, Redman CWG. Immunoregulation in normal pregnancy and pre-eclampsia: An overview. *Reprod BioMed Online*. (2006) 13:680–6. doi: 10.1016/S1472-6483(10)60659-1

7. Sun FR, Chen CQ, Yu M, Wang SC, Li DJ, Du MR. Galectin-9 Promotes Human Trophoblast Cell Invasion through Matrix Metalloproteinase-2 and p38 Signaling Pathway. *Reprod Dev Med*. (2018) 2:1–17. doi: 10.4103/2096-2924.232880/ASSET/6405C685-D230-4C38-BC38-43DBAE94C30F/ASSETS/GRAPHIC/2096-2924-02-01-101-F005.PNG

8. Vinketova K, Mourdjeva M, Oreshkova T. Human decidual stromal cells as a component of the implantation niche and a modulator of maternal immunity. *J Pregnancy*. (2016) 2016:1-17. doi: 10.1155/2016/8689436

9. Hannon T, Innes BA, Lash GE, Bulmer JN, Robson SC. Effects of local decidua on trophoblast invasion and spiral artery remodeling in focal placenta creta – An immunohistochemical study. *Placenta*. (2012) 33:998–1004. doi: 10.1016/J.PLACENTA.2012.09.004

10. Hecht JL, Karumanchi SA, Shainker SA. Immune cell infiltrate at the utero-placental interface is altered in placenta accreta spectrum disorders. *Arch Gynecol Obstet*. (2020) 301:499–507. doi: 10.1007/s00404-020-05453-1

11. Miller DA, Chollet JA, Goodwin TM. Clinical risk factors for placenta previa–placenta accreta. *Am J Obstet Gynecol*. (1997) 177:210–4. doi: 10.1016/S0002-9378(97)70463-0

12. Ferry BL, Starkey PM, Sargent IL, Watt GM, Jackson M, Redman CW. Cell populations in the human early pregnancy decidua: natural killer activity and response to interleukin-2 of CD56-positive large granular lymphocytes. *Immunology*. (1990) 70:446–52.

13. Crespo ÂC, Strominger JL, Tilburgs T. Expression of KIR2DS1 by decidual natural killer cells increases their ability to control placental HCMV infection. *Proc Natl Acad Sci*. (2016) 113:15072–7. doi: 10.1073/pnas.1617927114

14. Colucci F. The role of KIR and HLA interactions in pregnancy complications. *Immunogenetics*. (2017) 69:557–65. doi: 10.1007/s00251-017-1003-9

15. Flores AC, Marcos CY, Paladino N, Arruvito L, Williams F, Middleton D, et al. KIR receptors and HLA-C in the maintenance of pregnancy. *Tissue Antigens*. (2007) 69:112–3. doi: 10.1111/j.1399-0039.2006.762_8.x

16. Ponte M, Cantoni C, Biassoni R, Tradori-Cappai A, Bentivoglio G, Vitale C, et al. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc Natl Acad Sci U.S.A*. (1999) 96:5674–9. doi: 10.1073/pnas.96.10.5674

17. Marlin R, Duriez M, Berkane N, de Truchis C, Madec Y, Rey-Cuille M-A, et al. Dynamic shift from CD85j/ILT-2 to NKG2D NK receptor expression pattern on human decidual NK during the first trimester of pregnancy. *PloS One*. (2012) 7:e30017. doi: 10.1371/journal.pone.0030017

18. McMaster MT, Librach CL, Zhou Y, Lim KH, Janatpour MJ, DeMars R, et al. Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. *J Immunol*. (1995) 154:3771–8. doi: 10.4049/jimmunol.154.8.3771

19. Hutter H, Hammer A, Blaschitz A, Hartmann M, Ebbesen P, Dohr G, et al. Expression of HLA class I molecules in human first trimester and term placenta trophoblast. *Cell Tissue Res*. (1996) 286:439–47. doi: 10.1007/s004410050713

20. Pfeiffer KA, Rebmann V, Pässler M, van der Ven K, van der Ven H, Krebs D, et al. Soluble HLA levels in early pregnancy after in *vitro* fertilization. *Hum Immunol*. (2000) 61:559–64. doi: 10.1016/S0198-8859(00)00123-3

21. Hunt JS, Jadhav L, Chu W, Geraghty DE, Ober C. Soluble HLA-G circulates in maternal blood during pregnancy. *Am J Obstet Gynecol*. (2000) 183:682–8. doi: 10.1067/mob.2000.106762

22. Beneventi F, Locatelli E, De Amici M, Martinetti M, Spinillo A. Soluble HLA-G concentrations in obese women during pregnancy and in cord blood. *J Reprod Immunol*. (2017) 119:31–7. doi: 10.1016/j.jri.2016.11.005

23. Steinborn A, Varkonyi T, Scharf A, Bahlmann F, Klee A, Sohn C. Early detection of decreased soluble HLA-G levels in the maternal circulation predicts the occurrence of preeclampsia and intrauterine growth retardation during further course of pregnancy. *Am J Reprod Immunol*. (2007) 57:277–86. doi: 10.1111/j.1600-0897.2007.00475.x

24. Dunne MR, Elliott L, Hussey S, Mahmud N, Kelly J, Doherty DG, et al. Persistent changes in circulating and intestinal γδ T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease. *PloS One*. (2013) 8:e76008. doi: 10.1371/journal.pone.0076008

25. Gully BS, Rossjohn J, Davey MS. Our evolving understanding of the role of the γδ T cell receptor in γδ T cell mediated immunity. *Biochem Soc Trans*. (2021) 49:1985–95. doi: 10.1042/BST20200890

26. Terzieva A, Dimitrova V, Djerov L, Dimitrova P, Zapryanova S, Hristova I, et al. Early pregnancy human decidua is enriched with activated, fully differentiated and pro-inflammatory gamma/delta T cells with diverse TCR repertoires. *Int J Mol Sci*. (2019) 20:687. doi: 10.3390/ijms20030687

27. Tilburgs T, van der Mast BJ, Nagtzaam NMA, Roelen DL, Scherjon SA, Claas FHJ. Expression of NK cell receptors on decidual T cells in human pregnancy. *J Reprod Immunol*. (2009) 80:22–32. doi: 10.1016/J.JRI.2009.02.004

28. Fan DX, Duan J, Li MQ, Xu B, Li DJ, Jin LP. The decidual gamma-delta T cells up-regulate the biological functions of trophoblasts *via* IL-10 secretion in early human pregnancy. *Clin Immunol*. (2011) 141:284–92. doi: 10.1016/j.clim.2011.07.008

29. Moser G, Weiss G, Gauster M, Sundl M, Huppertz B. Evidence from the very beginning: endoglandular trophoblasts penetrate and replace uterine glands *in situ* and *in vitro*. *Hum Reprod*. (2015) 30:2747–57. doi: 10.1093/humrep/dev266

30. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, et al. Embryo implantation. *Dev Biol*. (2000) 223:217–37. doi: 10.1006/DBIO.2000.9767

31. Hempstock J, Cindrova-Davies T, Jauniaux E, Burton GJ. Endometrial glands as a source of nutrients, growth factors and cytokines during the first trimester of human pregnancy: A morphological and immunohistochemical study. *Reprod Biol Endocrinol*. (2004) 2:58. doi: 10.1186/1477-7827-2-58

32. Kaiser BK, Barahmand-pour F, Paulsene W, Medley S, Geraghty DE, Strong RK. Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics. *J Immunol*. (2005) 174:2878–84. doi: 10.4049/JIMMUNOL.174.5.2878

33. Navarro F, Llano M, Bellón T, Colonna M, Geraghty DE, López-Botet M. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *Eur J Immunol*. (1999) 29:277–83. doi: 10.1002/(SICI)1521-4141(199901)29:01<277::AID-IMMU277>3.0.CO;2-4

34. Yan WH, Fan LA. Residues Met76 and Gln79 in HLA-G α1 domain involved in KIR2DL4 recognition. *Cell Res*. (2005) 15:176–82. doi: 10.1038/sj.cr.7290283

35. Rajagopalan S. Endosomal signaling and a novel pathway defined by the natural killer receptor KIR2DL4 (CD158d). *Traffic*. (2010) 11:1381–90. doi: 10.1111/j.1600-0854.2010.01112.x

36. Rajagopalan S, Fu J, Long EO. Cutting edge: induction of IFN-γ Production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. *J Immunol.* (2001) 167:1877–81. doi: 10.4049/jimmunol.167.4.1877

37. Boyson JE, Rybalov B, Koopman LA, Exley M, Balk SP, Racke FK, et al. CD1d and invariant NKT cells at the human maternal-fetal interface. *Proc Natl Acad Sci U.S.A.* (2002) 99:13741–6. doi: 10.1073/pnas.162491699

38. Matsumoto J, Kawana K, Nagamatsu T, Schust DJ, Fujii T, Sato H, et al. Expression of surface CD1d in the extravillous trophoblast cells of early gestational placenta is downregulated in a manner dependent on trophoblast differentiation. *Biochem Biophys Res Commun.* (2008) 371:236–41. doi: 10.1016/j.bbrc.2008.04.051

39. Jenkinson HJ, Wainwright SD, Simpson KL, Perry ACF, Fotiadou P, Holmes CH. Expression of CD1D mRNA transcripts in human choriocarcinoma cell lines and placentally derived trophoblast cells. *Immunology.* (1999) 96:649–55. doi: 10.1046/j.1365-2567.1999.00726.x

40. Lesport E, Baudhuin J, Sousa S, Lemaoult J, Zamborlini A, Rouas-Freiss N, et al. Inhibition of human Vγ9Vδ2 T-cell antitumoral activity through HLA-G: Implications for immunotherapy of cancer. *Cell Mol Life Sci.* (2011) 68:3385–99. doi: 10.1007/s00018-011-0632-7

41. van der Zwan A, van Unen V, Beyrend G, Laban S, van der Keur C, Kapsenberg HJM, et al. Visualizing dynamic changes at the maternal-fetal interface throughout human pregnancy by mass cytometry. *Front Immunol.* (2020) 11:571300. doi: 10.3389/fimmu.2020.571300

42. Sandstrom A, Peigné C-M, Léger A, Crooks JE, Konczak F, Gesnel M-C, et al. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2 T cells. *Immunity.* (2014) 40:490–500. doi: 10.1016/j.immuni.2014.03.003

43. Rhodes DA, Chen H-C, Price AJ, Keeble AH, Davey MS, James LC, et al. Activation of human γδ T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin. *J Immunol.* (2015) 194:2390–8. doi: 10.4049/jimmunol.1401064

44. Harly C, Guillaume Y, Nedellec S, Peigné C-M, Mönkkönen H, Mönkkönen J, et al. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset. *Blood.* (2012) 120:2269–79. doi: 10.1182/blood-2012-05-430470

45. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells. *Sci (1979).* (2020) 367. doi: 10.1126/science.aay5516

46. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, Mcpherson KG, Lim RT, et al. CD1d-lipid antigen recognition by the γδ TCR. *Nat Immunol.* (2013) 14:1137–45. doi: 10.1038/ni.2713

47. Le Nours J, Gherardin NA, Ramarathinam SH, Awad W, Wiede F, Gully BS, et al. A class of gd T cell receptors recognize the underside of the antigen-presenting molecule MR1. *Sci (1979).* (2019) 366:1522–7. doi: 10.1126/science.aav3900

48. Roy S, Ly D, Castro CD, Li N-S, Hawk AJ, Altman JD, et al. Molecular analysis of lipid-reactive Vδ1 γδ T cells identified by CD1c tetramers. *J Immunol.* (2016) 196:1933–42. doi: 10.4049/jimmunol.1502202

49. Reijneveld JF, Ocampo TA, Shahine A, Gully BS, Vantourout P, Hayday AC, et al. Human γδ T cells recognize CD1b by two distinct mechanisms. *Proc Natl Acad Sci U.S.A.* (2020) 117:22944–52. doi: 10.1073/pnas.2010545117

50. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial γδ T cells. *Sci (1979).* (1998) 279:1737–40. doi: 10.1126/science.279.5357.1737

51. Cai D, Tang Y, Yao X. Changes of γδT cell subtypes during pregnancy and their influences in spontaneous abortion. *J Reprod Immunol.* (2019) 131:57–62. doi: 10.1016/j.jri.2019.01.003

52. Manchorova D, Papadopoulou M, Alexandrova M, Dimitrova V, Djerov L, Zapryanova S, et al. Human decidual gamma/delta T cells possess unique effector and TCR repertoire profiles during pregnancy. *Cell Immunol.* (2022) 382:104634. doi: 10.1016/J.CELLIMM.2022.104634

53. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, O'Shea D, et al. Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vδ3 T cells. *J Immunol.* (2013) 191:30–4. doi: 10.4049/JIMMUNOL.1300121

54. Braud VM, Allan DSJ, Wilson D, McMichael AJ. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr Biol.* (1998) 8:1–10. doi: 10.1016/S0960-9822(98)70014-4

55. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol.* (1997) 27:1164–9. doi: 10.1002/EJI.1830270517

56. Gao Z, Bai Y, Lin A, Jiang A, Zhou C, Cheng Q, et al. Gamma delta T-cell-based immune checkpoint therapy: attractive candidate for antitumor treatment. *Mol Cancer.* (2023) 22:1–23. doi: 10.1186/S12943-023-01722-0

57. Lin A, Yan WH. Intercellular transfer of HLA-G: its potential in cancer immunology. *Clin Transl Immunol.* (2019) 8. doi: 10.1002/CTI2.1077

58. Rajagopalan S, Long EO. Cellular senescence induced by CD158d reprograms natural killer cells to promote vascular remodeling. *Proc Natl Acad Sci U.S.A.* (2012) 109:20596–601. doi: 10.1073/pnas.1208248109

59. Zhang JY, Wu P, Chen D, Ning F, Lu Q, Qiu X, et al. Vitamin D promotes trophoblast cell induced separation of vascular smooth muscle cells in vascular remodeling *via* induction of G-CSF. *Front Cell Dev Biol.* (2020) 8:601043/BIBTEX. doi: 10.3389/fcell.2020.601043

60. Ding J, Yang C, Zhang Y, Wang J, Zhang S, Guo D, et al. M2 macrophage-derived G-CSF promotes trophoblasts EMT, invasion and migration *via* activating PI3K/Akt/Erk1/2 pathway to mediate normal pregnancy. *J Cell Mol Med.* (2021) 25:2136–47. doi: 10.1111/JCMM.16191

61. Paiva P, Hannan NJ, Hincks C, Meehan KL, Pruysers E, Dimitriadis E, et al. Human chorionic gonadotrophin regulates FGF2 and other cytokines produced by human endometrial epithelial cells, providing a mechanism for enhancing endometrial receptivity. *Hum Reprod.* (2011) 26:1153–62. doi: 10.1093/HUMREP/DER027

62. Sheridan MA, Fernando RC, Gardner L, Hollinshead MS, Burton GJ, Moffett A, et al. Establishment and differentiation of long-term trophoblast organoid cultures from the human placenta. *Nat Protoc* (2020), 3441–63. doi: 10.1038/s41596-020-0381-x

63. Islami D, Bischof P, Chardonnens D. Modulation of placental vascular endothelial growth factor by leptin and hCG. *Mol Hum Reprod.* (2003) 9:395–8. doi: 10.1093/MOLEHR/GAG053

64. Barrientos G, Toro A, Moschansky P, Cohen M, Garcia MG, Rose M, et al. Leptin promotes HLA-G expression on placental trophoblasts *via* the MEK/Erk and PI3K signaling pathways. *Placenta.* (2015) 36:419–26. doi: 10.1016/J.PLACENTA.2015.01.006

65. Chau K, Hennessy A, Makris A. Placental growth factor and pre-eclampsia. *J Hum Hypertension.* (2017) 31:782–6. doi: 10.1038/jhh.2017.61

66. Colucci F. Uterine NK cells ace an "A" in education: NKG2A sets up crucial functions at the maternal–fetal interface. *J Immunol.* (2022) 209:1421–5. doi: 10.4049/JIMMUNOL.2200384

67. Crespo ÂC, Mulik S, Dotiwala F, Ansara JA, Sen Santara S, Ingersoll K, et al. Decidual NK cells transfer granulysin to selectively kill bacteria in trophoblasts. *Cell.* (2020) 182:1125–1139.e18. doi: 10.1016/J.CELL.2020.07.019

68. Björkström NK, Ljunggren HG, Michaëlsson J. Emerging insights into natural killer cells in human peripheral tissues. *Nat Rev Immunol.* (2016) 16:310–20. doi: 10.1038/nri.2016.34

69. Llano M, Lee N, Navarro F, García P, Albar JP, Geraghty DE, et al. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: Preferential response to an HLA-G-derived nonamer. *Eur J Immunol.* (1998) 28:2854–63. doi: 10.1002/(SICI)1521-4141(199809)28:09<2854::AID-IMMU2854>3.0.CO;2-W

70. Ulbrecht M, Honka T, Person S, Johnson JP, Weiss EH. The HLA-E gene encodes two differentially regulated transcripts and a cell surface protein. *J Immunol.* (1992) 149:2945–53. doi: 10.4049/jimmunol.149.9.2945

71. Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol.* (1998) 160:160. doi: 10.4049/jimmunol.160.10.4951

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 19 April 2024
DOI 10.3389/fimmu.2024.1385762

Check for updates

OPEN ACCESS

EDITED BY
Alicia E. Damiano,
University of Buenos Aires, Argentina

REVIEWED BY
Reggie García-Robles,
Pontifical Javeriana University, Colombia
Victor Sánchez-Margalet,
Virgen Macarena University Hospital, Spain

*CORRESPONDENCE
Amir-Hassan Zarnani
zarnania@sina.tums.ac.ir;
zarnania@gmail.com
Mahmood Jeddi-Tehrani
mahjed@avicenna.ac.ir;
mahjed@yahoo.com

†These authors have contributed equally to this work

RECEIVED 13 February 2024
ACCEPTED 08 April 2024
PUBLISHED 19 April 2024

CITATION
Khorami-Sarvestani S, Vanaki N, Shojaeian S, Zarnani K, Stensballe A, Jeddi-Tehrani M and Zarnani A-H (2024) Placenta: an old organ with new functions.
Front. Immunol. 15:1385762.
doi: 10.3389/fimmu.2024.1385762

COPYRIGHT
© 2024 Khorami-Sarvestani, Vanaki, Shojaeian, Zarnani, Stensballe, Jeddi-Tehrani and Zarnani. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Placenta: an old organ with new functions

Sara Khorami-Sarvestani[1,2†], Negar Vanaki[3†], Sorour Shojaeian[4], Kayhan Zarnani[3], Allan Stensballe[5,6], Mahmood Jeddi-Tehrani[2*] and Amir-Hassan Zarnani[1,3*]

[1]Reproductive Immunology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran, [2]Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran, [3]Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, [4]Department of Biochemistry, School of Medical Sciences, Alborz University of Medical Sciences, Karaj, Iran, [5]Department of Health Science and Technology, Aalborg University, Aalborg, Denmark, [6]Clinical Cancer Research Center, Aalborg University Hospital, Aalborg, Denmark

The transition from oviparity to viviparity and the establishment of feto-maternal communications introduced the placenta as the major anatomical site to provide nutrients, gases, and hormones to the developing fetus. The placenta has endocrine functions, orchestrates maternal adaptations to pregnancy at different periods of pregnancy, and acts as a selective barrier to minimize exposure of developing fetus to xenobiotics, pathogens, and parasites. Despite the fact that this ancient organ is central for establishment of a normal pregnancy in eutherians, the placenta remains one of the least studied organs. The first step of pregnancy, embryo implantation, is finely regulated by the trophoectoderm, the precursor of all trophoblast cells. There is a bidirectional communication between placenta and endometrium leading to decidualization, a critical step for maintenance of pregnancy. There are three-direction interactions between the placenta, maternal immune cells, and the endometrium for adaptation of endometrial immune system to the allogeneic fetus. While 65% of all systemically expressed human proteins have been found in the placenta tissues, it expresses numerous placenta-specific proteins, whose expression are dramatically changed in gestational diseases and could serve as biomarkers for early detection of gestational diseases. Surprisingly, placentation and carcinogenesis exhibit numerous shared features in metabolism and cell behavior, proteins and molecular signatures, signaling pathways, and tissue microenvironment, which proposes the concept of "cancer as ectopic trophoblastic cells". By extensive researches in this novel field, a handful of cancer biomarkers has been discovered. This review paper, which has been inspired in part by our extensive experiences during the past couple of years, highlights new aspects of placental functions with emphasis on its immunomodulatory role in establishment of a successful pregnancy and on a potential link between placentation and carcinogenesis.

KEYWORDS

placenta, proteins, pregnancy, immunomodulation, embryo implantation, cancer, biomarker

Introduction

> "Yet, in spite of being the star of the show, the placenta has never quite managed to gain the attention it deserves. Compared to other organs of the body, the placenta comes very low down the list when measured on the scale of public awareness. It is not the placenta's fault that it has languished in such obscurity. No one has bothered to speak up on its behalf and put it firmly into the public domain." (1)
>
> - Y. W. Loke

Placental evolution in mammals

The way in which mammals reproduce is the major focus of evolutionary biology, and understanding these processes underpins much of the diversity in our lives. After many years of evolution from the egg-laying ancestor of vertebrates, viviparity converges and involves numerous anatomical, behavioral, genetic, and physiological changes to support internally-incubated embryos. The transition from oviparity to viviparity and the establishment of feto-maternal communications to provide nutrients, gases, and hormones to the developing fetuses introduced the 'placenta' as the anatomical mediator of exchange between fetal membranes. The time and approaches that viviparous mammals, marsupials, and eutherians invest in birth differ from one another. The brain size of the neonates in each species is considered one factor that may influence the type of maternal investment (1).

Egg-laying monotremes like the Platypus and Echidna lack a placenta and other reproductive compartments such as the uterus; however, marsupials like kangaroos, koalas, bandicoots, and opossums have a functional placenta. The gestation time in marsupials is as short as 12 days, so much of maternal investment focuses on lactation for the better development of the little joeys. It has been shown that the mammary glands of marsupials function as an alternative to the eutherian placenta, and the composition of milk gradually changes as the offspring grows (2). Delving into the transcriptome changes in the placenta of a small Australian marsupial, the tammar wallaby, researchers have found that marsupials have a eutherian-like placenta with similarly differentially expressed genes as the eutherian placenta in the early stages of pregnancy (3). This suggests that placental functions are the same in the two species, and the difference might lie in the way of compartmentalization and the type of maternal investment in each species.

Much of our knowledge on placenta development comes from animals in the eutherian lineage, which comprises over 5000 vertebrates? species and 20 phylogenetic orders. The different sizes, gross shapes, and histological structures of layers that separate maternal and fetal circulations result in various types of placentas. Regarding size and shape, placentas are classified as diffuse, cotyledonary, zonary, and discoid/bidiscoid types (Figure 1). In the diffuse type, as seen in horses and pigs, the entire surface of the allantochorion is involved in the formation of the placenta. The interaction of multiple patches of allantochorion with the endometrium results in cotyledonary placenta, observed in ruminants. Zonary is a complete or incomplete band of tissue that surrounds the fetus, found in carnivores. The last type is discoid/bidiscoid, confined to a roughly circular area and seen in primates, rodents, and rabbits (4).

Based on histological structure, four types of placentation have emerged, namely epitheliochorial, endotheliochorial, haemochorial, and synepitheliochorial (Figure 2). In species with non-invasive epitheliochorial placentation, such as horses and pigs, pockets of columnar trophoblasts loosely attach to the endometrial epithelium. Endotheliochorial placentation involves a medium degree of invasion, seen in carnivores. The most invasive type is haemochorial, as seen in humans, rats, and mice, in which the trophoblast is in direct contact with maternal blood. In synepitheliochorial placentation, trophoblast cells fuse with uterine epithelial cells to form syncytium. This type is observed in ruminants; however, some developmental biologists believe it has evolved from haemochorial placentation and named it secondary epitheliochorial placentation (5). The change in the type of placentation in ruminants might result from a positive pleiotropic effect, as described first for the relationship between placentation and cancer malignancy (see below for more information).

Some researchers posit that marsupials possess a non-invasive placenta; however, recent hypotheses have indicated that the placenta of marsupials is also invasive. The challenge lies in the brief gestation period, which precludes the observation of changes related to placental invasiveness. This limitation has been compensated for by a relatively longer lactation period to better nourish the joeys (6).

In addition to the gestational period, maternal homeostatic mechanisms that cope with embryo-induced inflammation also determine the degree of invasiveness. This means that the short period of fetus implantation in marsupials is followed by parturition. In contrast, in eutherians, severe inflammation associated with deep placental invasion is compensated for by anti-inflammatory mechanisms (7). Therefore, deep placental invasion occurs in species that have evolved mechanisms to handle severe inflammation. Among the most important compensatory mechanisms is the platelet/megakaryocyte system responsible for hemostasis. The degree of invasion is much more related to maternal balancing systems than the nature of the placenta itself. Hemostatic mechanisms are needed to repair the destruction of blood vessels upon hemochorial placentation in eutherians (8). The evolution of the megakaryocyte platelet system emerged before the evolution of deep placentation. This suggests that deep invasion occurs in species that have learned how to deal with undesired outcomes. This idea echoes the words of the evolutionary biologist Theodosius Dobzhansky: 'Nothing in biology makes sense except in the light of evolution.'"

An overview on placental development in humans and rodents

While human and mouse placentas share similar overall structures and molecular mechanisms underlying development, there are notable differences in their gross structures. Assuming the formation of the

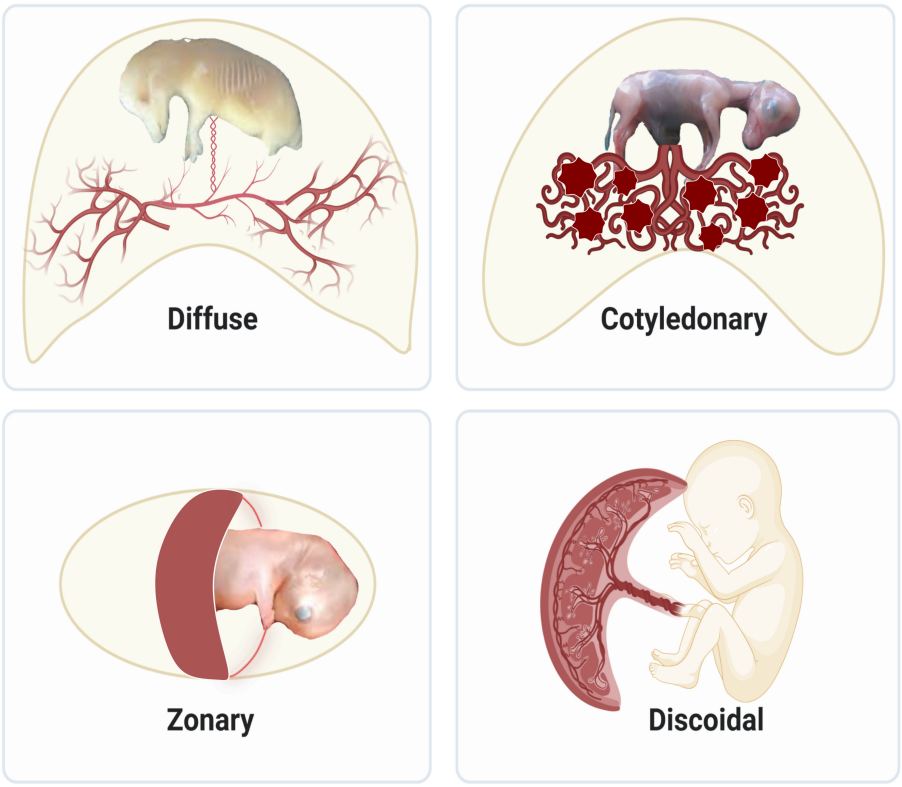


FIGURE 1
Structural diversity of placentas in mammals: Diffuse placentas have a uniform distribution of chorionic villi covering the chorion's surface and are seen in horses and pigs. Cotyledonary placentas have numerous button-like structures called cotyledon that join together using vascularized intervening areas to form placentome. This type of structure is seen in sheep, goat, and cattle. Zonary placentas are like a belt created around the chorionic sac and are seen in carnivores like dogs, cats, and seals, omnivores like bears, and herbivores like elephants. Discoidal placentas form disc-like structures and this placental structure is seen in rodents and primates like humans.

vaginal plug as day 0.5 of mouse pregnancy (9, 10), placental development begins at 3.5 days post conception (dpc) when the inner cell mass and outer trophoectoderm layers are established (10, 11). At the time of implantation (4.5 dpc), trophoblasts, corresponding to the area opposite and farthest from the inner cell mass, differentiate into polyploid trophoblast giant cells analogous to human extravillous cytotrophoblast cells (EVT) (12). Trophoectoderm cells adjacent to the inner cell mass, polar trophoectoderm, differentiate into the two main diploid cell types: the extraembryonic ectoderm and the ectoplacental cone (11).

In later stages of mouse placental development, the extraembryonic ectoderm forms the chorion layer and later the labyrinth. The ectoplacental cone differentiates into the spongiotrophoblasts, which form a compact layer between the labyrinth and the outer giant cell layer and is analogous to the column cytotrophoblast of the human placenta (10, 12). Subsequently, glycogen trophoblast cells are formed within the spongiotrophoblast layer (11). The allantois, formed at the posterior end of the developing embryo (8 dpc), is the origin of the vascular portion of the placenta (11).

The fusion of chorionic mesothelium and allantois occurs at 8.5 dpc, followed by the folding of the chorionic mesothelium, creating spaces into which fetal blood vessels grow from the allantois (11). This event coincides with the differentiation of chorionic trophoblast cells into two labyrinth cell types. In a process quite similar to human placental development, trophoblast cells of the labyrinth layer adjacent to the fetal endothelium fuse with each other, forming multinucleated syncytiotrophoblast cells (STB). The second cell type differentiated from labyrinth trophoblast cells is a mononuclear trophoblast cell that lines the maternal blood sinuses. Fetal vessels and labyrinth trophoblasts generate branched villi, analogous to human chorionic villi, which continue to grow and branch until 18.5-19.5 dpc. Notably, maternal and fetal blood circulate in opposite directions, maximizing nutrient transport (11).

Human placental development begins 6-7 days after conception, around the attachment of the blastocyst to the uterine wall (13). In humans, the polar trophoectoderm adheres to the endometrial epithelium, while in mice, it is the mural trophoectoderm that contacts the endometrium (14). Once attached, the polar trophoectoderm rapidly proliferates and forms a regional bilayer trophoblast, consisting of an inner layer of rapidly proliferating cytotrophoblasts (CTB) and an outer layer of multinucleated STB, differentiated by CTB fusion (15). Although STB are non-proliferative in essence, they can combine with the CTB basal cell layer and continue to proliferate throughout pregnancy (16). Trophoblast invasion into the maternal decidua

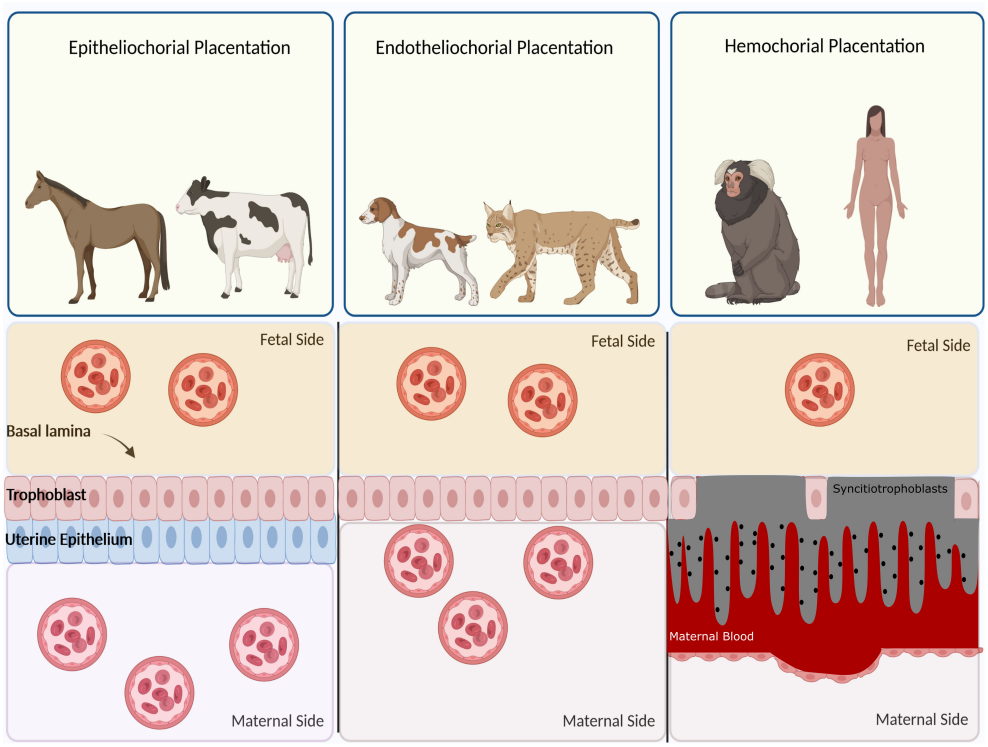


FIGURE 2
Classification of placentas based on cell layers comprising the maternal-fetal interface. In epitheliochorial placentation, as seen in cow, horse, and ruminants, the least intimate interactions occur between fetal tissue and maternal blood since a barrier containing maternal vascular endothelium and uterine epithelium exists. In endotheliochorial placentation, as seen in dogs and cats, trophoblasts invade the uterine epithelium and contact with maternal endothelium. In hemochorial placentation, as seen in primates and humans, the most intimate contact occurs between fetal trophoblasts and maternal circulation since both the epithelium and endothelium degrade and trophoblasts are soaked in the maternal blood.

is an essential step for the initiation of subsequent fetal development, including vasculature remodeling and crosstalk with endometrial decidual stromal cells (DSC) and immune cells residing in the decidua (17).

The development of human placental villi starts around 8 dpc through the proliferation of CTB and folding of the outer primitive STB layer into the decidua. The first villus mesenchymal cores are formed by the invasion of extraembryonic mesenchyme into the villi at around 12 dpc Endothelial cells can be identified within the villus core from 15 dpc. Similar to mouse villi, endothelial cords within human placenta villi are in close contact with the trophoblast basement membrane, implying that trophoblast cells may contribute to villous vascular network development through a paracrine manner. At around 18-20 dpc, the first fetal capillaries and placental macrophages (Hofbauer cells) appear in the stroma of placental villi. The development of placental villi capillaries continues, and at around 22 dpc, branching vessels are developed by connecting endothelial cell cords to each other and elongating parallel to the villus axis. The villi blood vessels connect to the fetal circulation via the umbilical cord around 32 dpc (18).

Villi structure in humans and mice is structurally similar, surrounded by a trophoblastic bilayer consisting of mononuclear trophoblasts and an overlying multinucleated STB layer. STB is in close contact with maternal blood and is the major site for the absorption of nutrients and oxygen and waste exchange. STB actively involves the production of human chorionic gonadotropin (hCG) and progesterone, necessary for fetal development. Around 15 dpc, CTB at the tips of anchoring villi rapidly proliferate, detach from villi columns, and differentiate into EVT (19). Two types of EVT can be identified: interstitial CTB (iCTB), which is highly invasive and invade decidual stroma, playing a fundamental role in shaping endometrial immune regulation (see below). Endovascular CTBs (eCTB) invade uterine spiral artery lumens and replace endothelial cells to ensure adequate placental perfusion as pregnancy proceeds. eCTB form trophoblastic plugs during the first weeks of pregnancy until around 7 weeks of gestation and prevent blood flow in the spiral arteries. This event provides a hypoxic microenvironment and is thought to play a major role in placental development (18). In the second and third trimesters of pregnancy, placental villi continue to mature, characterized by a densely packed stroma. In parallel, placental villi vasculature undergoes extensive expansion through branching angiogenesis. In the third trimester, vascular branching stops, and capillary loops are formed, further increasing exchange between maternal and fetal circulations.

The role of placenta in maintenance of pregnancy

The placenta performs numerous vital biological functions, including nutrient and waste transfer, gas exchange, and the

transmission of maternal immunoglobulins to the fetus, providing passive immunity (20). Additionally, it contributes to fetal development through hormone secretion (21). The proximity of the placenta and decidual blood arteries is carefully regulated to meet the developing fetus's higher metabolic needs by adjusting the endometrial circulation appropriately (22). It's noteworthy that the human placenta possesses safeguards preventing the passage of potentially harmful compounds. Export pumps in the STB's maternal-facing membrane, along with the expression of cytochrome P450 (CYP) genes involved in xenobiotic metabolism during the first trimester of pregnancy, contribute to these protective characteristics (23, 24).

The placenta also acts as a safeguard against the vertical transmission of infectious agents. While few pathogens can infect the placenta and fetus in humans, maternal infection doesn't guarantee placental or fetal infection (25). The placenta employs physical and immunological mechanisms to resist infection. The STB layer, consisting of almost 60 billion nuclei, lacks cell junctions, forming a barrier against microbial attachment and invasion. The microvasculature of fetal blood vessels restricts access of microorganisms to fetal blood. The maternal decidua, rich in leukocytes, serves as an effective first-line immunological defense. Various cells, such as decidual natural killer (dNK) cells and DSCs, contribute to placental innate immune defenses. The maternal decidua is a leukocyte-rich layer and constitutes an effective first-line immunological defense at the maternal–fetal interface. As an example, dNK cells could transfer granulysin to trophoblasts and confer resistance without actively killing these cells. DSCs, as the most frequent decidual cells, have a major role in placental innate immune defenses. Villous trophoblast, in addition to providing a physical defense, also acts as a chemical barrier to the vertical transmission of microorganisms. For instance, human trophoblasts secrete antiviral interferons (type III) that restrict infection in both an autocrine and a paracrine manner. Interestingly, placental extracellular vesicles (EV) contained antiviral microRNAs with broad antiviral activity. Inflammasome-associated cytokines such as Interleukin (IL)-1β, IL-18, and IL-1α have been reported to protect placental cell infection with L. monocytogenes (25). As an example of an effective function of the placenta against infection, it is noteworthy that only 1.5-2% of pregnancies in HIV-positive women appear to result in the vertical transmission of HIV across the placenta (26).

Additionally, the placenta is a source of autocrine, paracrine, and endocrine factors. Placental hormones can be broadly categorized into those acting at the feto-maternal interface, mediating communication between the mother and developing fetus, and those exerting regulatory effects in remote targets in maternal compartments. These regulatory effects are essential for maternal physiological adaptations during pregnancy, such as changes in maternal circulation, development of lactating glands, provision of hemotroph to the embryo, and parturition. Sex hormones, including estrogen and progesterone, play a crucial role in various functions such as endometrial differentiation, myometrial quiescence, cervical closure, local immunotolerance in the pregnant uterus (27–29), trophoblast differentiation, autoregulation of placental steroidogenesis, regulation of the maternal cardiovascular system, uteroplacental blood flow, placental neovascularization and mammary gland development (30). Growth hormone (GH) and prolactin (PRL) are well-studied placental hormones with significant activity in mediating maternal metabolic adaptations to pregnancy. Placental hormones also impact maternal neuroendocrine organs, including the brain, hypothalamus, and pituitary glands, facilitating maternal adaptation to pregnancy and maintaining homeostasis. Neuroactive hormones like melatonin, serotonin, and oxytocin are involved in these activities. Other placental hormones, such as activins, relaxin, leptin, and parathyroid hormone-related protein (PTHrP), play roles in mediating changes in maternal vascular function and metabolism (26). The immunoregulatory role of leptin in the human placenta has been discussed further in the next sections.

Placenta function in decidualization and implantation

In species with hemochorial placentation, the placenta must invade the endometrial spiral arteries to create sufficient blood supply for nourishing the developing embryo. This invasion, completed at the end of the first trimester, is regulated by the local decidual environment (31). According to an evolutionary hypothesis, known as the precondition hypothesis, the uterus undergoes various cellular and molecular alterations to be adapted for potential pregnancy. One mechanism for such adaptations is the extensive structural and molecular changes in endometrial stromal cells under the control of ovarian steroid hormones, collectively referred to as decidualization (32, 33). Decidualization occurs in the mid-to-late secretory phase of the menstrual cycle in menstruating species; however, in non-menstruating species, it occurs only after implantation. The decidual environment consists of DSCs, immune cells, and extracellular matrix (34).

Decidualization serves to protect the endometrium from oxidative stress and hyper-inflammation during the deep invasion of the placenta in hemochorial placentation (32, 34). In humans, decidualization coincides with the recruitment of uterine natural killer cells (uNK) to the uterus via local expression of chemokines such as CCL4, CXCL9, and CXCL10. This cell type is a rich source of growth and angiogenic factors and is the most frequent immune cells of the uterus that modulate T cell functions through the expression of glycodelin-A and galectin-1 (33). The endometrial immune system plays a fundamental role in immune-based uterine preconditioning necessary for blastocyst implantation. The activation of redox-sensitive signaling pathways, resistance to cell death, and the expression of angiogenic factors such as vascular endothelial cell growth factor (VEGF) are among the most important mechanisms for the immune-based uterine preconditioning process (32).

Decidualization has both protective and supportive roles in placental development. It provides cytokines and growth factors necessary for remodeling the implantation site, while simultaneously limiting the degree of placental invasion to prevent complications such as placenta accreta. The depth of trophoblast invasion in mammals, which differs according to the type of placentation, correlates with the extent of decidualization (35).

The connection between the placenta and decidua is bidirectional, with the secreted factors of each tissue regulating the function of the other tissue (Figure 3). For example, a recently-discovered small peptide secreted by viable embryos called pre-implantation factor (PIF) enhances decidualization of Endometrial Stromal Cells (ESCs) (36). In addition, Profilin-1 (PFN1), an actin-binding protein secreted by EVT, enhances the proliferation and decidualization of ESCs *in vitro*. HCG, produced by trophoblasts, maintains progesterone production by ovaries, which is necessary for decidualization. Reciprocally, conditioned media from DSCs increased the secretion of PFN1 by EVTs (37). Interestingly, EVs derived from endometrial stromal cells promote not only the decidualization process but also the differentiation of trophoblast stem cells into the EVT lineage. The increased expression of human leukocyte antigen (HLA)-G, matrix metalloproteinase 2 (MMP2), and integrin alpha (ITGAV) as markers of EVT differentiation validate the effect of stromal-derived EV (38). Moreover, these EVs could upregulate the expression of N-cadherin in trophoblasts, which is a critical marker of invasion (39).

Regardless of decidualization, ESCs themselves could regulate the invasion and migration of trophoblasts. Recent evidence has pointed out that the inflammatory environment generated by ESCs promotes the migration and invasive capacity of trophoblasts in a process modulated by tumor necrosis factor (TNF)-α signaling (40). The process of trophoblast invasion, as directed by DSCs, is a necessary step for blastocyst implantation. The implantation process consists of three stages: apposition, adhesion, and invasion. All phases of implantation are controlled by trophoblasts in different stages of differentiation. The apposition and adhesion phases are promoted by the stable connections between STBs and uterine epithelium. Then, with the penetration of STBs through the epithelium, the invasion phase initiates; however, successful implantation is not solely controlled by the trophoblast itself. It requires interconnections between the trophoblasts and uterine cells. These dialogs include

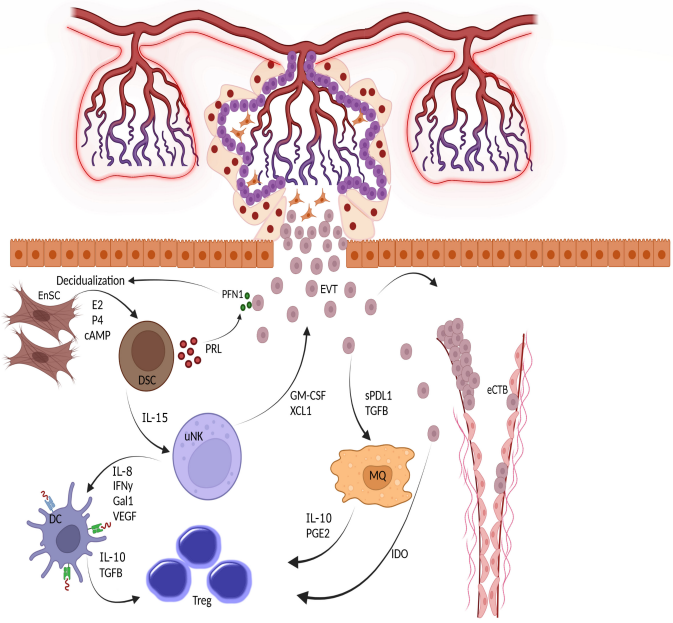


FIGURE 3
Triad interactions between trophoblast, decidual stromal cells and immune cells. Following implantation, the placental cytotrophoblasts in anchoring villi fuse together to form multinucleated syncitiotrophoblasts, which further differentiated to invasive extravillous trophoblasts (EVTs). The EVTs then invade the uterine tissues up to inner third of the myometrium. Some of the EVTs replace the endothelial cells and are called endovascular cytotrophoblasts (eCTBs). Secreted products from the invaded trophoblasts then modulate the function of endometrial stromal and immune cells. For example, Profilin-1 (PFN1) secreted from trophoblasts enhances the differentiation of endometrial stromal cells (EnSCs) into the epitheloid secretory decidual stromal cells (DSCs) in a process called decidualization through the action of estrogen (E2), progesterone (P4) and cyclic adenosine monophosphate (cAMP). Prolactin (PRL) is an important secreted product of DSCs and increases the production of PFN1 by trophoblasts. DSCs are master regulator of endometrial immune cells and help the function and survival of uterine natural killer cells (uNK), the most frequent immune cells in endometrium, by producing IL-15. The uNKs then produce factors such as IFN-γ, IL-18, Galectin-1, and VEGF to induce a tolerogenic phenotype in dendritic cells (DCs). Tolerogenic DCs are responsible for induction of regulatory T cells (Tregs) to maintain a pregnancy-friendly environment in the endometrium. These Tregs can also be induced by macrophages (MQs) under the influence of soluble products secreted from trophoblasts. Trophoblasts can directly induce Tregs using indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades tryptophan. The available *in vitro* data has shown that the immune cells like uNKs can enhance the migration of trophoblasts through the secretion of chemokines. Hence, interaction between trophoblasts, EnSCs and uterine immune cells form the endometrial environment, in which each cell type modulate the function of the others. GM-CSF, Granulocyte-macrophage colony stimulating factor; XCL1, X-C motif chemokine ligand 1; IL, Interleukin; IFN, Interferon; Gal1, Galectin 1; VEGF, Vascular endothelial growth factor; TGF, Transforming growth factor; PGE2, Prostaglandin E2.

several molecules and mediators. Among them are integrins, which are critical for the attachment of trophoblast to uterine epithelium. Like endometrial cells that express integrins on their surfaces, trophoblasts express α3, α5, β1, β3, β4, and β5 integrins, and their repertoire is modulated during invasion and differentiation of trophoblast cells (41–43). The process of trophoblast attachment to the uterine epithelium is also modulated through their interaction with extracellular matrix (ECM) components. Evidence suggests that, in addition to uterine cells, trophoblasts also produce ECM components like laminin, fibronectin, and vitronectin to increase their production of MMP, which is necessary for their invasion in an autocrine manner (44). Growth factors like epidermal growth factor (EGF) are also produced by trophoblasts to induce their invasion, differentiation, and proliferation (45). Transforming growth factor (TGF)-β, expressed by trophoblasts, was found to inhibit trophoblast invasion and proliferation through reducing MMP-9 and stimulating MMP inhibitors like tissue inhibitor of metalloproteinase (TIMP) (46). Trophoblasts also produce hCG, which increases MMP-9 and activates adenylate cyclase to produce cyclic adenosine monophosphate (cAMP) to boost their invasiveness (44). cAMP is also necessary to initiate endometrial stromal decidualization (47). In addition to the critical role of trophoblasts in implantation, the successful development of the placenta is dependent on implantation and decidualization. Taken together, decidualization, placentation, and implantation are three sides of a triangle that are necessary for maintaining a successful pregnancy.

Bidirectional crosstalk between endometrial immune system and placenta

The coexistence of two genetically distinct individuals throughout gestation was pointed out by Peter Medawar following his studies on skin grafts. This scientific view of pregnancy, known as 'Nature's transplant,' helped researchers better understand the interaction between the fetus and the maternal immune system (48). As an interface between fetal and maternal circulations, the placenta modulates the endometrial immune system. Placenta-derived factors help shape the function of immune cells in the uterus, and postnatal immune outcomes have also been associated with placental immune functions (49). Conversely, placental development and function are regulated by the local maternal immune system.

The first evidence for the regulation of placentation by the immune system comes from studies on one of the pregnancy complications known as preeclampsia (PE). Shallow trophoblast invasion in this syndrome causes maternal endothelial cell dysfunction and hypoxia in the developing fetus. Li and Wi, reported that women who have experienced preeclampsia in a previous pregnancy might exhibit protection when conceiving with a new partner. This finding could be aligned with the typical attributes of the immune system and highlight the central role of immunological specificity and memory against paternal antigens during pregnancy (50, 51).

There are two sites where fetal cells meet decidual immune cells: the large contact area between STB and maternal blood and the site of EVT-maternal decidua interaction. Endometrial immune cells have evolved mechanisms to simultaneously tolerate fetal antigens and regulate the invasion of trophoblasts (48). Bidirectional crosstalk between maternal immune cells and the placenta is exemplified by the unique functions of uNKs, the dominant leukocyte population in the first few weeks of pregnancy. Activation of Killer Immunoglobulin-like Receptor (KIR)-DS1 on uNKs by HLA-C2 on trophoblasts stimulates the production of soluble Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and XCL1 from uNKs leading to increased migration of trophoblasts *in vitro* (52). This interaction also stimulates uNKs to secrete pro-inflammatory, angiogenic, and proteolytic mediators to help trophoblast invasion. Recent evidence has shown that extracellular matrix proteins such as fibronectin and the pro-inflammatory cytokine Interferon, (IFN)- γ, upregulate the expression of non-classical HLA molecules on trophoblasts. Interestingly, only first-trimester trophoblasts but not term trophoblasts respond to this stimulation (53). uNK cells can also orchestrate hemochorial placentation by regulating hypoxia, the key intrinsic regulator of placentation. In uNK-depleted mice, decreased oxygen tension stabilized hypoxia-inducible factor (HIF)-1 protein causing trophoblasts to become more invasive (54). Moreover, uNKs regulate the development of uterine spiral arteries by the production of VEGF (55). Mutually, the placenta also shapes the uterine immune system, and in this regard, uNKs play a crucial role. Classical HLA-C, and non-classical HLA-E and HLA-G are among the HLA molecules expressed by trophoblasts; however, trophoblasts do not express classical HLA-A and B (56). Both HLA-E and HLA-C can inhibit the cytotoxic response of NK cells to the trophoblasts. HLA-G in the soluble form induces a senescent phenotype with low cytotoxicity in NK cells (57).

It has been reported that HLA-G can be induced by leptin, a placental protein which is expressed by human trophoblasts (58). Recent evidence has shown that in addition to trophoblasts, leptin induces HLA-G expression on myocytes and inhibits contraction and differentiation of these cells. Moreover, leptin at low concentration can inhibit the production of reactive oxygen species from macrophages. This tocolytic effect of leptin can be used in prevention of preterm labor (59). Leptin level has been increased during the human pregnancy. Placenta is the second leptin-producing tissue after adipose tissue in humans during pregnancy (58). In humans, leptin is induced by beta-hCG, estradiol, and insulin and mediates immune tolerance in placenta (60–62). Although leptin has also a crucial role in rodent pregnancy, the main trigger for leptin induction in mice is unknown. In pregnant mice, adipose tissue is the main source that produces leptin and placenta is not responsible for producing this hormone; however, its receptor named OB-R or leptin receptor is expressed in

placenta of both species. It is reported that mice lacking leptin receptor have fewer pups and adverse parturition outcomes (63). This implies that leptin expression with its broad immunoregulatory and functional roles has an evolutionary advantage in humans and mice as well as other primates.

Macrophages are the second most frequent immune cell population in the decidua. Modulation of macrophage polarization and function by trophoblasts is conducted via paracrine-secreted factors or cell-cell contacts. There are two subpopulations of macrophages: M1 and M2. M1 macrophages participate in extensive tissue remodeling via the secretion of matrix metalloproteinases upon trophoblast invasion, while M2 macrophages are activated by secreted factors from trophoblasts such as placental-specific glycoproteins (PSGs), M-CSF, TGF-β, IL-10, IL-34, and sHLA-G5 and support angiogenesis through the secretion of VEGF. In addition, M2 macrophages are involved in tissue repair and immune tolerance to the developing fetus (64). Recent evidence has pointed out that the phenotype of the macrophages in decidua basalis and parietalis, two anatomically different parts of the placenta, are different from each other and the secreted products of trophoblasts are responsible for this event. Macrophages of basalis layer highly express CD11c and are efficient inducers of regulatory T cells (Tregs) which secrete high levels of CXCL1, CXCL5, M-CSF, and IL-10. Whereas, macrophages of parietalis layer are motile and characterized by induced expression HLA class II for activation of T cell. It has shown that the conditioned media that collected from the culture of trophoblasts can convert the phenotype of decidua parietalis-associated macrophages into the decidua basalis phenotype (65).

Trophoblasts lack Major histocompatibility complex (MHC)-II molecules and are not able to prime maternal T cell activation; instead, decidual myeloid cells uptake EVT antigens and present them to maternal T cells in uterine draining lymph nodes (56). To avoid maternal T cell activation against fetal antigens, trophoblasts regulate T cell function by expressing indoleamine 2, 3 dioxygenases (IDO), an enzyme that inhibits T cell proliferation by depleting tryptophan (66–68). Similarly, trophoblasts control uterine T cells by Programmed cell Death Ligand (PDL)1 and PDL2 expression. This interaction is crucial for maternal tolerance during pregnancy since the blocking of PD1-PDL1 interaction in the decidua results in the expansion of T helper (Th)-1 and Th17 subtypes in favor of embryo resorption (69).

Trophoblasts also secrete TGF-β, which induces the differentiation of T cells to Treg. Moreover, placental TGF-β, IL-10, TNF-related apoptosis-inducing ligand (TRAIL) as well as galectin-1 help Tregs to expand (70). Progesterone produced by the early placenta is critical for the development of DSCs and differentiation of Tregs (71). Other examples of uterine immune stem modulation by trophoblasts are the expression of HLA-G dimers by trophoblasts which induce a tolerogenic phenotype in dendritic cells (DC) (72).

Besides trophoblasts, amniotic epithelial cells (AEC) also have a profound effect on modulation of immune cells. Human AEC (hAEC) exerted an inhibitor effect on proliferation of naive $CD4^+$ T cells and production of Th1 and Th17 cytokines, while induced production of Th2 cytokines and augmented differentiation of Tregs and production of TGF-β1 and IL-10 (73, 74).

There is an increasing body of evidence suggesting a noticeable link between a pro-senescent decidual response in peri-implantation endometrium and recurrent pregnancy loss (75). Defective decidualization has been postulated to impair proper modulation of endometrial immune cells in a pregnancy-friendly manner, which could potentially lead to implantation failure or miscarriage (76). This finding shows that the endometrial stromal cells are one of the main elements of the regulation of the endometrial immune system and seriously challenges the immune system defect as the primary cause of repeated miscarriage and infertility (77–79). There is a growing body of evidence showing that mesenchymal stem cell therapy of female CBA/J mice protects fetuses from resorption in CBA/J x DBA/2 abortion model and induces Treg and Th2 type cytokines profile (80–82).Considering that highly regenerative endometrial stroma harbors multipotent endometrial stem cells with properties and phenotype similar to bone marrow and adipose tissue MSC and possibly are responsible for regenerating the endometrial stroma in each menstrual cycle (83), it would be of utmost interest to investigate whether mesenchymal stem cell therapy in CBA/J mice could potentially affect decidualization.

In the past decade, exosomes have emerged as novel mediators of intercellular communication by transfer of signaling molecules (84). All the aforementioned functions of the placenta can be mediated by exosomes. Placental exosomes can be detected in maternal blood as early as 6 weeks after conception and gradually increase until peaking at term (85). Evidence suggests that placental exosomes can be internalized by NK cells *in vitro* and *in vivo* and have diverse functions such as apoptosis induction and cytotoxicity reduction (86). These placental exosomes also educate classical phagocytic monocytes to the phenotype with impaired migratory and pro-inflammatory capacities (87). Placental exosomes can also affect apoptosis, differentiation, and cytotoxicity of maternal T cells. For instance, placental exosomes carrying NKG2D ligands such as MHC class I chain-related (MIC) and UL-16 binding protein (ULBP) as their cargo can decrease the cytotoxic action of CD8+ and γδ T cells (88).

Although, with a default conception in mind of the immunoregulatory activity of trophoblasts, most researchers have come to the conclusion that these cells exert suppressive activity on the uterine immune system, interpretation of these results should be made with caution. Pregnancy is a continuous spectrum starting from implantation and ending with parturition and involves different phases with inflammatory or anti-inflammatory natures. Many cell types and mediators from maternal origin are involved and work in concert in uterine immune modulation (89–95). In this regard, the immunoregulatory activity of trophoblasts could not tell us the whole story of endometrial immune regulation. There are three-direction interactions between the placenta, maternal immune cells, and the endometrium that shape the uterine immune fate during pregnancy and, in this regard, endometrial stromal cells are among the master regulators of uterine immune system (93).

The story of the link between placenta development and carcinogenesis

The concept of evolutionary similarities between cancer and the placenta originates from a hypothesis proposed by Scottish embryologist John Beard. He characterized cancer as ectopic trophoblastic cells (96). While not entirely comprehensive and lacking robust evidence, this hypothesis has spurred significant research in the field. Placentation and carcinogenesis exhibit numerous shared features, broadly categorized into four levels: metabolism and cell behavior, proteins and molecular signatures, signaling pathways, and tissue microenvironment (Table 1) (6, 144–146). The ability to invade the surrounding stroma is a crucial characteristic shared by trophoblasts and cancer cells. This similarity has led to the hypothesis that metastasis is a negative consequence of invasive placentation, termed "antagonistic pleiotropy." Advocates of this theory argue that cancer occurs late in a human's lifetime, making this negative consequence tolerable. Furthermore, the advantages of invasive placentation, particularly in nourishing the fetus, outweigh its negative costs (5).

The observation that marsupials' placentas lack the hallmarks of mammalian invasive placentation but still develop invasive skin cancer later in life challenges the accuracy of antagonistic pleiotropy. To address this discrepancy, an alternative hypothesis called "positive pleiotropy" suggests that it is not the nature of the trophoblast itself that influences invasiveness, but rather the endometrium that controls trophoblast invasion. According to this view, animals like ruminants with less-invasive placentation have evolved to suppress trophoblast invasion, reducing the risk of malignancy (5); however, the reliability of this hypothesis is questioned by shared molecular mechanisms with other biological phenomena such as wound healing. A more detailed theory, the Evolved Level of Invasibility (ELI) theory, emphasizes the role of endometrial fibroblast cells. ELI proposes that the permissiveness or resistance of endometrial stromal fibroblasts to placental invasion in each species determines the level of malignancy and metastasis. Contrary to human embryonic stem cells (ESCs), bovine ESCs resist trophoblast invasion. Supporting this hypothesis, similar behavior is observed in skin fibroblasts of each species in response to cancer cell invasion. Tissue fibroblasts in each species, with different types of placentation, have evolved to respond to invasive cancer cells similarly to how their endometrial fibroblasts react to placental invasion (147).

As trophoblasts undergo differentiation into their lineages, they undergo an epithelial-mesenchymal transition (EMT)-like process and cell-cell fusion, akin to processes observed in cancer. Differentiation of CTB into EVT and the formation of STB from CTB involve EMT and cell-cell fusion, respectively. EMT is considered a crucial step for metastasis and invasion of tumor cells. Additionally, tumor cells respond to therapeutic intervention-induced cell damage through cell-cell fusion (148, 149).

Immune escape is another aspect of similarity between trophoblasts and tumor cells. Among the shared mechanisms of immune escape is the expression of cell surface antigens to induce immune tolerance. Proliferation, vasculogenic mimicry (VM), and angiogenesis are other aspects of similarity between placentation and carcinogenesis (144). Both tumor cells and trophoblasts are highly proliferative due to high telomerase activity, overexpression of anti-apoptotic proteins such as survivin, and activation of the insulin-like growth factor (IGF) signaling pathway (150, 151). In the VM process, tumor cells and trophoblasts form vascular structures upon invasion, providing an additional blood supply different from endothelial-dependent vasculature (132, 133). Both cell types use the same genes and signaling pathways in this process, including the overexpression of matrix glycoprotein-binding galectin-3 and Mig-7, key factors for the development of an endothelial phenotype (127). Apart from VM, the expression of key angiogenic factors such as angiopoietins, VEGF, and Placental Growth Factor (PGF), which are crucial for spiral artery remodeling in placentation and tumor cell growth, is a critical step for increasing blood supply (144).

The microenvironment of cancer and the placenta exerts profound effects on the behavior of cancer cells and trophoblasts. The decidua, as the maternal side of the placenta, affects gene expression reprogramming in trophoblasts. Similarly, the tumor microenvironment (TME) can induce epigenetic alterations in cancer cells, affecting the behavior of cancer cells, such as the promotion of cancer stemness. Immune regulation mechanisms are also shared in decidua and TME. The expression of HLA-G by both trophoblasts and tumor cells to prevent NK-mediated cytolysis, production of immunosuppressive factors to inhibit the function of cytotoxic immune cells, and the recruitment of immunosuppressive cells such as Treg and myeloid-derived suppressor cells (MDSC) are among these mechanisms. The use of the same ECM proteins and cell-secreted products and the induction of hypoxia have been observed in both environments. MMPs secreted by trophoblasts and cancer cells can shape the ECM, triggering its remodeling and facilitating invasion and the recruitment of immune cells. On the other hand, hypoxia induces EMT and invasion in CTB and VM, invasion, and angiogenesis in tumor cells (152).

Pregnancy and cancer are associated with profound systemic metabolic changes. Examples of these metabolic changes include increased blood volume and hyperglycemia to meet energy requirements during pregnancy, and abnormal metabolism in a process named cachexia, which involves the loss of skeletal muscle in cancer. In both conditions, insulin resistance (IR), defined by the inefficient function of insulin to promote glucose uptake, takes place. Inflammatory mediators, including TNF-α, IL-1, and IL-6, are key factors in the induction of IR. Nevertheless, the potential signaling pathways behind this IR remain to be further elucidated. After delivery or surgical removal of the tumor, IR gradually disappears (144).

Based on the aforementioned similarities between carcinogenesis and placentation, it could be inferred that the placenta can be considered a physiological tumor (125). In this context, the expression of placenta-specific antigens in different types of cancers has motivated many researchers to target them for cancer therapy (153–158). Moreover, biological products derived from the placenta can remarkably hinder the growth, proliferation, and progression of cancers (159, 160). For example, conditioned media from the term placenta can induce apoptosis and reduce proliferation in non-small

TABLE 1 Different aspects of similarity between placenta and cancer.

	Placenta	Ref	Cancer	Ref
Metabolic phenomena				
Insulin resistance (IR)	Human placental growth hormone secreted from placenta induces mild IR through increasing fatty acids that interfere with glucose entrance to the cells for fetal consumption.	(97)	Inflammation induces cachexia in cancer, which induce production of pro-inflammatory mediators. Long term exposure to these mediators leads to IR.	(98)
Warburg effect	The Warburg effect is necessary for rapid growth of the placental villus.	(99)	Cancer cells prefer to metabolize glucose anaerobically rather than aerobically, even under normoxia, which contributes to chemoresistance.	(100)
Protein signature				
Human leukocyte antigen (HLA)-G	HLA-G expressed by trophoblasts interacts with inhibitory signals on NK cells to control their cytotoxicity and its expression correlates with trophoblast invasiveness.	(101)	HLA-G expression promotes tumor progression, metastasis and is associated with poor clinical outcome.	(102)
Placental growth factor (PlGF)	Its main action is developing angiogenesis and maintaining the blood vessels through direct action, and indirectly by VEGF.	(103)	PlGF is overexpressed in some tumors and promotes angiogenesis. Cancer patients with higher expression of PlGF have poor prognosis and lower survival.	(104)
Human chorionic gonadotropin (hCG)	hCG is an important mediator for the endometrial implantation of trophoblasts and stimulates myometrial cell proliferation.	(105)	hCG promotes cancer cell invasion and is associated with poor prognosis in cancer patients. It induces angiogenesis and serves as a diagnostic biomarker in some types of cancer.	(106)
Indoleamine 2,3-dioxygenase (IDO)	Increased kynurnine to tryptophan ratio or enhanced IDO activity has been reported in placenta and is associated with gestational age	(67, 107)	Enhanced IDO activity has been reported in several malignancies. It helps tumor cells to escape from the cytotoxicity of cytotoxic T cells and γσT cells.	(108, 109)
Galectins	Galectins regulate maternal-fetal immune tolerance and promote placental angiogenesis.	(110)	Galectins are involved in cancer biology, where GAL-1 and GAL-7 are protumorigenic, while GAL-4 and Gal-8 act as a tumor suppressor.	(111)
Insulin receptor	Placenta respond to insulin for growth.	(112)	The growth of cancer cells is effectively induced by insulin.	(113)
Mucin 1 (MUC1)	MUC1 has been found in human trophoblasts, and its expression is increased during placental development.	(114)	MUC1 protects cancer cells from apoptosis by direct binding to the P53 regulatory domain 36.	(115)
α5β1 integrin	The expression of integrins such as α5β1 gives the ability of migration and invasion to the trophoblast.	(116)	α5β1 expression in tumor cells promotes invasion and metastasis.	(117)
The chemokine ligand 2 (CCL2)	Expressed by Decidual fibroblasts, macrophages and EVTs, can help recruit T cells to the maternal-fetal interface.	(118)	CCL2, secreted by cancer cells or CAFs, recruit monocytes, MDSCs, and Treg into TME.	(119)
Transforming growth factor beta (TGF)-β	TGF-β can both enhance and decrease the invasion of trophoblasts through Smad and ERK signaling pathways, respectively.	(120)	In different stages of cancer, TGF-β can both enhance and decrease the proliferation and invasion of cancer cells	(121)
Molecular and Epigenetic similarities				
Methylation status	Global DNA methylation level is lower in placenta compared to other organs.	(122)	Global hypomethylation status exists in cancer including hypomethylation of LINE-1 elements.	(122)
Activation of normally silenced retrotransposons	Transposable elements that are normally silenced in somatic tissues are reactivated in placenta.	(123)	Transposable elements that are normally silenced in somatic tissues are reactivated in cancer.	(124)
Toleration of gene mutation	The placenta can tolerate gene mutation without causing maternal harm.	(125)	Cancer cells also tolerate chromosomal aberrations but with undesired consequences to the host.	(126)
Signaling pathways				
HIF, MAPK, PI3K/AKT, MMP, and growth factor signaling pathways like IGF-1, TGF,	These signaling pathways involved in invasion, growth, proliferation, angiogenesis and immune evasion in trophoblasts	(127)	These signaling pathways involved in invasion, growth, proliferation, angiogenesis and immune evasion in cancer	(127)

(Continued)

TABLE 1 Continued

	Placenta	Ref	Cancer	Ref
Signaling pathways				
PDGF, FGF, PGF, VEGF, etc.				
Tissue Microenvironment Cell behavior (Trophoblasts and cancer cells)				
Epithelial-Mesenchymal transition (EMT)	In placental development, cytotrophoblasts (CTBs) can undergo pseudo-EMT and convert to extravillous trophoblasts (EVTs).	(128)	EMT is an essential step for invasion and metastasis of different cancers.	(129)
Cell-Cell fusion	Cell-Cell fusion in placenta may help syncitiotrophoblast (STB) to defend against oxidative stress-induced damage caused by blood flow from the uterine spiral artery	(130)	Occurs in cancer to respond to gene damage after chemotherapy or radiation	(131)
Vasculogenic mimicry (VM)	The trophoblasts that remodel the uterine spiral artery and the vessels that form in the placenta are thought to be formed by a kind of VM.	(132)	The tumor blood supply does not depend on invasion of the vascular endothelium, and the tumor itself forms channels through the process of VM.	(133)
Cell transformation	Decidual fibroblasts experience mesenchymal-epithelial transition (MET) during decidualization.	(134)	Cancer-associated fibroblasts developed from macrophages through a process of macrophage-myofibroblast transition (MMT).	(135)
Tissue Microenvironment Phenomena				
Hypoxia	A moderately hypoxic environment is believed to promote CTB exit from the cell cycle, induction of EMT, and acquisition of invasive ability.	(136)	Hypoxia can induce many tumor processes, such as invasion, vasculogenesis, angiogenesis and VM, to generate a nutrient supply system.	(137)
Immune escape: Downregulation of HLA class I expression on surface	Suppression of HLA-A and -B genes prevent the recognition of trophoblast cells by the mother's cytotoxic T cells. This suppression is complemented by expression of HLA-C and HLA-G.	(138)	The downregulation or loss of HLA class I molecules can prevent tumor cells from being recognized by CTL.	(139)
Immune escape: Expression of Programmed cell death ligand 1 (PD-L1)	PD-L1 is expressed on the outer surface of placental syncytiotrophoblasts, which is assumed to induce maternal immune tolerance to fetal tissue via programmed death-1 (PD-1) receptors on T cell.	(140)	Expression of PD-L1 by the tumor is a way for the tumor to evade the PD-1 positive T cells.	(141)
Extracellular matrix (ECM) remodeling	Differentiation of fibroblasts to decidual stromal cells is associated with extensive ECM remodeling.	(142)	ECM stiffening and degradation drive cancer.	(143)

cell lung cancer. The cellular and molecular mechanisms behind this observation involve the up-regulation of caspase 3 and suppression of the proliferation marker ki67 in tumor tissues and cell lines (161). It has also been shown that placental lysate extracts are able to suppress proliferation and induce differentiation of tumor cells *in vitro* (162). Besides *in vitro* experiments, immunization with human placenta-derived antigens has been used as an effective approach for preventive and therapeutic aims in animal cancer models (157, 163).

The idea of "immunoplacental therapy" dates back to the 1970s when a Muscovite oncoimmunologist, Valentin I. Govallo, immunized cancer patients with extracts of term placental chorionic villi. He found his therapeutic approach effective in solid tumors and published his findings in the book "Immunology of Pregnancy and Cancer." According to Govallo's opinion, cancer vaccination creates an immunological state similar to spontaneous abortion in pregnant women, in which immunologically foreign placental tissue would be rejected. In patients receiving placental extracts selected from various types of cancers, the overall three-, five-, and ten-year survival rates significantly increased, and metastasis was also resolved. Since Govallo's observations, researchers have focused on using placental antigens for cancer vaccination (164).

The application of placenta-derived antigens in designing prophylactic cancer vaccines has been demonstrated in research on placental heat shock protein, glycoprotein-96 (gp96), for cancer immunotherapy. Immunization of mice with placental gp96, one week prior to challenge with melanoma cells, decreased tumor volume to 45% for about one month after the injection. In addition, higher cytotoxicity of CD8+ cells was observed against melanoma cells after immunization. Similar results were reported in gp96-immunized mice after a challenge with breast cancer cells. Comparing the tumor inhibitory effects of placental gp96 vaccine

with tumor cell lysate-loaded DC-based vaccine has shown similar results in melanoma and breast cancer mouse models (163). The underlying idea of using placental antigens as effective cancer vaccines led to our recent observation that immunization with placenta-specific 1 (PLAC1), a membrane-associated protein, delays tumor onset and increases survival in a mouse model of melanoma. This immunization increased humoral and cellular immune responses, as shown by higher titers of antibodies and increased killing capacity of plac1-specific CD107a+ cytotoxic T cells (157). Using an anti-PLAC1 antibody-drug conjugate has also been shown to be effective in prostate cancer immunotherapy (158).

The anti-cancer effects of placenta-derived cells, such as hAEC, have also been the focus of much research. In a mouse model of colorectal cancer, an expanded population of systemic and splenic cytotoxic T cells and reduced tumor burden were observed after vaccination with hAEC (165). In addition to hAEC itself, secreted products derived from these cells exerted similar cytotoxic and anti-proliferative effects against cancer cell lines *in vitro*. Vaccination with hAEC-derived EVs can also induce apoptosis in cancer cells by triggering the Warburg effect and arginine consumption (160).

While the antigenic similarity between placenta and cancer could be viewed as a framework for effective targeted cancer immunotherapy, lessons from other aspects of similarity are no less important for controlling cancer cell growth. Shared cell behavior, signaling pathways, metabolic activity, and, most importantly, a common network of immunosuppressive microenvironment shed light on the future direction in the field of cancer immunotherapy. Nonetheless, the central role of the endometrium in controlling trophoblast invasion should not be ignored. According to the positive pleiotropy hypothesis, it is the endometrium and its controlling mechanisms that determine the degree of trophoblast invasion and dictate the type of placentation in eutherian mammals (5). In this context, a comprehensive view of the placenta and pregnancy decidua could better shape our understanding of the pathogenesis of cancer development and provide new clues for cancer treatment.

Placental development and pregnancy-related diseases

The placenta plays crucial protective roles for the fetus, but its structure and function are also associated with pregnancy complications such as IR, preeclampsia, and eclampsia (166–168). Malfunctioning placenta can affect the fetus, leading to preterm birth, fetal growth issues (169), and neurodevelopmental abnormalities (170, 171). Research on fetal nutrition and its essential role in healthy fetal development (172, 173) has laid the groundwork for the concept that many fetal and adult disorders originate in the placenta. In fact, future cardiovascular disorders may be both predicted and caused by placental function (174, 175).

Insufficient placental function, as reported, can influence early growth and obesity, which are associated with an earlier age at menarche. Additionally, measurements of the fetus's size at 35 weeks of gestation closely correspond with the rate of placental growth between 17 and 20 weeks of gestation (176). Compared to infants of normal weight, the placentas of newborns with intrauterine growth restriction have smaller diameters, higher placental coefficients, and lower weights and volumes (177). Intrahepatic cholestasis of pregnancy (ICP), a liver disease during pregnancy, typically affects women in their third trimester. ICP is linked to a higher risk of spontaneous preterm birth, fetal distress, and sudden intrauterine death, causing complications for both mother and fetus. Although the exact origin of the disease is presently unknown, research suggests that maternal bile acid levels play a role. Studies indicate that high bile acid concentrations are linked to placental apoptosis. Additionally, the placenta's function in protecting the fetus from the negative effects of potentially harmful endogenous compounds involves mitigating the effects of bile acids on the fetus (178).

Placental disorders are often categorized into ischemic and nonischemic types. Preeclampsia, and intrauterine growth retardation (IUGR), fall under conditions where the fetus lacks sufficient blood perfusion and are thus categorized as ischemic placental disorders. Although preterm labor (PTL) was once classified outside this category (179), about one-third of preterm births are associated with changes in placental ultrastructure typically seen in ischemic disease processes (180). This finding suggests a significant overlap in the mechanisms responsible for each type of pregnancy complication (22).Critical in determining life expectancy are patterns of intrauterine development and size at delivery, impacting adult rates of morbidity and mortality as well as neonatal viability. Low birth weight, according to human epidemiological studies, is linked to an increased risk of adult-onset cardiovascular and metabolic diseases such as hypertension, coronary heart disease, obesity, and type 2 diabetes, as well as a higher incidence of reproductive and neurological disorders (170, 171, 174, 175, 181, 182). Changes in the placenta's surface area, affecting both diffusion and transporter-mediated processes, directly influence the capacity for nutrition transfer (183). In many species, there is a positive correlation between fetal and placental weight (22, 184–187). In human IUGR cases, the placental volume is on average 92.66 cm^2 smaller in comparison to the placenta in normal pregnancy (188).

Placenta proteome in health and disease

Placental proteins in normal pregnancy

Transcriptome analysis indicates that 65% of all human proteins are expressed in the placenta. Within this subset, the placenta exhibits higher expression levels for 354 of these genes, the majority of which encode secreted proteins (189).

Placental proteins are not only fundamental for pregnancy maintenance and development of the fetus, but also exerts critical function in bidirectional crosstalk with endometrial immune system and establishment of maternal tolerance during pregnancy; however, our knowledge of placental proteome is still limited and

these aspects must be studied in more detail to understand the dynamics of proteins in pregnancy, fetal growth and complications during pregnancy. Indeed, this information is central for our understanding of molecular mechanisms involved in placental and gestational disorders. In this regard, proteomics technology has fundamentally advanced our knowledge of placental proteins. In this context, we recently compared normal first-trimester and full-term human placenta proteomes. A total of 120 differentially expressed proteins were identified in the first trimester versus term placenta. Among these, 20 proteins with expression fold changes higher than 2 or less than 0.5 were analyzed. Accordingly, GRP78, PDIA3, ENOA, ECH1, PRDX4, ERP29, and ECHM vs. TRFE, ALBU, K2C1, ACTG, CSH2, PRDX2, FABP5, FABP4, K2C8, MESD, K1C9, MYDGF, HBG1 had significantly higher expression in the first trimester and full-term placenta, respectively. Interestingly, we found two proteins (MESD, MYDGF) that were exclusively expressed in the first-trimester placenta. These proteins play significant roles in biological quality control, programmed cell death, hemostasis and catabolic processes, protein folding, cellular oxidant detoxification, coagulation, and retinal homeostasis. They also play essential roles in reactions to chemical stimuli and stress (190). In a comprehensive study conducted by Mushahary et al., 117 proteins in the term placenta were reported. The functions of the reported proteins were categorized into eight distinct groups, including metabolism, cell stress, cytoskeletal, transport, signal transduction, nuclear proteins, translation, cell cycle, and growth (191). In a recent study, an anatomical approach was employed to unravel the proteome profile of different sub-anatomical regions of the human placenta (192). The results showed 1081, 1086, and 1101 proteins in maternal, middle, and fetal sub-anatomical regions respectively with 374 differentially expressed proteins between sample site locations and sub-anatomical regions. Notably, proteins with the anti-senescent function were decreased in the maternal sub-anatomical region, while proteins associated with a switch from ATP to fatty acid consumption were increased in the middle and fetal sub-anatomical regions.

Apart from high throughput proteomics approaches, there are some studies with a focus on isolated placental proteins. A specific placental protein, placental protein 13 (PP13), binds to β-galactoside residues on the cell surface, cytoskeleton, and extracellular matrix, generating various responses such as blood vessel adaptation, immune responses, and influencing functions like apoptosis and molecular recognition (193). PLAC1 is the product of a paternally imprinted X-linked gene (*Plac1*) with limited normal tissue expression, which plays fundamental roles in placental function and development. It is expressed in both human and mouse placenta and is necessary for placental and embryonic development. Knockout and heterozygous placentae of the Plac1-null allele inherited from the mother weighed roughly 100% more than wildtype placentae, though the corresponding embryos weighed 7-12% less (194, 195). Retrotransposon Gag-like-1 (RTL-1), also known as Peg11, is another placental protein with a known function in placenta and fetal development. Its expression during neonatal period has to be finely regulated. Inheritance of the *Peg11/RTL1* KO allele from father causes late fetal lethality due to late fetal growth retardation, whereas maternal transmission leads to overexpression of *Peg11/Rtl1* and neonatal lethality (196, 197). This protein is preferentially expressed in the labyrinth layer of the mouse placenta by the endothelial cells. In both the *Peg11/Rtl1* paternal and maternal KO placenta, severe abnormalities of the fetal capillaries were observed. This finding is in favor of the critical role of PEG11/RTL1 protein in maintaining the integrity of the feto–maternal interface of the placenta during pregnancy (198). The placenta expresses approximately 100 placental-specific proteins. Table 2 provides examples of placental proteins and their related biological functions.

Modification of placental protein in preeclampsia

Dr. David Barker initially introduced the concept of fetal origins of adult disease (FOAD), which has garnered significant attention since its inception. The FOAD hypothesis posits that events during early development profoundly impact the risk of developing adult diseases in the future. Low birth weight, serving as a surrogate marker for poor fetal growth and nutrition, is associated with conditions such as coronary artery disease, hypertension, obesity, and IR (220–227). Epidemiological evidence now establishes a connection between an individual's susceptibility to chronic diseases in adulthood and events during their intrauterine phase of development. Influences during this period can permanently alter the functional capacity and structure of organ systems, as well as the activity of enzyme systems and endocrine axes. These effects set the stage for a diverse array of diseases that may manifest many years later, often in response to secondary environmental stressors.

The foundation of fetal development lies in the placenta, which plays a crucial role in nutrient and oxygen delivery to the fetus. Impaired placental function or its inability to adapt may compromise fetal development (175). There is accumulating undeniable evidence in the past decade regarding the fundamental role of placental phenotype, structure, physiology, and function in a variety of postnatal diseases; however, we will specifically focus here on the modification of the placental proteome in preeclampsia, a leading cause of maternal and neonatal morbidity and mortality. This information is essential for establishing novel biomarkers for the non-invasive diagnosis of this disease.

Survivors of preeclampsia face reduced life expectancy with increased risks of diabetes, stroke, and cardiovascular diseases. Indeed, babies born from preeclamptic pregnancies are at higher risk of preterm birth, perinatal death, and postnatal diseases such as neurodevelopmental disability, and cardiovascular and metabolic diseases later in life. Preeclampsia is a syndrome driven by placental dysfunction causing maternal endothelial dysfunction and systemic inflammation (228).

Only a limited number of studies have delved into alterations in placental tissue proteins related to pregnancy-related disorders. Among a total of unique placental proteins reported by Mushahary et al. (191), twenty-five proteins (mainly cell stress proteins) have been associated with PE (229–235), early pregnancy loss [ubiquitin-conjugating enzyme E2 N, calgizzarin (S100

TABLE 2 Examples of proteins expressed in different placental cell types.

Protein Name	Expressing cell	Description	Biological Process	References
PEG10 PAGE4 PEG11/RTL1	Cytotrophoblasts	Paternally expressed 10 Prostate-associated gene 4 Paternally expressed 11/ Retrotransposon-like 1	Apoptosis, Differentiation, Transport Transcription regulation Maintaining placental fetal capillaries	(199, 200) (201, 202) (203)
CSH1 KISS1 GCM1 PLAC1	Syncitiotrophoblasts	Chorionic somatomammotropin hormone 1 KiSS-1 metastasis suppressor Glial cells missing transcription factor 1 Placenta-Specific 1	Stimulating lactation, fetal growth and metabolism Metastasis Suppressor Transcription regulation Placental establishment and maintenance	(204, 205) (206, 207) (208, 209) (195, 210, 211)
PAPPA2 PRG2 HLA-G	Extravillous trophoblasts	Pappalysin 2 Proteoglycan 2 Human leukocyte antigen G	Hydrolase, Metalloprotease, Protease Immunity Immune Tolerance	(212, 213) (214) (215, 216)
FCGR2B LIN28B FBN2	Placental blood vessels and stroma	Fc fragment of IgG receptor IIb Lin-28 homolog B Fibrillin 2	Transcytosis of IgG across placental endothelium RNA-mediated gene silencing Promote trophoblast invasiveness and has glucogenic action	(217) (218) (219)

calcium-binding protein A11), galectin-1 (236), PTL (cytoskeletal proteins) (237) and assisted reproductive technology [fascin] (238).

In a systematic review, twelve studies employing mass spectrometry-based techniques were examined, comparing samples from preeclamptic and normotensive pregnancies. Across all studies, 401 proteins with significantly altered expression in PE were identified. Comparison between studies identified 52 proteins as significant in two or more studies, which were then enriched for 22 pathways, including those previously implicated in PE such as hemostasis, immune response, and lipid metabolism. In particular, the proteins complement component 4 and apolipoprotein E displayed abnormal expression at week 12 before the clinical diagnosis of PE, suggesting their value as clinical biomarkers (239). Downregulation of antioxidant proteins (peroxiredoxin two and peroxiredoxin 3) and altered expression of stress response proteins (Hsc) 70, Heat shock protein (Hsp) gp96, and protein disulfide isomerase) has also proposed in the pathogenesis of PE (235). To assess the effect of elevated levels of neurokinin B (NKB), a tachykinin linked to systemic symptoms of PE, on the placental proteome, the proteome profile of cultured human term CTB was assessed in response to NKB treatment. The results revealed a statistically significant decrease in 20 proteins, including those crucial in antioxidant defenses (240). Table 3 summarized alterations of placental proteins in women with PE.

Placental proteins as biomarkers for early diagnosis of Preeclampsia

There is a necessity to identify and establish gestational biomarkers that can be employed for monitoring pregnancies, and predicting adverse outcomes. While utilizing proteomic biomarkers in placental tissue is one approach for assessing health, nutritional status, and disease, it is practically challenging

TABLE 3 Alterations in the placental protein expression in PE.

Name	Description	Expression Change	References
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Up	(241–243)
ANXA4	Annexin A4	Up	(243–245)
CLIC3	Chloride intracellular channel protein 3	Up	(235, 241, 246–248)
PRDX2	Peroxiredoxin-2	Up	(247, 249–251)
HSPB1	Heat shock protein beta-1	Up	(242–244, 246, 247)
ALB	Albumin	Down	(241, 244, 247–249)
PLG	Plasminogen	Up	(241, 249, 252)
HBZ	Hemoglobin subunit zeta	Up	(241, 248, 250)
FGB	Fibrinogen beta chain	Down	(244, 249, 250)
FGG	Fibrinogen gamma chain	Down	(248–250)
CAT	Catalase	Up	(242, 249–251)
CYP11A1	Cholesterol side-chain cleavage enzyme, mitochondrial	Up	(241, 248, 253)
ANXA6	Annexin A6	Up	(241, 249, 252)
HSPA5	Endoplasmic reticulum chaperone BiP	Up	(243, 246, 247, 249)
ATIC	Bifunctional purine biosynthesis protein ATIC	Up	(241, 248, 251)
ACTG1	Actin, cytoplasmic 2	Down	(235, 244, 254)
PAPPA2	Pappalysin-2	Up	(241, 245, 248)
FLT1	Vascular endothelial growth factor receptor 1	Up	(245, 248, 249)
FTH1	Ferritin Heavy chain	Down	(255, 256)

for the early diagnosis of gestational diseases. Therefore, recent trends primarily focus on the non-invasive examination of placental proteins in peripheral blood. Human placenta harbors numerous proteins, most of which are secreted to the maternal circulation during pregnancy. Notable examples of these secreted proteins include human chorionic gonadotropin, human placental lactogen (hPL), human GH variant or placental growth hormone, leptin, corticotropin-releasing hormone, placental growth factor (PlGF) all of which are secreted by the placenta into the mother's bloodstream (31, 58, 178, 257–265).

The composition of maternal plasma proteome is steadily altered throughout gestation, which is in part due to the changes in the placental proteins secreted to the maternal circulation. This has offered a novel perspective for the precise timing of placental protein expression relative to gestational age, the "placental proteomic clock". Keeping this concept in mind, several researchers have unveiled changes in the placental proteome during different gestational periods. In a cohort study, Degnet et al. identified five placenta-derived proteins capable of predicting conceptional age, including chorionic somatomammotropin (CSH1/2), biglycan (BGN), glypican 3 (GPC3), inter-alpha-trypsin inhibitor heavy chain H5 (ITIH5), and lysosomal alpha-glucosidase (GAA) (266). In this study, Degnes et.al., among nearly 5000 measured proteins in maternal circulation, described 256 placenta-derived proteins and 101 proteins absorbed by the placenta. Of these, 101 placenta-derived proteins were deemed placenta-specific, forming two clusters with distinct developmental patterns throughout gestation. This innovative concept may serve to monitor deviations in the developmental patterns of placenta-derived proteins across gestation, indicating potential placental dysfunction.

PlGF, a member of the VEGF family predominantly expressed in the placenta, is crucial for normal pregnancy. PlGF concentrations are initially low in the first trimester of an uncomplicated pregnancy, increasing from week 11 to 12 and peaking at week 30, followed by a decline. Decreased PlGF expression in the placenta is associated with disruptions in placental development, maturation, and the onset of conditions like PE. Since PlGF's primary role outside the placenta is angiogenesis in response to pathological ischemia or injury, a low level of PlGF serves as an indicator of abnormal placentation. This is supported by observations that women without preeclampsia who give birth to small for gestational age babies also exhibit low PlGF levels early in pregnancy (267). Similarly, serum levels of inhibin A, a glycoprotein of human STB, have been explored for predicting and evaluating the severity of PE. The deficient trophoblastic invasion in PE leads to ischemic damage to the STB's surface layer. This modification is suggested to contribute to the increased release of inhibin A into the maternal circulation. In comparison to the group without adverse pregnancy outcomes, elevated serum levels of inhibin A during the second trimester not only pose a risk for preeclampsia but also for gestational diabetes mellitus, preterm delivery, and low birth weight (268, 269).

PP13 has emerged as a biomarker for predicting preeclampsia. Lower levels of maternal serum PP13 in the first trimester are associated with the development of PE and intrauterine growth restriction (193, 270, 271).

Measurement of maternal serum levels of PlGF and Pregnancy-associated Plasma Protein-A (PAPP-A), combined with maternal risk factors (e.g., obesity, hypertension, maternal age, etc.) and uterine artery measures by Doppler ultrasound, is proposed to have sensitivity of about 95% for detection of early-onset PE (diagnosed < 34 weeks) (272). In addition to alterations in placental protein expression, there are reports indicating that placental stress can lead to maternal physiological maladaptation to pregnancy by causing abnormal glycosylation of placental-derived factors. Endoplasmic reticulum (ER) stress leads to diminished complexity and sialylation of trophoblast protein N-glycosylation, and aberrant glycosylation of VEGF leading to its reduced bioactivity. ER stress affects the expression of 66 out of 146 genes annotated with 'protein glycosylation' and diminishes the expression of sialyltransferases (273). Table 4 summarizes the serum proteins associated with early onset PE.

Of note, most of the evaluated markers have been found when the diagnosis of PE has been already established. Despite the identification of proteins that are differentially expressed in patients with PE, few studies have been conducted to find a specific and reliable marker for early prediction of the disease onset. Using a machine-learning approach, a recent study combined multi-omics datasets (transcriptome, proteome, etc.) of plasma, urine and vaginal swab, collected from pregnant women during the first 16 weeks of pregnancy to find a panel of markers for early diagnosis of PE. Plasma proteome and urine metabolome were the most accurate predictive models for PE and, in each model, top proteins and metabolites were identified. Adenine, isovalerylglutamic acid, uric acid ribonucleoside, 1,5-anhydroglucitol, dehydroepiandrosterone, sialyllactose, Nε-acetyl-L-lysine, and nonanoylcarnitine were the top urine metabolites that predict PE with high accuracy. Besides, serum proteins leptin (LEP), VEGF, L-selectin (SELL), E-selectin (SELE), IL-24, IL-22, tyrosine-protein kinase transmembrane receptor (ROR1), C-X-C motif chemokine ligand 10 (CXCL10), and SPARC-like 1 (SPARCL1) were found to have the top predictive value for PE. Along with the plasma proteome and urine metabolome, the immune cell dynamics of the single cells isolated from the patient's blood between the first and second trimesters can also predict the onset of PE and correlated with the aforementioned markers in plasma and urine. Basal pSTAT1 (phosphorylated signal transducer and activator of transcription) and STAT5 signaling as well as basal NF-κB (Nuclear factor kappa B) levels in immune cells were the immune features that correlated with top predictive plasma proteins. There was also significant correlation

TABLE 4 Serum proteins associated with early onset PE.

Name	Description	Expression change	Reference
HP	Haptoglobin	Down	(274–276)
IGKV1-9	Immunoglobulin kappa variable cluster	Down	(275, 276)
CST3	Cystatin C	Up	(276, 277)
ITIH3	Inter alpha trypsin inhibitor heavy chain 3	Up	(275, 276)
FN1	Fibronectin 1	Up	(275, 276)
C4B	Complement protein	Down	(275, 277)

between the top predictive proteins and metabolites with clinical data of the patients. Upon final univariate analysis of all the predictive features, the LEP, CCL23, and FAM3D had significant association with PE outcome (278).

Placental proteins as biomarkers for cancer diagnosis and target discovery

Based on investigations into differentially expressed proteins in cancer, certain proteins have been identified with high expression in the testis. Coined as "cancer-testes antigens" (CTAs) by Old and Chen in 1997 (279, 280), these proteins form over 70 families with more than 170 members, extensively documented in various cancer databases (281). Intriguingly, some of these proteins also exhibit elevated expression in placental tissues, leading to the emergence of a new term, "cancer placental antigens" (CPAs) or more comprehensively, "cancer testes placental antigens" (CTPAs). Figure 4 illustrates several CPAs, their cellular compartmentalization, and enrichment in diverse biological pathways. Certain markers, like cancer antigen 125 (CA-125) and carcinoembryonic antigen cellular adhesion molecule-5 (CEACAM-5), already serve as serum tumor markers for pancreatic and ovarian cancers (282, 283); however, their limited specificity and sensitivity hinder their reliability for accurate diagnoses (155). Notably, Melanoma-associated antigen 3 (MAGE-A3), a CTPA, has proven to be an attractive target in undifferentiated sarcoma (284).

PlGF, utilized as a biomarker for predicting and diagnosing PE, has demonstrated overexpression in cancer and its associated stromal cells (104). Trophoblast surface glycoproteins TROP2 and 5T4 hold prognostic significance in at least 13 different types of cancers (285, 286). Antibodies against survivin, livin, and PLAC1 are detected in sera across various cancer types (287–290). Recent mining of transcriptomic databases has identified MMP11 and ADAM metallopeptidase domain 12 (ADAM12) as potential CPAs, with MMP11 exhibiting high transcript levels in 25 different types of cancers (155). PLAC1, an X-linked trophoblast antigen, is ectopically expressed in a large set of cancer cell lines and several cancers, including melanoma, breast, cervical, prostate, ovary, liver, and colorectal cancers (156, 158, 195, 291, 292). More recently, RTL1 has been introduced as a placenta-specific protein with ectopic expression in cancer, particularly evident in hepatocellular carcinoma (HCC) (293), melanoma (294), and breast cancer (295). In melanoma, RTL1 promotes cell proliferation through the Wnt/β-Catenin signaling pathway (294). Preliminary observations indicate high RTL1 expression in breast cancer cell lines and tissues, correlating significantly with histological grade and vascular invasion (295). Further research is required to uncover the cancer-modulatory effects of RTL1 *in vivo*.

Growth/differentiation factor-15 (GDF-15) is a placental protein, which is barely detected in most somatic tissues in humans. It is a member of the TGF-β superfamily and is highly expressed across various cancer types (296, 297). GDF-15 is induced under stress conditions to maintain cell and tissue homeostasis. Reportedly, high blood levels of GDF-15 are associated with inflammatory conditions, which is the hallmark of myocardial ischemia, and especially cancer (297).

Numerous studies have concentrated on tumor-associated autoantibodies as potential cancer biomarkers (298–301). Considering common antigens expressed in both placenta and various cancers (CPAs), autoantibodies against placenta proteins could be viewed as potential serologic cancer biomarkers. We recently reported that patients with breast, gastric, and colorectal cancers produce detectable levels of placenta-reactive autoantibodies. This was extremely the case for breast cancer, in which sera from early stage breast cancer patients contained antibodies reactive with the first-trimester placenta and reacted with a scattered subpopulation of cells, probably trophoblast stem cells (153).This approach, could be utilized for non-invasive cancer screening at early stages, paving the way for new strategies in targeted immunotherapy for cancer patients. Profiling of autoantigens by protein array technology for discovery and point-of-care type investigations are a promising approach for prognostics and diagnostics (302).

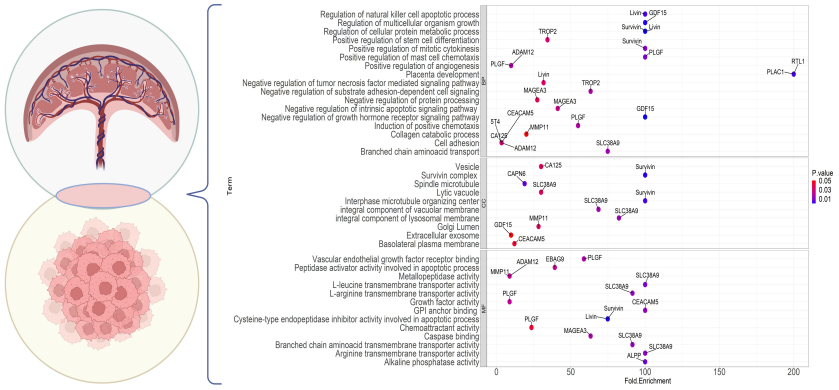


FIGURE 4
Gene Ontology enrichment analysis of the genes commonly expressed in cancer and placenta. The dot plot shows the top enrichment terms for the markers with a shared expression between the placenta and tumor cells. The color of each dot indicates the enrichment P value and the x-axis shows the enrichment fold. Each part points to one of the GO sections; BP, Biological Processes; CC, Cellular Component; MF, Molecular Function.

Concluding remarks

Despite the undeniable importance of the placenta in eutherian pregnancy, this vital organ has not been studied as much as it deserves. New findings show that the placenta, in addition to being the major anatomical site to provide nutrients to the developing fetus, has an undeniable role in regulating the microenvironment of the endometrium and preparing it for a successful pregnancy. Despite the conventional belief of the main role of maternal factors, placental cells, and its secretory factors exert a central role in regulating the maternal immune system and inducing immunological tolerance of pregnancy. Placental proteome and circulating levels of placental proteins are reliable sources for investigating and identifying gestational diseases. Interestingly, there is a very fascinating connection between placentation and carcinogenesis. Accordingly, the type of placentation in eutherian mammals and adaptation of endometrium to placental invasion are determining factors in the prevalence of the type of invasive cancers. In this context, a deep knowledge about the placenta and pregnancy decidua could better shape our understanding of the pathogenesis of cancer development and provide new clues for cancer treatment. In spite of being the star of the evolution of mammals, yet placenta is generally seen as a waste organ! It is not the placenta's fault that it has languished in such obscurity. No one has bothered to speak up on its behalf.

Author contributions

SK-S: Writing – original draft, Writing – review & editing. NV: Software, Writing – review & editing. SS: Conceptualization, Data curation, Writing – review & editing. KZ: Writing – original draft, Formal analysis, Methodology. AS: Data curation, Formal analysis, Investigation, Writing – review & editing. MJ-T: Project administration, Supervision, Writing – review & editing. A-HZ: Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This review paper was supported by the National Institute for Medical Research Development (NIMAD) (Grant No: 973140), Tehran, Iran, and in part by Iran National Science Foundation (INSF) (Grant No: 96013023), Tehran, Iran.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Roberts RM, Green JA, Schulz LC. The evolution of the placenta. *Reproduction*. (2016) 152:R179–89. doi: 10.1530/rep-16-0325

2. Weisbecker V, Goswami A. Brain size, life history, and metabolism at the marsupial/placental dichotomy. *Proc Natl Acad Sci*. (2010) 107:16216–21. doi: 10.1073/pnas.0906486107

3. Guernsey MW, Chuong EB, Cornelis G, Renfree MB, Baker JC. Molecular conservation of marsupial and eutherian placentation and lactation. *eLife*. (2017) 6: e27450. doi: 10.7554/eLife.27450

4. Whittington CM, Van Dyke JU, Liang SQT, Edwards SV, Shine R, Thompson MB, et al. Understanding the evolution of viviparity using intraspecific variation in reproductive mode and transitional forms of pregnancy. *Biol Rev*. (2022) 97:1179–92. doi: 10.1111/brv.12836

5. D'Souza AW, Wagner GP. Malignant cancer and invasive placentation: A case for positive pleiotropy between endometrial and Malignancy phenotypes. *Evolution medicine Public Health*. (2014) 2014:136–45. doi: 10.1093/emph/eou022

6. Wagner GP, Kshitiz, Dighe A, Levchenko A. The coevolution of placentation and cancer. *Annu Rev Anim Biosci*. (2022) 10:259–79. doi: 10.1146/annurev-animal-020420-031544

7. Griffith OW, Chavan AR, Protopapas S, Maziarz J, Romero R, Wagner GP. Embryo implantation evolved from an ancestral inflammatory attachment reaction. *Proc Natl Acad Sci U.S.A*. (2017) 114:E6566–e75. doi: 10.1073/pnas.1701129114

8. Martin JF, Wagner GP. The origin of platelets enabled the evolution of eutherian placentation. *Biol Lett*. (2019) 15. doi: 10.1098/rsbl.2019.0374

9. Duan Y, Wang H, Mitchell-silbaugh K, Cai S, Fan F, Li Y, et al. Heat shock protein 60 regulates yolk sac erythropoiesis in mice. *Cell Death Dis*. (2019) 10:766. doi: 10.1038/s41419-019-2014-2

10. Fonseca BM, Correia-da-Silva G, Teixeira NA. The rat as an animal model for fetoplacental development: A reappraisal of the post-implantation period. *Reprod Biol*. (2012) 12:97–118. doi: 10.1016/s1642-431x(12)60080-1

11. Watson ED, Cross JC. Development of structures and transport functions in the mouse placenta. *Physiol (Bethesda)*. (2005) 20:180–93. doi: 10.1152/physiol.00001.2005

12. Rossant J, Cross JC. Placental development: lessons from mouse mutants. *Nat Rev Genet*. (2001) 2:538–48. doi: 10.1038/35080570

13. Knöfler M, Haider S, Saleh L, Pollheimer J, Gamage T, James J. Human placenta and trophoblast development: key molecular mechanisms and model systems. *Cell Mol Life Sci*. (2019) 76:3479–96. doi: 10.1007/s00018-019-03104-6

14. Weberling A, Zernicka-Goetz M. Trophectoderm mechanics direct epiblast shape upon embryo implantation. *Cell Rep*. (2021) 34:108655. doi: 10.1016/j.celrep.2020.108655

15. Chang CW, Wakeland AK, Parast MM. Trophoblast lineage specification, differentiation and their regulation by oxygen tension. *J Endocrinol*. (2018) 236:R43–r56. doi: 10.1530/joe-17-0402

16. James JL, Lissaman A, Nursalim YNS, Chamley LW. Modelling human placental villous development: designing cultures that reflect anatomy. *Cell Mol Life Sci*. (2022) 79:384. doi: 10.1007/s00018-022-04407-x

17. Velicky P, Knöfler M, Pollheimer J. Function and control of human invasive trophoblast subtypes: intrinsic vs. Maternal control. *Cell Adh Migr*. (2016) 10:154–62. doi: 10.1080/19336918.2015.1089376

18. Boss AL, Chamley LW, James JL. Placental formation in early pregnancy: how is the centre of the placenta made? *Hum Reprod Update*. (2018) 24:750–60. doi: 10.1093/humupd/dmy030

19. Meakin C, Barrett ES, Aleksunes LM. Extravillous trophoblast migration and invasion: impact of environmental chemicals and pharmaceuticals. *Reprod Toxicol*. (2022) 107:60–8. doi: 10.1016/j.reprotox.2021.11.008

20. Ciobanu AM, Dumitru AE, Gica N, Botezatu R, Peltecu G, Panaitescu AM. Benefits and risks of igg transplacental transfer. *Diagnostics (Basel)*. (2020) 10. doi: 10.3390/diagnostics10080583

21. Costa MA. The endocrine function of human placenta: an overview. *Reprod BioMed Online*. (2016) 32:14–43. doi: 10.1016/j.rbmo.2015.10.005

22. Maltepe E, Fisher SJ. Placenta: the forgotten organ. *Annu Rev Cell And Dev Biol*. (2015) 31:523–52. doi: 10.1146/annurev-cellbio-100814-125620

23. Kammala AK, Lintao RCV, Vora N, Mosebarger A, Khanipov K, Golovko G, et al. Expression of cyp450 enzymes in human fetal membranes and its implications in xenobiotic metabolism during pregnancy. *Life Sci*. (2022) 307:120867. doi: 10.1016/j.lfs.2022.120867

24. Joshi AA, Vaidya SS, St-Pierre MV, Mikheev AM, Desino KE, Nyandege AN, et al. Placental ABC transporters: biological impact and pharmaceutical significance. *Pharm Res*. (2016) 33:2847–78. doi: 10.1007/s11095-016-2028-8

25. Megli CJ, Coyne CB. Infections at the maternal–fetal interface: an overview of pathogenesis and defence. *Nat Rev Microbiol*. (2022) 20:67–82. doi: 10.1038/s41579-021-00610-y

26. Napso T, Yong HEJ, Lopez-Tello J, Sferruzzi-Perri AN. The role of placental hormones in mediating maternal adaptations to support pregnancy and lactation. *Front Physiol*. (2018) 9:1091. doi: 10.3389/fphys.2018.01091

27. Chwalisz K, Garfield RE. Regulation of the uterus and cervix during pregnancy and labor. Role of progesterone and nitric oxide. *Ann N Y Acad Sci*. (1997) 828:238–53. doi: 10.1111/j.1749-6632.1997.tb48545.x

28. Spencer TE, Johnson GA, Burghardt RC, Bazer FW. Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol Reprod*. (2004) 71:2–10. doi: 10.1095/biolreprod.103.024133

29. Arck P, Hansen PJ, Mulac Jericevic B, Piccinni MP, Szekeres-Bartho J. Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress. *Am J Reprod Immunol*. (2007) 58:268–79. doi: 10.1111/j.1600-0897.2007.00512.x

30. Pepe GJ, Albrecht ED. Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr Rev*. (1995) 16:608–48. doi: 10.1210/edrv-16-5-608

31. Soares MJ, Varberg KM, Iqbal K. Hemochorial placentation: development, function, and adaptations. *Biol Reprod*. (2018) 99:196–211. doi: 10.1093/biolre/ioy049

32. Evans J, Salamonsen LA, Winship A, Menkhorst E, Nie G, Gargett CE, et al. Fertile ground: human endometrial programming and lessons in health and disease. *Nat Rev Endocrinol*. (2016) 12:654–67. doi: 10.1038/nrendo.2016.116

33. Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med*. (2003) 198:1201–12. doi: 10.1084/jem.20030305

34. Brosens JJ, Parker MG, McIndoe A, Pijnenborg R, Brosens IA. A role for menstruation in preconditioning the uterus for successful pregnancy. *Am J Obstet Gynecol*. (2009) 200:615. doi: 10.1016/j.ajog.2008.11.037

35. Muter J, Kong C-S, Brosens JJ. The role of decidual subpopulations in implantation, menstruation and miscarriage. *Front Reprod Health*. (2021) 3:804921. doi: 10.3389/frph.2021.804921

36. Santos ED, Moindjie H, Sérazin V, Arnould L, Rodriguez Y, Fathallah K, et al. Preimplantation factor modulates trophoblastic invasion throughout the decidualization of human endometrial stromal cells. *Reprod Biol Endocrinol*. (2021) 19:96. doi: 10.1186/s12958-021-00774-5

37. Menkhorst EM, Van Sinderen ML, Rainczuk K, Cuman C, Winship A, Dimitriadis E. Invasive trophoblast promote stromal fibroblast decidualization via profilin 1 and alox5. *Sci Rep*. (2017) 7:8690. doi: 10.1038/s41598-017-05947-0

38. Ma Q, Beal JR, Bhurke A, Kannan A, Yu J, Taylor RN, et al. Extracellular vesicles secreted by human uterine stromal cells regulate decidualization, angiogenesis, and trophoblast differentiation. *Proc Natl Acad Sci*. (2022) 119:e2200252119. doi: 10.1073/pnas.2200252119

39. Liu M, Chen X, Chang QX, Hua R, Wei YX, Huang LP, et al. Decidual small extracellular vesicles induce trophoblast invasion by upregulating N-cadherin. *Reprod (Cambridge England)*. (2020) 159:171–80. doi: 10.1530/rep-18-0616

40. You Y, Stelzl P, Joseph DN, Aldo PB, Maxwell AJ, Dekel N, et al. Tnf-A Regulated endometrial stroma secretome promotes trophoblast invasion. *Front Immunol*. (2021) 12.

41. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. *Science*. (1994) 266:1508–18. doi: 10.1126/science.7985020

42. Male V. Medawar and the immunological paradox of pregnancy: in context. *Oxford Open Immunol*. (2021) 2:iqaa006. doi: 10.1093/oxfimm/iqaa006

43. Geldenhuys J, Rossouw TM, Lombaard HA, Ehlers MM, Kock MM. Disruption in the regulation of immune responses in the placental subtype of preeclampsia. *Front Immunol*. (2018) 9. doi: 10.3389/fimmu.2018.01659

44. Staun-Ram E, Goldman S, Gabarin D, Shalev E. Expression and importance of matrix metalloproteinase 2 and 9 (Mmp-2 and -9) in human trophoblast invasion. *Reprod Biol Endocrinol*. (2004) 2:59. doi: 10.1186/1477-7827-2-59

45. Bass KE, Morrish D, Roth I, Bhardwaj D, Taylor R, Zhou Y, et al. Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. *Dev Biol*. (1994) 164:550–61. doi: 10.1006/dbio.1994.1223

46. Graham CH, Connelly I, MacDougall JR, Kerbel RS, Stetler-Stevenson WG, Lala PK. Resistance of Malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta. *Exp Cell Res*. (1994) 214:93–9. doi: 10.1006/excr.1994.1237

47. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev*. (2014) 35:851–905. doi: 10.1210/er.2014-1045

48. Moffett A, Shreeve N. Local immune recognition of trophoblast in early human pregnancy: controversies and questions. *Nat Rev Immunol*. (2022). doi: 10.1038/s41577-022-00777-2

49. Ding J, Maxwell A, Adzibolosu N, Hu A, You Y, Liao A, et al. Mechanisms of immune regulation by the placenta: role of type I interferon and interferon-stimulated genes signaling during pregnancy. *Immunol Rev*. (2022) 308:9–24. doi: 10.1111/imr.13077

50. Moffett A, Hiby SE, Sharkey AM. The role of the maternal immune system in the regulation of human birthweight. *Philos Trans R Soc Lond B Biol Sci*. (2015) 370:20140071. doi: 10.1098/rstb.2014.0071

51. Li DK, Wi S. Changing paternity and the risk of preeclampsia/eclampsia in the subsequent pregnancy. *Am J Epidemiol*. (2000) 151:57–62. doi: 10.1093/oxfordjournals.aje.a010122

52. Xiong S, Sharkey AM, Kennedy PR, Gardner L, Farrell LE, Chazara O, et al. Maternal uterine nk cell-activating receptor Kir2ds1 enhances placentation. *J Clin Invest*. (2013) 123:4264–72. doi: 10.1172/jci68991

53. Tilburgs T, Crespo ÂC, van der Zwan A, Rybalov B, Raj T, Stranger B, et al. Human hla-G+ Extravillous trophoblasts: immune-activating cells that interact with decidual leukocytes. *Proc Natl Acad Sci U.S.A*. (2015) 112:7219–24. doi: 10.1073/pnas.1507977112

54. Chakraborty D, Rumi MA, Konno T, Soares MJ. Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions. *Proc Natl Acad Sci U.S.A*. (2011) 108:16295–300. doi: 10.1073/pnas.1109478108

55. Robson A, Harris LK, Innes BA, Lash GE, Aljunaidy MM, Aplin JD, et al. Uterine natural killer cells initiate spiral artery remodeling in human pregnancy. *FASEB J*. (2012) 26:4876–85. doi: 10.1096/fj.12-210310

56. Wang XQ, Li DJ. The mechanisms by which trophoblast-derived molecules induce maternal-fetal immune tolerance. *Cell Mol Immunol*. (2020) 17:1204–7. doi: 10.1038/s41423-020-0460-5

57. Rajagopalan S. Hla-G-mediated NK cell senescence promotes vascular remodeling: implications for reproduction. *Cell Mol Immunol*. (2014) 11:460–6. doi: 10.1038/cmi.2014.53

58. Henson MC, Castracane VD. Leptin in pregnancy: an update. *Biol Reprod*. (2006) 74:218–29. doi: 10.1095/biolreprod.105.045120

59. Wendremaire M, Lopez TE, Barrichon M, Zhang H, Hadi T, Ye XY, et al. Leptin-induced HLA-G inhibits myometrial contraction and differentiation. *Cells*. (2022) 11. doi: 10.3390/cells11060954

60. Maymó JL, Pérez Pérez A, Sánchez-Margalet V, Dueñas JL, Calvo JC, Varone CL. Up-regulation of placental leptin by human chorionic gonadotropin. *Endocrinology*. (2009) 150:304–13. doi: 10.1210/en.2008-0522

61. Gambino YP, Maymó JL, Pérez-Pérez A, Dueñas JL, Sánchez-Margalet V, Calvo JC, et al. 17beta-estradiol enhances leptin expression in human placental cells through genomic and nongenomic actions. *Biol Reprod*. (2010) 83:42–51. doi: 10.1095/biolreprod.110.083535

62. Pérez-Pérez A, Maymó J, Gambino Y, Guadix P, Dueñas JL, Varone C, et al. Insulin enhances leptin expression in human trophoblastic cells. *Biol Reprod*. (2013) 89:20. doi: 10.1095/biolreprod.113.109348

63. Pollock KE, Talton OO, Schulz LC. Morphology and gene expression in mouse placentas lacking leptin receptors. *Biochem Biophys Res Commun*. (2020) 528:336–42. doi: 10.1016/j.bbrc.2020.03.104

64. Ding J, Zhang Y, Cai X, Diao L, Yang C, Yang J. Crosstalk between trophoblast and macrophage at the maternal-fetal interface: current status and future perspectives. *Front Immunol*. (2021) 12:758281. doi: 10.3389/fimmu.2021.758281

65. Vondra S, Höbler AL, Lackner AI, Raffetseder J, Mihalic ZN, Vogel A, et al. The human placenta shapes the phenotype of decidual macrophages. *Cell Rep*. (2023) 42:111977. doi: 10.1016/j.celrep.2022.111977

66. Zarnani AH, Dokouhaki P, Jeddi-Tehrani M. Indoleamine 2,3-dioxygenase and immunological tolerance during pregnancy. *Iranian J Immunol*. (2004) 1:143–53.

67. Shayda H, Mahmood JT, Ebrahim T, Jamileh G, Golnaz Ensieh KS, Parivash D, et al. Indoleamine 2,3-dioxygenase (Ido) is expressed at feto-placental unit throughout mouse gestation: an immunohistochemical study. *J Reprod Infertil*. (2009) 10:177–83.

68. Nikoo S, Moazzeni S, Bozorgmehr M, Zarnani A. Effect of pregnant mouse serum on induction of indolamine 2, 3- dioxygenase in dendritic cells. *J Reprod Infertility*. (2006) 7:187–98.

69. Meggyes M, Miko E, Szigeti B, Farkas N, Szereday L. The importance of the PD-1/PD-L1 pathway at the maternal-fetal interface. *BMC Pregnancy Childbirth*. (2019) 19:74. doi: 10.1186/s12884-019-2218-6

70. Papuchova H, Kshirsagar S, Xu L, Bougleux Gomes HA, Li Q, Iyer V, et al. Three types of HLA-G+ Extravillous trophoblasts that have distinct immune regulatory properties. *Proc Natl Acad Sci U.S.A*. (2020) 117:15772–7. doi: 10.1073/pnas.2000484117

71. Yang Q, Li M, Zhao M, Lu F, Yu X, Li L, et al. Progesterone modulates CD4(+) CD25(+) FoxP3(+) regulatory T cells and TGF-B1 in the maternal-fetal interface of the late pregnant mouse. *Am J Reprod Immunol*. (2022) 88:e13541. doi: 10.1111/aji.13541

72. Liang S, Ristich V, Arase H, Dausset J, Carosella ED, Horuzsko A. Modulation of dendritic cell differentiation by HLA-G and ILT4 requires the IL-6–STAT3 signaling pathway. *Proc Natl Acad Sci U.S.A*. (2008) 105:8357–62. doi: 10.1073/pnas.0803341105

73. Motedayyen H, Zarnani AH, Tajik N, Ghotloo S, Rezaei A. Immunomodulatory effects of human amniotic epithelial cells on naive CD4(+) T cells from women with unexplained recurrent spontaneous abortion. *Placenta*. (2018) 71:31–40. doi: 10.1016/j.placenta.2018.06.008

74. Motedayyen H, Rezaei A, Zarnani AH, Tajik N. Human amniotic epithelial cells inhibit activation and pro-inflammatory cytokines production of naive CD4+ T cells from women with unexplained recurrent spontaneous abortion. *Reprod Biol*. (2018) 18:182–8. doi: 10.1016/j.repbio.2018.04.002

75. Lucas ES, Vrljicak P, Muter J, Diniz-da-Costa MM, Brighton PJ, Kong C-S, et al. Recurrent pregnancy loss is associated with a pro-senescent decidual response during the peri-implantation window. *Commun Biol*. (2020) 3:37. doi: 10.1038/s42003-020-0763-1

76. Xu L, Li Y, Sang Y, Li D-J, Du M. Crosstalk between trophoblasts and decidual immune cells: the cornerstone of maternal-fetal immunotolerance. *Front Immunol*. (2021) 12:642392. doi: 10.3389/fimmu.2021.642392

77. Idali F, Rezaii-Nia S, Golshahi H, Fatemi R, Naderi MM, Goli LB, et al. Adoptive cell therapy with induced regulatory T cells normalises the abortion rate in abortion-prone mice. *Reprod Fertil Dev*. (2021) 33:220–8. doi: 10.1071/rd20063

78. Wang W-J, Liu F-J, Xin L, Hao C-F, Bao H-C, Qu Q-L, et al. Adoptive transfer of pregnancy-induced CD4+CD25+ Regulatory T cells reverses the increase in abortion rate caused by interleukin 17 in the CBA/J×BALB/c mouse model. *Hum Reprod*. (2014) 29:946–52. doi: 10.1093/humrep/deu014

79. Bonney EA, Brown SA. To drive or be driven: the path of a mouse model of recurrent pregnancy loss. *Reproduction*. (2014) 147:R153–67. doi: 10.1530/rep-13-0583

80. Salek Farrokhi A, Zarnani AH, Rezaei Kahmini F, Moazzeni SM. Mesenchymal stem cells induce expansion of regulatory T cells in abortion-prone mice. *Reproduction*. (2021) 161:477–87. doi: 10.1530/rep-20-0320

81. Salek Farrokhi A, Zarnani AH, Moazzeni SM. Mesenchymal stem cells therapy protects fetuses from resorption and induces Th2 type cytokines profile in abortion prone mouse model. *Transpl Immunol*. (2018) 47:26–31. doi: 10.1016/j.trim.2018.01.002

82. Sadighi-Moghaddam B, Salek Farrokhi A, Namdar Ahmadabad H, Barati M, Moazzeni SM. Mesenchymal stem cell therapy prevents abortion in CBA/J × DBA/2 mating. *Reprod Sci*. (2018) 25:1261–9. doi: 10.1177/1933719117737848

83. Gargett CE, Masuda H. Adult stem cells in the endometrium. *Mol Hum Reprod*. (2010) 16:818–34. doi: 10.1093/molehr/gaq061

84. Claridge B, Kastaniegaard K, Stensballe A, Greening DW. Post-translational and transcriptional dynamics – regulating extracellular vesicle biology. *Expert Rev Proteomics*. (2019) 16:17–31. doi: 10.1080/14789450.2019.1551135

85. Mitchell MD, Peiris HN, Kobayashi M, Koh YQ, Duncombe G, Illanes SE, et al. Placental exosomes in normal and complicated pregnancy. *Am J Obstet Gynecol*. (2015) 213:S173–81. doi: 10.1016/j.ajog.2015.07.001

86. Czernek L, Düchler M. Exosomes as messengers between mother and fetus in pregnancy. *Int J Mol Sci*. (2020) 21. doi: 10.3390/ijms21124264

87. Bai K, Lee C-L, Liu X, Li J, Cao D, Zhang L, et al. Human placental exosomes induce maternal systemic immune tolerance by reprogramming circulating monocytes. *J Nanobiotechnology*. (2022) 20:86. doi: 10.1186/s12951-022-01283-2

88. Hedlund M, Stenqvist AC, Nagaeva O, Kjellberg L, Wulff M, Baranov V, et al. Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. *J Immunol*. (2009) 183:340–51. doi: 10.4049/jimmunol.0803477

89. Ghanavatinejad A, Bozorgmehr M, Shokri M-R, Aleahmad M, Tavakoli M, Shokri F, et al. Menscs exert a supportive role in establishing a pregnancy-friendly microenvironment by inhibiting TH17 polarization. *J Reprod Immunol*. (2021) 144:103252. doi: 10.1016/j.jri.2020.103252

90. Shokri M-R, Bozorgmehr M, Ghanavatinejad A, Falak R, Aleahmad M, Kazemnejad S, et al. Human menstrual blood-derived stromal/stem cells modulate functional features of natural killer cells. *Sci Rep*. (2019) 9:10007. doi: 10.1038/s41598-019-46316-3

91. Aleahmad M, Ghanavatinejad A, Bozorgmehr M, Shokri MR, Nikoo S, Tavakoli M, et al. Menstrual blood-derived stromal stem cells augment CD4+ T cells proliferation. *Avicenna J Med Biotechnol*. (2018) 10:183–91.

92. Bozorgmehr M, Moazzeni SM, Salehnia M, Sheikhian A, Nikoo S, Zarnani AH. Menstrual blood-derived stromal stem cells inhibit optimal generation and maturation of human monocyte-derived dendritic cells. *Immunol Lett*. (2014) 162:239–46. doi: 10.1016/j.imlet.2014.10.005

93. Bozorgmehr M, Gurung S, Darzi S, Nikoo S, Kazemnejad S, Zarnani AH, et al. Endometrial and menstrual blood mesenchymal stem/stromal cells: biological properties and clinical application. *Front Cell Dev Biol*. (2020) 8:497. doi: 10.3389/fcell.2020.00497

94. Hosseini S, Shokri F, Tokhmechy R, Savadi-Shiraz E, Jeddi-Tehrani M, Rahbari M, et al. Menstrual blood contains immune cells with inflammatory and anti-inflammatory properties. *J Obstet Gynaecol Res*. (2015) 41:1803–12. doi: 10.1111/jog.12801

95. Hosseini S, Shokri F, Pour SA, Khoshnoodi J, Jeddi-Tehrani M, Zarnani AH. Diminished frequency of menstrual and peripheral blood NKT-like cells in patients with unexplained recurrent spontaneous abortion and infertile women. *Reprod Sci*. (2019) 26:97–108. doi: 10.1177/1933719118766261

96. Ross CA. The trophoblast model of cancer. *Nutr Cancer*. (2015) 67:61–7. doi: 10.1080/01635581.2014.956257

97. Barbour LA, Shao J, Qiao L, Pulawa LK, Jensen DR, Bartke A, et al. Human placental growth hormone causes severe insulin resistance in transgenic mice. *Am J Obstet Gynecol*. (2002) 186:512–7. doi: 10.1067/mob.2002.121256

98. Masi T, Patel BM. Altered glucose metabolism and insulin resistance in cancer-induced cachexia: A sweet poison. *Pharmacol Rep*. (2021) 73:17–30. doi: 10.1007/s43440-020-00179-y

99. Zhu Y, Liu Q, Liao M, Diao L, Wu T, Liao W, et al. Overexpression of lncRNA EPB41L4A-AS1 induces metabolic reprogramming in trophoblast cells and placenta tissue of miscarriage. *Mol Ther Nucleic Acids*. (2019) 18:518–32. doi: 10.1016/j.omtn.2019.09.017

100. Liberti MV, Locasale JW. The warburg effect: how does it benefit cancer cells? *Trends Biochem Sci*. (2016) 41:211–8. doi: 10.1016/j.tibs.2015.12.001

101. Rajagopalan S, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I, et al. Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PloS Biol*. (2006) 4:e9. doi: 10.1371/journal.pbio.0040009

102. Lin A, Yan WH. Human leukocyte antigen-G (Hla-G) expression in cancers: roles in immune evasion, metastasis and target for therapy. *Mol Med*. (2015) 21:782–91. doi: 10.2119/molmed.2015.00083

103. Nejabati HR, Latifi Z, Ghasemnejad T, Fattahi A, Nouri M. Placental growth factor (Plgf) as an angiogenic/inflammatory switcher: lesson from early pregnancy losses. *Gynecol Endocrinol*. (2017) 33:668–74. doi: 10.1080/09513590.2017.1318375

104. Kim KJ, Cho CS, Kim WU. Role of placenta growth factor in cancer and inflammation. *Exp Mol Med*. (2012) 44:10–9. doi: 10.3858/emm.2012.44.1.023

105. Staun-Ram E, Shalev E. Human trophoblast function during the implantation process. *Reprod Biol Endocrinol*. (2005) 3:56. doi: 10.1186/1477-7827-3-56

106. Jankowska A, Szczerba A. Chapter 4.2 - human chorionic gonadotropin in cancer—Where are we? In: Cole LA, Butler SA, editors. *100 years of human chorionic gonadotropin*. Elsevier (2020). p. 255–60.

107. Kudo Y, Koh I, Sugimoto J. Localization of indoleamine 2,3-dioxygenase-1 and indoleamine 2,3-dioxygenase-2 at the human maternal-fetal interface. *Int J Tryptophan Res*. (2020) 13:1178646920984163. doi: 10.1177/1178646920984163

108. Meireson A, Devos M, Brochez L. Ido expression in cancer: different compartment, different functionality? *Front Immunol*. (2020) 11:531491. doi: 10.3389/fimmu.2020.531491

109. Jonescheit H, Oberg HH, Gonnermann D, Hermes M, Sulaj V, Peters C, et al. Influence of indoleamine-2,3-dioxygenase and its metabolite kynurenine on Γδ T cell cytotoxicity against ductal pancreatic adenocarcinoma cells. *Cells*. (2020) 9. doi: 10.3390/cells9051140

110. Balogh A, Toth E, Romero R, Parej K, Csala D, Szenasi NL, et al. Placental galectins are key players in regulating the maternal adaptive immune response. *Front Immunol*. (2019) 10:1240. doi: 10.3389/fimmu.2019.01240

111. Chou FC, Chen HY, Kuo CC, Sytwu HK. Role of galectins in tumors and in clinical immunotherapy. *Int J Mol Sci*. (2018) 19. doi: 10.3390/ijms19020430

112. Desoye G, Hartmann M, Jones CJ, Wolf HJ, Kohnen G, Kosanke G, et al. Location of insulin receptors in the placenta and its progenitor tissues. *Microsc Res Tech*. (1997) 38:63–75.

113. Malaguarnera R, Belfiore A. The insulin receptor: A new target for cancer therapy. *Front Endocrinol (Lausanne)*. (2011) 2:93. doi: 10.3389/fendo.2011.00093

114. Shyu MK, Lin MC, Liu CH, Fu YR, Shih JC, Lee CN, et al. Muc1 expression is increased during human placental development and suppresses trophoblast-like cell invasion *in vitro*. *Biol Reprod*. (2008) 79:233–9. doi: 10.1095/biolreprod.108.067629

115. Nath S, Mukherjee P. Muc1: A multifaceted oncoprotein with a key role in cancer progression. *Trends Mol Med*. (2014) 20:332–42. doi: 10.1016/j.molmed.2014.02.007

116. Rattila S, Dunk CEE, Im M, Grichenko O, Zhou Y, Yanez-Mo M, et al. Interaction of pregnancy-specific glycoprotein 1 with integrin A5β1 is a modulator of extravillous trophoblast functions. *Cells*. (2019) 8. doi: 10.3390/cells8111369

117. Hou J, Yan D, Liu Y, Huang P, Cui H. The roles of integrin A5β1 in human cancer. *Onco Targets Ther*. (2020) 13:13329–44. doi: 10.2147/ott.S273803

118. Lin Z, Shi JL, Chen M, Zheng ZM, Li MQ, Shao J. Ccl2: an important cytokine in normal and pathological pregnancies: A review. *Front Immunol.* (2022) 13:1053457. doi: 10.3389/fimmu.2022.1053457

119. O'Connor T, Heikenwalder M. Ccl2 in the tumor microenvironment. *Adv Exp Med Biol.* (2021) 1302:1–14. doi: 10.1007/978-3-030-62658-7_1

120. Chuva de Sousa Lopes SM, Alexdottir MS, Valdimarsdottir G. The TGFβ Family in human placental development at the fetal-maternal interface. *Biomolecules.* (2020) 10. doi: 10.3390/biom10030453

121. Drabsch Y, ten Dijke P. TGF-β Signaling in breast cancer cell invasion and bone metastasis. *J Mammary Gland Biol Neoplasia.* (2011) 16:97–108. doi: 10.1007/s10911-011-9217-1

122. Nordor AV, Nehar-Belaid D, Richon S, Klatzmann D, Bellet D, Dangles-Marie V, et al. The early pregnancy placenta foreshadows DNA methylation alterations of solid tumors. *Epigenetics.* (2017) 12:793–803. doi: 10.1080/15592294.2017.1342912

123. Keighley LM, Lynch-Sutherland CF, Almomani SN, Eccles MR, Macaulay EC. Unveiling the hidden players: the crucial role of transposable elements in the placenta and their potential contribution to pre-eclampsia. *Placenta.* (2023) 141:57–64. doi: 10.1016/j.placenta.2023.05.017

124. Jang HS, Shah NM, Du AY, Dailey ZZ, Pehrsson EC, Godoy PM, et al. Transposable elements drive widespread expression of oncogenes in human cancers. *Nat Genet.* (2019) 51:611–7. doi: 10.1038/s41588-019-0373-3

125. Burki TK. Placenta can provide new insights on the genetics of cancer. *Lancet Oncol.* (2021) 22:591. doi: 10.1016/s1470-2045(21)00208-4

126. Alfieri F, Caravagna G, Schaefer MH. Cancer genomes tolerate deleterious coding mutations through somatic copy number amplifications of wild-type regions. *Nat Commun.* (2023) 14:3594. doi: 10.1038/s41467-023-39313-8

127. Holtan SG, Creedon DJ, Haluska P, Markovic SN. Cancer and pregnancy: parallels in growth, invasion, and immune modulation and implications for cancer therapeutic agents. *Mayo Clin Proc.* (2009) 84:985–1000. doi: 10.1016/s0025-6196(11)60669-1

128. DaSilva-Arnold S, James JL, Al-Khan A, Zamudio S, Illsley NP. Differentiation of first trimester cytotrophoblast to extravillous trophoblast involves an epithelial–mesenchymal transition. *Placenta.* (2015) 36:1412–8. doi: 10.1016/j.placenta.2015.10.013

129. Georgakopoulos-Soares I, Chartoumpekis DV, Kyriazopoulou V, Zaravinos A. Emt factors and metabolic pathways in cancer. *Front Oncol.* (2020) 10:499. doi: 10.3389/fonc.2020.00499

130. Renaud SJ, Jeyarajah MJ. How Trophoblasts Fuse: An in-Depth Look into Placental Syncytiotrophoblast Formation. *Cell Mol Life Sci.* (2022) 79:433. doi: 10.1007/s00018-022-04475-z

131. Wang HF, Xiang W, Xue BZ, Wang YH, Yi DY, Jiang XB, et al. Cell fusion in cancer hallmarks: current research status and future indications. *Oncol Lett.* (2021) 22:530. doi: 10.3892/ol.2021.12791

132. Rai A, Cross JC. Development of the hemochorial maternal vascular spaces in the placenta through endothelial and vasculogenic mimicry. *Dev Biol.* (2014) 387:131–41. doi: 10.1016/j.ydbio.2014.01.015

133. Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer.* (2003) 3:411–21. doi: 10.1038/nrc1092

134. Zhang XH, Liang X, Liang XH, Wang TS, Qi QR, Deng WB, et al. The mesenchymal-epithelial transition during *in vitro* decidualization. *Reprod Sci.* (2013) 20:354–60. doi: 10.1177/1933719112472738

135. Tang PC, Chung JY, Xue VW, Xiao J, Meng XM, Huang XR, et al. Smad3 promotes cancer-associated fibroblasts generation via macrophage-myofibroblast transition. *Adv Sci (Weinh).* (2022) 9:e2101235. doi: 10.1002/advs.202101235

136. Genbacev O, Joslin R, Damsky CH, Polliotti BM, Fisher SJ. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion *in vitro* and models the placental defects that occur in preeclampsia. *J Clin Invest.* (1996) 97:540–50. doi: 10.1172/JCI118447

137. Carmeliet P, Dor Y, Herbert J-M, Fukumura D, Brusselmans K, Dewerchin M, et al. Role of hif-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature.* (1998) 394:485–90. doi: 10.1038/28867

138. Tersigni C, Meli F, Neri C, Iacoangeli A, Franco R, Lanzone A, et al. Role of human leukocyte antigens at the feto-maternal interface in normal and pathological pregnancy: an update. *Int J Mol Sci.* (2020) 21. doi: 10.3390/ijms21134756

139. DhatChinamoorthy K, Colbert JD, Rock KL. Cancer immune evasion through loss of mhc class I antigen presentation. *Front Immunol.* (2021) 12:636568. doi: 10.3389/fimmu.2021.636568

140. Veras E, Kurman RJ, Wang TL, Shih IM. Pd-L1 expression in human placentas and gestational trophoblastic diseases. *Int J Gynecol Pathol.* (2017) 36:146–53. doi: 10.1097/pgp.0000000000000305

141. Han Y, Liu D, Li L. Pd-1/pd-L1 pathway: current researches in cancer. *Am J Cancer Res.* (2020) 10:727–42.

142. Favaro R, Abrahamsohn PA, Zorn MT. 11 - decidualization and endometrial extracellular matrix remodeling. In: Croy BA, Yamada AT, DeMayo FJ, Adamson SL, editors. *The guide to investigation of mouse pregnancy*. Academic Press, Boston (2014). p. 125–42.

143. Deng B, Zhao Z, Kong W, Han C, Shen X, Zhou C. Biological role of matrix stiffness in tumor growth and treatment. *J Trans Med.* (2022) 20:540. doi: 10.1186/s12967-022-03768-y

144. Pang H, Lei D, Guo Y, Yu Y, Liu T, Liu Y, et al. Three categories of similarities between the placenta and cancer that can aid cancer treatment: cells, the microenvironment, and metabolites. *Front Oncol.* (2022) 12:977618. doi: 10.3389/fonc.2022.977618

145. Lala PK, Nandi P, Hadi A, Halari C. A crossroad between placental and tumor biology: what have we learnt? *Placenta.* (2021) 116:12–30. doi: 10.1016/j.placenta.2021.03.003

146. Costanzo V, Bardelli A, Siena S, Abrignani S. Exploring the links between cancer and placenta development. *Open Biol.* (2018) 8:180081. doi: 10.1098/rsob.180081

147. Kshitiz, Afzal J, Maziarz JD, Hamidzadeh A, Liang C, Erkenbrack EM, et al. Evolution of placental invasion and cancer metastasis are causally linked. *Nat Ecol Evol.* (2019) 3:1743–53. doi: 10.1038/s41559-019-1046-4

148. Dittmar T, Hass R. Intrinsic signalling factors associated with cancer cell-cell fusion. *Cell Communication Signaling.* (2023) 21:68. doi: 10.1186/s12964-023-01085-5

149. ED J, Pollheimer J, Yong HE, Kokkinos MI, Kalionis B, Knöfler M, et al. Epithelial-mesenchymal transition during extravillous trophoblast differentiation. *Cell Adh Migr.* (2016) 10:310–21. doi: 10.1080/19336918.2016.1170258

150. Fest S, Brachwitz N, Schumacher A, Zenclussen ML, Khan F, Wafula PO, et al. Supporting the hypothesis of pregnancy as a tumor: survivin is upregulated in normal pregnant mice and participates in human trophoblast proliferation. *Am J Reprod Immunol.* (2008) 59:75–83. doi: 10.1111/j.1600-0897.2007.00557.x

151. Lala PK, Lee BP, Xu G, Chakraborty C. Human placental trophoblast as an *in vitro* model for tumor progression. *Can J Physiol Pharmacol.* (2002) 80:142–9. doi: 10.1139/y02-006

152. Krstic J, Deutsch A, Fuchs J, Gauster M, Gorsek Sparovec T, Hiden U, et al. (Dis)Similarities between the decidual and tumor microenvironment. *Biomedicines.* (2022) 10. doi: 10.3390/biomedicines10051065

153. Khorami Sarvestani S, Shojaeian S, Sarrami-Forooshani R, Yekaninejad MS, Gilany K, Ghaderi A, et al. Cancer is associated with the emergence of placenta-reactive autoantibodies. *Biomedicines.* (2023) 11. doi: 10.3390/biomedicines11020316

154. Jasti S, Farahbakhsh M, Nguyen S, Petroff BK, Petroff MG. Immune response to a model shared placenta/tumor-associated antigen reduces cancer risk in parous mice. *Biol Reprod.* (2017) 96:134–44. doi: 10.1095/biolreprod.116.144907

155. Bradley SD, Talukder AH, Lai I, Davis R, Alvarez H, Tiriac H, et al. Vestigial-like 1 is a shared targetable cancer-placenta antigen expressed by pancreatic and basal-like breast cancers. *Nat Commun.* (2020) 11:5332. doi: 10.1038/s41467-020-19141-w

156. Gholami P, Asgarian-Omran H, Yaghmaie M, Mahmudian J, Kianersi S, Salari S, et al. Investigation of expression profile of placenta-specific 1 (Plac1) in acute myeloid and lymphoid leukemias. *Avicenna J Med Biotechnol.* (2023) 15:167–72. doi: 10.18502/ajmb.v15i3.12926

157. Rahdan S, Razavi SA, Shojaeian S, Shokri F, Amiri MM, Zarnani A-H. Immunization with placenta-specific 1 (Plac1) induces potent anti-tumor responses and prolongs survival in a mouse model of melanoma. *Adv Med Sci.* (2022) 67:338–45. doi: 10.1016/j.advms.2022.08.002

158. Nejadmoghaddam M-R, Zarnani A-H, Ghahremanzadeh R, Ghods R, Mahmoudian J, Yousefi M, et al. Placenta-specific1 (Plac1) is a potential target for antibody-drug conjugate-based prostate cancer immunotherapy. *Sci Rep.* (2017) 7:13373. doi: 10.1038/s41598-017-13682-9

159. Babajani A, Manzari-Tavakoli A, Jamshidi E, Tarasi R, Niknejad H. Anti-cancer effects of human placenta-derived amniotic epithelial stem cells loaded with paclitaxel on cancer cells. *Sci Rep.* (2022) 12:18148. doi: 10.1038/s41598-022-22562-w

160. Bolouri MR, Ghods R, Zarnani K, Vafaei S, Falak R, Zarnani AH. Human amniotic epithelial cells exert anti-cancer effects through secretion of immunomodulatory small extracellular vesicles (Sev). *Cancer Cell Int.* (2022) 22:329. doi: 10.1186/s12935-022-02755-z

161. Marwitz S, Zeiser T, Schultz H, Kähler D, Abdullah M, Hauber HP, et al. The human placenta releases substances that drive lung cancer into apoptosis. *Diagn Pathol.* (2009) 4:27. doi: 10.1186/1746-1596-4-27

162. Marleau AM, McDonald G, Koropatnick J, Chen CS, Koos D. Reduction of tumorigenicity by placental extracts. *Anticancer Res.* (2012) 32:1153–61.

163. Zhao B, Wang Y, Wu B, Liu S, Wu E, Fan H, et al. Placenta-derived gp96 as a multivalent prophylactic cancer vaccine. *Sci Rep.* (2013) 3:1947. doi: 10.1038/srep01947

164. Harandi A. Immunoplacental therapy, a potential multi-epitope cancer vaccine. *Med Hypotheses.* (2006) 66:1182–7. doi: 10.1016/j.mehy.2005.12.011

165. Tabatabaei M, Mosaffa N, Ghods R, Nikoo S, Kazemnejad S, Khanmohammadi M, et al. Vaccination with human amniotic epithelial cells confer effective protection in a murine model of colon adenocarcinoma. *Int J Cancer.* (2018) 142:1453–66. doi: 10.1002/ijc.31159

166. Kampmann U, Knorr S, Fuglsang J, Ovesen P. Determinants of maternal insulin resistance during pregnancy: an updated overview. *J Diabetes Res.* (2019) 2019:5320156. doi: 10.1155/2019/5320156

167. Skeith L, Blondon M, Ní Áinle F. Understanding and preventing placenta-mediated pregnancy complications. *Hamostaseologie.* (2020) 40:356–63. doi: 10.1055/a-1184-8388

168. Kozlosky D, Barrett E, Aleksunes LM. Regulation of placental efflux transporters during pregnancy complications. *Drug Metab Dispos*. (2022) 50:1364–75. doi: 10.1124/dmd.121.000449

169. Morgan TK. Role of the placenta in preterm birth: A review. *Am J Perinatol*. (2016) 33:258–66. doi: 10.1055/s-0035-1570379

170. Kundu S, Maurer SV, Stevens HE. Future horizons for neurodevelopmental disorders: placental mechanisms. *Front Pediatr*. (2021) 9:653230. doi: 10.3389/fped.2021.653230

171. Leon RL, Mir IN, Herrera CL, Sharma K, Spong CY, Twickler DM, et al. Neuroplacentology in congenital heart disease: placental connections to neurodevelopmental outcomes. *Pediatr Res*. (2022) 91:787–94. doi: 10.1038/s41390-021-01521-7

172. Thayer ZM, Rutherford J, Kuzawa CW. The maternal nutritional buffering model: an evolutionary framework for pregnancy nutritional intervention. *Evol Med Public Health*. (2020) 2020:14–27. doi: 10.1093/emph/eoz037

173. Harding JE. The nutritional basis of the fetal origins of adult disease. *Int J Epidemiol*. (2001) 30:15–23. doi: 10.1093/ije/30.1.15

174. Thornburg KL, O'Tierney PF, Louey S. Review: the placenta is a programming agent for cardiovascular disease. *Placenta*. (2010) 31 Suppl:S54–9. doi: 10.1016/j.placenta.2010.01.002

175. Burton GJ, Fowden AL, Thornburg KL. Placental origins of chronic disease. *Physiol Rev*. (2016) 96:1509–65. doi: 10.1152/physrev.00029.2015

176. Thame M, Osmond C, Bennett F, Wilks R, Forrester T. Fetal growth is directly related to maternal anthropometry and placental volume. *Eur J Clin Nutr*. (2004) 58:894–900. doi: 10.1038/sj.ejcn.1601909

177. Oliveira LH, Xavier CC, Lana AM. [Changes in placental morphology of small for gestational age newborns]. *J Pediatr (Rio J)*. (2002) 78:397–402. doi: 10.2223/JPED.887

178. Arrese M, Reyes H. Intrahepatic cholestasis of pregnancy: A past and present riddle. *Ann Hepatol*. (2006) 5:202–5. doi: 10.1016/S1665-2681(19)32012-5

179. Gyamfi-Bannerman C, Ananth CV. Trends in spontaneous and indicated preterm delivery among singleton gestations in the United States, 2005-2012. *Obstet Gynecol*. (2014) 124:1069–74. doi: 10.1097/aog.0000000000000546

180. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. *Science*. (2014) 345:760–5. doi: 10.1126/science.1251816

181. Grillo MA, Mariani G, Ferraris JR. Prematurity and low birth weight in neonates as a risk factor for obesity, hypertension, and chronic kidney disease in pediatric and adult age. *Front Med (Lausanne)*. (2021) 8:769734. doi: 10.3389/fmed.2021.769734

182. Malhotra A, Allison BJ, Castillo-Melendez M, Jenkin G, Polglase GR, Miller SL. Neonatal morbidities of fetal growth restriction: pathophysiology and impact. *Front Endocrinol (Lausanne)*. (2019) 10:55. doi: 10.3389/fendo.2019.00055

183. Fowden AL, Ward JW, Wooding FP, Forhead AJ, Constancia M. Programming placental nutrient transport capacity. *J Physiol*. (2006) 572:5–15. doi: 10.1113/jphysiol.2005.104141

184. Panja S, Paria BC. Development of the mouse placenta. *Adv Anat Embryol Cell Biol*. (2021) 234:205–21. doi: 10.1007/978-3-030-77360-1_10

185. Aguilera N, Salas-Pérez F, Ortíz M, Álvarez D, Echiburú B, Maliqueo M. Rodent models in placental research. Implications for fetal origins of adult disease. *Anim Reprod*. (2022) 19:e20210134. doi: 10.1590/1984-3143-ar2021-0134

186. Ishikawa H, Seki R, Yokonishi S, Yamauchi T, Yokoyama K. Relationship between fetal weight, placental growth and litter size in mice from mid- to late-gestation. *Reprod Toxicol*. (2006) 21:267–70. doi: 10.1016/j.reprotox.2005.08.002

187. Michael K, Ward BS, Moore WM. Relationship of fetal to placental size: the pig model. *Eur J Obstet Gynecol Reprod Biol*. (1983) 16:53–62. doi: 10.1016/0028-2243(83)90220-4

188. Pomorski M, Zimmer M, Florjanski J, Michniewicz J, Wiatrowski A, Fuchs T, et al. Comparative analysis of placental vasculature and placental volume in normal and IUGR pregnancies with the use of three-dimensional power doppler. *Arch Gynecol Obstet*. (2012) 285:331–7. doi: 10.1007/s00404-011-1968-9

189. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. *Science*. (2015) 347:1260419. doi: 10.1126/science.1260419

190. Khorami Sarvestani S, Shojaeian S, Vanaki N, Ghresi-Fard B, Amini M, Gilany K, et al. Proteome profiling of human placenta reveals developmental stage-dependent alterations in protein signature. *Clin Proteomics*. (2021) 18:18. doi: 10.1186/s12014-021-09324-y

191. Mushahary D, Gautam P, Sundaram CS, Sirdeshmukh R. Expanded protein expression profile of human placenta using two-dimensional gel electrophoresis. *Placenta*. (2013) 34:193–6. doi: 10.1016/j.placenta.2012.11.015

192. Manna S, Scheel J, Noone A, McElwain CJ, Scaife C, Gupta S, et al. A proteomic profile of the healthy human placenta. *Clin Proteomics*. (2023) 20:1. doi: 10.1186/s12014-022-09388-4

193. Huppertz B, Meiri H, Gizurarson S, Osol G, Sammar M. Placental protein 13 (Pp13): A new biological target shifting individualized risk assessment to personalized drug design combating pre-eclampsia. *Hum Reprod Update*. (2013) 19:391–405. doi: 10.1093/humupd/dmt003

194. Jackman SM, Kong X, Fant ME. Plac1 (Placenta-specific 1) is essential for normal placental and embryonic development. *Mol Reprod Dev*. (2012) 79:564–72. doi: 10.1002/mrd.22062

195. Mahmoudian J, Ghods R, Nazari M, Jeddi-Tehrani M, Ghahremani MH, Ghaffari-Tabrizi-Wizsy N, et al. Plac1: biology and potential application in cancer immunotherapy. *Cancer Immunol Immunother*. (2019) 68:1039–58. doi: 10.1007/s00262-019-02350-8

196. Sekita Y, Wagatsuma H, Nakamura K, Ono R, Kagami M, Wakisaka N, et al. Role of retrotransposon-derived imprinted gene, Rtl1, in the feto-maternal interface of mouse placenta. *Nat Genet*. (2008) 40:243–8. doi: 10.1038/ng.2007.51

197. Kitazawa M, Tamura M, Kaneko-Ishino T, Ishino F. Severe damage to the placental fetal capillary network causes mid- to late fetal lethality and reduction in placental size in Peg11/Rtl1 ko mice. *Genes to Cells*. (2017) 22:174–88. doi: 10.1111/gtc.12465

198. Kaneko-Ishino T, Ishino F. Retrovirus-derived RTL/SIRH: their diverse roles in the current eutherian developmental system and contribution to eutherian evolution. *Biomolecules*. (2023) 13. doi: 10.20944/preprints202308.1012.v1

199. Li CM, Margolin AA, Salas M, Memeo L, Mansukhani M, Hibshoosh H, et al. Peg10 is a C-myc target gene in cancer cells. *Cancer Res*. (2006) 66:665–72. doi: 10.1158/0008-5472.Can-05-1553

200. Chen H, Sun M, Zhao G, Liu J, Gao W, Si S, et al. Elevated expression of peg10 in human placentas from preeclamptic pregnancies. *Acta Histochem*. (2012) 114:589–93. doi: 10.1016/j.acthis.2011.11.003

201. Salgia R, Jolly MK, Dorff T, Lau C, Weninger K, Orban J, et al. Prostate-associated gene 4 (Page4): leveraging the conformational dynamics of a dancing protein cloud as a therapeutic target. *J Clin Med*. (2018) 7. doi: 10.3390/jcm7060156

202. Marsh B, Zhou Y, Kapidzic M, Fisher S, Blelloch R. Regionally distinct trophoblast regulate barrier function and invasion in the human placenta. *Elife*. (2022) 11. doi: 10.7554/eLife.78829

203. Shiura H, Kitazawa M, Ishino F, Kaneko-Ishino T. Roles of retrovirus-derived PEG10 and PEG11/RTL1 in mammalian development and evolution and their involvement in human disease. *Front Cell Dev Biol*. (2023) 11:1273638. doi: 10.3389/fcell.2023.1273638

204. Spencer TE. Biological roles of uterine glands in pregnancy. *Semin Reprod Med*. (2014) 32:346–57. doi: 10.1055/s-0034-1376354

205. Wei J, Lau SY, Blenkiron C, Chen Q, James JL, Kleffmann T, et al. Trophoblastic debris modifies endothelial cell transcriptome *in vitro*: A mechanism by which fetal cells might control maternal responses to pregnancy. *Sci Rep*. (2016) 6:30632. doi: 10.1038/srep30632

206. Babwah AV. Uterine and placental kiss1 regulate pregnancy: what we know and the challenges that lie ahead. *Reproduction*. (2015) 150:R121–8. doi: 10.1530/rep-15-0252

207. Nash KT, Welch DR. The kiss1 metastasis suppressor: mechanistic insights and clinical utility. *Front Biosci*. (2006) 11:647–59. doi: 10.2741/1824

208. Jeyarajah MJ, Jaju Bhattad G, Kelly RD, Baines KJ, Jaremek A, Yang FP, et al. The multifaceted role of gcm1 during trophoblast differentiation in the human placenta. *Proc Natl Acad Sci U.S.A*. (2022) 119:e2203071119. doi: 10.1073/pnas.2203071119

209. Wu YH, Lo HF, Chen SH, Chen H. Caspase-14 suppresses gcm1 acetylation and inhibits placental cell differentiation. *FASEB J*. (2013) 27:2818–28. doi: 10.1096/fj.12-224279

210. Fant M, Farina A, Nagaraja R, Schlessinger D. Plac1 (Placenta-specific 1): A novel, X-linked gene with roles in reproductive and cancer biology. *Prenat Diagn*. (2010) 30:497–502. doi: 10.1002/pd.2506

211. Chang WL, Wang H, Cui L, Peng NN, Fan X, Xue LQ, et al. Plac1 is involved in human trophoblast syncytialization. *Reprod Biol*. (2016) 16:218–24. doi: 10.1016/j.repbio.2016.07.001

212. Wang HY, Zhang Z, Yu S. Expression of pappa2 in human fetomaternal interface and involvement in trophoblast invasion and migration. *Genet Mol Res*. (2016) 15. doi: 10.4238/gmr.15038075

213. Wang J, Qiu Q, Haider M, Bell M, Gruslin A, Christians JK. Expression of pregnancy-associated plasma protein A2 during pregnancy in human and mouse. *J Endocrinol*. (2009) 202:337–45. doi: 10.1677/joe-09-0136

214. Zhang ET, Hannibal RL, Badillo Rivera KM, Song JHT, McGowan K, Zhu X, et al. PRG2 and AQPEP are misexpressed in fetal membranes in placenta previa and percreta†. *Biol Reprod*. (2021) 105:244–57. doi: 10.1093/biolre/ioab068

215. Hackmon R, Pinnaduwage L, Zhang J, Lye SJ, Geraghty DE, Dunk CE. Definitive class I human leukocyte antigen expression in gestational placentation: HLA-F, HLA-E, HLA-C, and HLA-G in extravillous trophoblast invasion on placentation, pregnancy, and parturition. *Am J Reprod Immunol*. (2017) 77. doi: 10.1111/aji.12643

216. Apps R, Gardner L, Moffett A. A critical look at HLA-G. *Trends Immunol*. (2008) 29:313–21. doi: 10.1016/j.it.2008.02.012

217. Ishikawa T, Takizawa T, Iwaki J, Mishima T, Ui-Tei K, Takeshita T, et al. Fc gamma receptor IIb participates in maternal IgG trafficking of human placental endothelial cells. *Int J Mol Med*. (2015) 35:1273–89. doi: 10.3892/ijmm.2015.2141

218. Ali A, Bouma GJ, Anthony RV, Winger QA. The role of LIN28-let-7-ARID3B pathway in placental development. *Int J Mol Sci*. (2020) 21. doi: 10.3390/ijms21103637

219. Yu Y, He JH, Hu LL, Jiang LL, Fang L, Yao GD, et al. Placensin is a glucogenic hormone secreted by human placenta. *EMBO Rep*. (2020) 21:e49530. doi: 10.15252/embr.201949530

220. Malassiné A, Frendo JL, Evain-Brion D. A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update*. (2003) 9:531–9. doi: 10.1093/humupd/dmg043

221. Sardet C, Paix A, Prodon F, Dru P, Chenevert J. From oocyte to 16-cell stage: cytoplasmic and cortical reorganizations that pattern the ascidian embryo. *Dev Dyn*. (2007) 236:1716–31. doi: 10.1002/dvdy.21136

222. Eckersley-Maslin MA, Alda-Catalinas C, Reik W. Dynamics of the epigenetic landscape during the maternal-to-zygotic transition. *Nat Rev Mol Cell Biol*. (2018) 19:436–50. doi: 10.1038/s41580-018-0008-z

223. Rayon T, Stamataki D, Perez-Carrasco R, Garcia-Perez L, Barrington C, Melchionda M, et al. Species-specific pace of development is associated with differences in protein stability. *Science*. (2020) 369. doi: 10.1126/science.aba7667

224. Serman A, Serman L. Development of placenta in a rodent–model for human placentation. *Front Biosci (Elite Ed)*. (2011) 3:233–9. doi: 10.2741/e238

225. Welsh AO, Enders AC. Trophoblast-decidual cell interactions and establishment of maternal blood circulation in the parietal yolk sac placenta of the rat. *Anat Rec*. (1987) 217:203–19. doi: 10.1002/ar.1092170213

226. Pereda J, Monge JI, Niimi G. Two different pathways for the transport of primitive and definitive blood cells from the yolk sac to the embryo in humans. *Microsc Res Tech*. (2010) 73:803–9. doi: 10.1002/jemt.20823

227. Calkins K, Devaskar SU. Fetal origins of adult disease. *Curr Probl Pediatr Adolesc Health Care*. (2011) 41:158–76. doi: 10.1016/j.cppeds.2011.01.001

228. Dimitriadis E, Rolnik DL, Zhou W, Estrada-Gutierrez G, Koga K, Francisco RPV, et al. Pre-eclampsia. *Nat Rev Dis Primers*. (2023) 9:8. doi: 10.1038/s41572-023-00417-6

229. Hoang VM, Foulk R, Clauser K, Burlingame A, Gibson BW, Fisher SJ. Functional proteomics: examining the effects of hypoxia on the cytotrophoblast protein repertoire. *Biochemistry*. (2001) 40:4077–86. doi: 10.1021/bi0023910

230. Sun LZ, Yang NN, De W, Xiao YS. Proteomic analysis of proteins differentially expressed in preeclamptic trophoblasts. *Gynecologic Obstetric Invest*. (2007) 64:17–23. doi: 10.1159/000098399

231. Ishioka S, Ezaka Y, Umemura K, Hayashi T, Endo T, Saito T. Proteomic analysis of mechanisms of hypoxia-induced apoptosis in trophoblastic cells. *Int J Med Sci*. (2006) 4:36–44. doi: 10.7150/ijms.4.36

232. Hu R, Jin H, Zhou S, Yang P, Li X. Proteomic analysis of hypoxia-induced responses in the syncytialization of human placental cell line bewo. *Placenta*. (2007) 28:399–407. doi: 10.1016/j.placenta.2006.07.005

233. Vorum H, Østergaard M, Hensechke P, Enghild JJ, Riazati M, Rice GE. Proteomic analysis of hyperoxia-induced responses in the human choriocarcinoma cell line jeg-3. *Proteomics*. (2004) 4:861–7. doi: 10.1002/pmic.200300639

234. Webster RP, Pitzer BA, Roberts VH, Brockman D, Myatt L. Differences in the proteome profile in placenta from normal term and preeclamptic preterm pregnancies. *Proteomics Clin Appl*. (2007) 1:446–56. doi: 10.1002/prca.200600745

235. Gharesi-Fard B, Zolghadri J, Kamali-Sarvestani E. Proteome differences of placenta between pre-eclampsia and normal pregnancy. *Placenta*. (2010) 31:121–5. doi: 10.1016/j.placenta.2009.11.004

236. Liu AX, Jin F, Zhang WW, Zhou TH, Zhou CY, Yao WM, et al. Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. *Biol Reprod*. (2006) 75:414–20. doi: 10.1095/biolreprod.105.049379

237. Butt RH, Lee MW, Pirshahid SA, Backlund PS, Wood S, Coorssen JR. An initial proteomic analysis of human preterm labor: placental membranes. *J Proteome Res*. (2006) 5:3161–72. doi: 10.1021/pr060282n

238. Zhang Y, Zhang YL, Feng C, Wu YT, Liu AX, Sheng JZ, et al. Comparative proteomic analysis of human placenta derived from assisted reproductive technology. *Proteomics*. (2008) 8:4344–56. doi: 10.1002/pmic.200800294

239. Wong F, Cox B. Proteomics analysis of preeclampsia, a systematic review of maternal and fetal compartments. *J Proteomics Bioinform S*. (2014) 10:2. doi: 10.4172/jpb

240. Sawicki G, Dakour J, Morrish DW. Functional proteomics of neurokinin B in the placenta indicates a novel role in regulating cytotrophoblast antioxidant defences. *Proteomics*. (2003) 3:2044–51. doi: 10.1002/pmic.200300537

241. Wang F, Shi Z, Wang P, You W, Liang G. Comparative proteome profile of human placenta from normal and preeclamptic pregnancies. *PloS One*. (2013) 8: e78025. doi: 10.1371/journal.pone.0078025

242. Zhang HH, Wang YP, Chen DB. Analysis of nitroso-proteomes in normotensive and severe preeclamptic human placentas. *Biol Reprod*. (2011) 84:966–75. doi: 10.1095/biolreprod.110.090688

243. Baig S, Kothandaraman N, Manikandan J, Rong L, Ee KH, Hill J, et al. Proteomic analysis of human placental syncytiotrophoblast microvesicles in preeclampsia. *Clin Proteomics*. (2014) 11:40. doi: 10.1186/1559-0275-11-40

244. Yang JI, Kong TW, Kim HS, Kim HY. The proteomic analysis of human placenta with pre-eclampsia and normal pregnancy. *J Korean Med Sci*. (2015) 30:770–8. doi: 10.3346/jkms.2015.30.6.770

245. Sun X, Qu T, He X, Yang X, Guo N, Mao Y, et al. Screening of differentially expressed proteins from syncytiotrophoblast for severe early-onset preeclampsia in women with gestational diabetes mellitus using tandem mass tag quantitative proteomics. *BMC Pregnancy Childbirth*. (2018) 18:437. doi: 10.1186/s12884-018-2066-9

246. Jin H, Ma KD, Hu R, Chen Y, Yang F, Yao J, et al. Analysis of expression and comparative profile of normal placental tissue proteins and those in preeclampsia patients using proteomic approaches. *Anal Chim Acta*. (2008) 629:158–64. doi: 10.1016/j.aca.2008.09.015

247. Shin JK, Baek JC, Kang MY, Park JK, Lee SA, Lee JH, et al. Proteomic analysis reveals an elevated expression of heat shock protein 27 in preeclamptic placentas. *Gynecol Obstet Invest*. (2011) 71:151–7. doi: 10.1159/000315162

248. Zhang W, Chen X, Yan Z, Chen Y, Cui Y, Chen B, et al. Detergent-insoluble proteome analysis revealed aberrantly aggregated proteins in human preeclampsia placentas. *J Proteome Res*. (2017) 16:4468–80. doi: 10.1021/acs.jproteome.7b00352

249. Mary S, Kulkarni MJ, Malakar D, Joshi SR, Mehendale SS, Giri AP. Placental proteomics provides insights into pathophysiology of pre-eclampsia and predicts possible markers in plasma. *J Proteome Res*. (2017) 16:1050–60. doi: 10.1021/acs.jproteome.6b00955

250. Jin X, Xu Z, Cao J, Shao P, Zhou M, Qin Z, et al. Proteomics analysis of human placenta reveals glutathione metabolism dysfunction as the underlying pathogenesis for preeclampsia. *Biochim Biophys Acta Proteins Proteom*. (2017) 1865:1207–14. doi: 10.1016/j.bbapap.2017.07.003

251. Xu Z, Jin X, Cai W, Zhou M, Shao P, Yang Z, et al. Proteomics analysis reveals abnormal electron transport and excessive oxidative stress cause mitochondrial dysfunction in placental tissues of early-onset preeclampsia. *Proteomics Clin Appl*. (2018) 12:e1700165. doi: 10.1002/prca.201700165

252. Ma K, Jin H, Hu R, Xiong Y, Zhou S, Ting P, et al. A proteomic analysis of placental trophoblastic cells in preeclampsia-eclampsia. *Cell Biochem Biophys*. (2014) 69:247–58. doi: 10.1007/s12013-013-9792-4

253. Shi Z, Long W, Zhao C, Guo X, Shen R, Ding H. Comparative proteomics analysis suggests that placental mitochondria are involved in the development of pre-eclampsia. *PloS One*. (2013) 8:e64351. doi: 10.1371/journal.pone.0064351

254. Johnstone ED, Sawicki G, Guilbert L, Winkler-Lowen B, Cadete VJ, Morrish DW. Differential proteomic analysis of highly purified placental cytotrophoblasts in pre-eclampsia demonstrates a state of increased oxidative stress and reduced cytotrophoblast antioxidant defense. *Proteomics*. (2011) 11:4077–84. doi: 10.1002/pmic.201000505

255. He A, Wang J, Yang X, Liu J, Yang X, Wang G, et al. Screening of differentially expressed proteins in placentas from patients with late-onset preeclampsia. *Proteomics Clin Appl*. (2022) 16:e2100053. doi: 10.1002/prca.202100053

256. Wang H, Shi Y, Ma J, Wang W, Gao J, Zhao L, et al. Integrated proteomic and N-glycoproteomic profiling of placental tissues of patients with preeclampsia. *Int J Womens Health*. (2023) 15:59–68. doi: 10.2147/ijwh.S387672

257. Kalousová M, Muravská A, Zima T. Pregnancy-associated plasma protein a (Papp-a) and preeclampsia. *Adv Clin Chem*. (2014) 63:169–209. doi: 10.1016/b978-0-12-800094-6.00005-4

258. Handwerger S, Freemark M. The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. *J Pediatr Endocrinol Metab*. (2000) 13:343–56. doi: 10.1515/jpem.2000.13.4.343

259. Alsat E, Guibourdenche J, Couturier A, Evain-Brion D. Physiological role of human placental growth hormone. *Mol Cell Endocrinol*. (1998) 140:121–7. doi: 10.1016/s0303-7207(98)00040-9

260. Alsat E, Guibourdenche J, Luton D, Frankenne F, Evain-Brion D. Human placental growth hormone. *Am J Obstetrics Gynecology*. (1997) 177:1526–34. doi: 10.1016/S0002-9378(97)70103-0

261. Sasaki A, Shinkawa O, Margioris AN, Liotta AS, Sato S, Murakami O, et al. Immunoreactive corticotropin-releasing hormone in human plasma during pregnancy, labor, and delivery. *J Clin Endocrinol Metab*. (1987) 64:224–9. doi: 10.1210/jcem-64-2-224

262. Burton GJ, Charnock-Jones DS, Jauniaux E. Regulation of vascular growth and function in the human placenta. *Reproduction*. (2009) 138:895–902. doi: 10.1530/rep-09-0092

263. Leguy MC, Brun S, Pidoux G, Salhi H, Choiset A, Menet MC, et al. Pattern of secretion of pregnancy-associated plasma protein-a (Papp-a) during pregnancies complicated by fetal aneuploidy, *in vivo* and *in vitro*. *Reprod Biol Endocrinol*. (2014) 12:129. doi: 10.1186/1477-7827-12-129

264. Degnes ML, Westerberg AC, Zucknick M, Powell TL, Jansson T, Henriksen T, et al. Placenta-derived proteins across gestation in healthy pregnancies-a novel approach to assess placental function? *BMC Med*. (2022) 20:227. doi: 10.1186/s12916-022-02415-z

265. Zhang T, Zhao C, Luo L, Xiang J, Cheng J, Wang T, et al. High concentraction of taurocholic acid induced apoptosis in HTR-8/SVneo cells via overexpression of ERp29 and activation of P38. *Placenta*. (2014) 35:496–500. doi: 10.1016/j.placenta.2014.03.023

266. Degnes M-HL, Westerberg AC, Zucknick M, Powell TL, Jansson T, Henriksen T, et al. Placenta-derived proteins across gestation in healthy pregnancies—a novel approach to assess placental function? *BMC Med*. (2022) 20:227. doi: 10.1186/s12916-022-02415-z

267. Chau K, Hennessy A, Makris A. Placental growth factor and pre-eclampsia. *J Hum Hypertens*. (2017) 31:782–6. doi: 10.1038/jhh.2017.61

268. Kim SY, Ryu HM, Yang JH, Kim MY, Ahn HK, Shin JS, et al. Maternal serum and amniotic fluid inhibin a levels in women who subsequently develop severe preeclampsia. *J Korean Med Sci*. (2006) 21:452–6. doi: 10.3346/jkms.2006.21.3.452

269. Yue CY, Zhang CY, Ni YH, Ying CM. Are serum levels of inhibin a in second trimester predictors of adverse pregnancy outcome? *PloS One*. (2020) 15:e0232634. doi: 10.1371/journal.pone.0232634

270. Khalil A, Cowans NJ, Spencer K, Goichman S, Meiri H, Harrington K. First trimester maternal serum placental protein 13 for the prediction of pre-eclampsia in women with a priori high risk. *Prenat Diagn*. (2009) 29:781–9. doi: 10.1002/pd.2287

271. Gonen R, Shahar R, Grimpel YI, Chefetz I, Sammar M, Meiri H, et al. Placental protein 13 as an early marker for pre-eclampsia: A prospective longitudinal study. *Bjog*. (2008) 115:1465–72. doi: 10.1111/j.1471-0528.2008.01902.x

272. Poon LC, Nicolaides KH. Early prediction of preeclampsia. *Obstet Gynecol Int*. (2014) 2014:297397. doi: 10.1155/2014/297397

273. Yung HW, Zhao X, Glover L, Burrin C, Pang PC, Jones CJP, et al. Perturbation of placental protein glycosylation by endoplasmic reticulum stress promotes maladaptation of maternal hepatic glucose metabolism. *iScience*. (2023) 26:105911. doi: 10.1016/j.isci.2022.105911

274. Liu LY, Yang T, Ji J, Wen Q, Morgan AA, Jin B, et al. Integrating multiple 'Omics' Analyses identifies serological protein biomarkers for preeclampsia. *BMC Med*. (2013) 11:236. doi: 10.1186/1741-7015-11-236

275. Ji Q, Zhang S, Jiang W, Wang J, Luan Y, Xin Q. Serum protein profile analysis via label-free quantitation proteomics in patients with early-onset preeclampsia. *J Obstet Gynaecol*. (2023) 43:2259982. doi: 10.1080/01443615.2023.2259982

276. Chen H, Aneman I, Nikolic V, Karadzov Orlic N, Mikovic Z, Stefanovic M, et al. Maternal plasma proteome profiling of biomarkers and pathogenic mechanisms of early-onset and late-onset preeclampsia. *Sci Rep*. (2022) 12:19099. doi: 10.1038/s41598-022-20658-x

277. Tu C, Tao F, Qin Y, Wu M, Cheng J, Xie M, et al. Serum proteins differentially expressed in early- and late-onset preeclampsia assessed using iTRAQ proteomics and bioinformatics analyses. *PeerJ*. (2020) 8:e9753. doi: 10.7717/peerj.9753

278. Marić I, Contrepois K, Moufarrej MN, Stelzer IA, Feyaerts D, Han X, et al. Early prediction and longitudinal modeling of preeclampsia from multiomics. *Patterns*. (2022) 3:100655. doi: 10.1016/j.patter.2022.100655

279. Chen ME, Lin SH, Chung LW, Sikes RA. Isolation and characterization of PAGE-1 and GAGE-7. New genes expressed in the LNCaP prostate cancer progression model that share homology with melanoma-associated antigens. *J Biol Chem*. (1998) 273:17618–25. doi: 10.1074/jbc.273.28.17618

280. Chen YT, Scanlan MJ, Sahin U, Türeci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U.S.A*. (1997) 94:1914–8. doi: 10.1073/pnas.94.5.1914

281. Nin DS, Deng L-W. Biology of cancer-testis antigens and their therapeutic implications in cancer. *Cells*. (2023) 12. doi: 10.3390/cells12060926

282. Liu L, Xu HX, Wang WQ, Wu CT, Xiang JF, Liu C, et al. Serum CA125 is a novel predictive marker for pancreatic cancer metastasis and correlates with the metastasis-associated burden. *Oncotarget*. (2016) 7:5943–56. doi: 10.18632/oncotarget.6819

283. Blumenthal RD, Leon E, Hansen HJ, Goldenberg DM. Expression patterns of ceacam5 and ceacam6 in primary and metastatic cancers. *BMC Cancer*. (2007) 7:2. doi: 10.1186/1471-2407-7-2

284. Conley AP, Wang WL, Livingston JA, Ravi V, Tsai JW, Ali A, et al. Mage-A3 is a clinically relevant target in undifferentiated pleomorphic sarcoma/myxofibrosarcoma. *Cancers (Basel)*. (2019) 11. doi: 10.3390/cancers11050677

285. Shvartsur A, Bonavida B. Trop2 and its overexpression in cancers: regulation and clinical/therapeutic implications. *Genes Cancer*. (2015) 6:84–105. doi: 10.18632/genesandcancer.40

286. Harrop R, O'Neill E, Stern PL. Cancer stem cell mobilization and therapeutic targeting of the 5T4 oncofetal antigen. *Ther Adv Vaccines Immunother*. (2019) 7:2515135518821623. doi: 10.1177/2515135518821623

287. Yagihashi A, Ohmura T, Asanuma K, Kobayashi D, Tsuji N, Torigoe T, et al. Detection of autoantibodies to survivin and livin in sera from patients with breast cancer. *Clin Chim Acta*. (2005) 362:125–30. doi: 10.1016/j.cccn.2005.06.009

288. Dong XY, Peng JR, Ye YJ, Chen HS, Zhang LJ, Pang XW, et al. Plac1 is a tumor-specific antigen capable of eliciting spontaneous antibody responses in human cancer patients. *Int J Cancer*. (2008) 122:2038–43. doi: 10.1002/ijc.23341

289. Silva WA Jr., Gnjatic S, Ritter E, Chua R, Cohen T, Hsu M, et al. Plac1, a trophoblast-specific cell surface protein, is expressed in a range of human tumors and elicits spontaneous antibody responses. *Cancer Immun*. (2007) 7:18.

290. Liu FF, Dong XY, Pang XW, Xing Q, Wang HC, Zhang HG, et al. The specific immune response to tumor antigen CP1 and its correlation with improved survival in colon cancer patients. *Gastroenterology*. (2008) 134:998–1006. doi: 10.1053/j.gastro.2008.01.029

291. Mahmoudian J, Ghods R, Nazari M, Jeddi-Tehrani M, Ghahremani MH, Ostad SN, et al. Expression profiling of plac1 in murine cancer cell lines. *Exp Oncol*. (2019) 41:7–13. doi: 10.32471/10.32471/exp-oncology.2312-8852.vol-41-no-1

292. Ghods R, Ghahremani MH, Madjd Z, Asgari M, Abolhasani M, Tavasoli S, et al. High placenta-specific 1/low prostate-specific antigen expression pattern in high-grade prostate adenocarcinoma. *Cancer Immunol Immunother*. (2014) 63:1319–27. doi: 10.1007/s00262-014-1594-z

293. Riordan JD, Keng VW, Tschida BR, Scheetz TE, Bell JB, Podetz-Pedersen KM, et al. Identification of rtl1, a retrotransposon-derived imprinted gene, as a novel driver of hepatocarcinogenesis. *PloS Genet*. (2013) 9:e1003441. doi: 10.1371/journal.pgen.1003441

294. Fan G, Ye D, Zhu S, Xi J, Guo X, Qiao J, et al. RTL1 promotes melanoma proliferation by regulating Wnt/B-catenin signalling. *Oncotarget*. (2017) 8:106026–37. doi: 10.18632/oncotarget.22523

295. Mahmoudi AR, Ghods R, Madjd Z, Abolhasani M, Saeednejad Zanjani L, Safaei M, et al. Expression profiling of rtl1 in human breast cancer tissues and cell lines. *Exp Mol Pathol*. (2021) 121:104654. doi: 10.1016/j.yexmp.2021.104654

296. Welsh JB, Sapinoso LM, Kern SG, Brown DA, Liu T, Bauskin AR, et al. Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. *Proc Natl Acad Sci*. (2003) 100:3410–5. doi: 10.1073/pnas.0530278100

297. Wischhusen J, Melero I, Fridman WH. Growth/differentiation factor-15 (Gdf-15): from biomarker to novel targetable immune checkpoint. *Front Immunol*. (2020) 11:951. doi: 10.3389/fimmu.2020.00951

298. Yang R, Han Y, Yi W, Long Q. Autoantibodies as biomarkers for breast cancer diagnosis and prognosis. *Front Immunol*. (2022) 13:1035402. doi: 10.3389/fimmu.2022.1035402

299. Macdonald IK, Parsy-Kowalska CB, Chapman CJ. Autoantibodies: opportunities for early cancer detection. *Trends Cancer*. (2017) 3:198–213. doi: 10.1016/j.trecan.2017.02.003

300. Tan HT, Low J, Lim SG, Chung MC. Serum autoantibodies as biomarkers for early cancer detection. *FEBS J*. (2009) 276:6880–904. doi: 10.1111/j.1742-4658.2009.07396.x

301. Zaenker P, Ziman MR. Serologic autoantibodies as diagnostic cancer biomarkers–a review. *Cancer Epidemiol Biomarkers Prev*. (2013) 22:2161–81. doi: 10.1158/1055-9965.Epi-13-0621

302. Poulsen TBG, Karamehmedovic A, Aboo C, Jørgensen MM, Yu X, Fang X, et al. Protein array-based companion diagnostics in precision medicine. *Expert Rev Mol Diagnostics*. (2020) 20:1183–98. doi: 10.1080/14737159.2020.1857734

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 07 May 2024
DOI 10.3389/fimmu.2024.1401738

Check for updates

OPEN ACCESS

EDITED BY
Marie-Pierre Piccinni,
University of Florence, Italy

REVIEWED BY
Uday Kishore,
United Arab Emirates University, United Arab Emirates
Yohei Sato,
Jikei University School of Medicine, Japan

*CORRESPONDENCE
Shigeru Saito
s30saito@adm.u-toyama.ac.jp
Akitoshi Nakashima
akinaka@med.u-toyama.ac.jp

†These authors have contributed equally to this work and share last authorship

RECEIVED 15 March 2024
ACCEPTED 23 April 2024
PUBLISHED 07 May 2024

CITATION
Tsuda S, Shichino S, Tilburgs T, Shima T, Morita K, Yamaki-Ushijima A, Roskin K, Tomura M, Sameshima A, Saito S and Nakashima A (2024) CD4$^+$ T cell heterogeneity in gestational age and preeclampsia using single-cell RNA sequencing. *Front. Immunol.* 15:1401738. doi: 10.3389/fimmu.2024.1401738

COPYRIGHT
© 2024 Tsuda, Shichino, Tilburgs, Shima, Morita, Yamaki-Ushijima, Roskin, Tomura, Sameshima, Saito and Nakashima. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

CD4$^+$ T cell heterogeneity in gestational age and preeclampsia using single-cell RNA sequencing

Sayaka Tsuda[1,2], Shigeyuki Shichino[3], Tamara Tilburgs[2], Tomoko Shima[1], Keiko Morita[1], Akemi Yamaki-Ushijima[1], Krishna Roskin[4], Michio Tomura[5], Azusa Sameshima[1,6], Shigeru Saito[7*†] and Akitoshi Nakashima[1*†]

[1]Department of Obstetrics and Gynecology, University of Toyama, Toyama, Japan, [2]Division of Immunobiology, Center for Inflammation and Tolerance, Cincinnati Children's Hospital, Cincinnati, OH, United States, [3]Division of Molecular Regulation of Inflammatory and Immune Diseases, Research Institute of Biomedical Sciences, Tokyo University of Science, Chiba, Japan, [4]Divisions of Biomedical Informatics & Immunobiology, Cincinnati Children's Hospital, Cincinnati, OH, United States, [5]Laboratory of Immunology, Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan, [6]Ladies' Clinic We! Toyama, Toyama, Japan, [7]University of Toyama, Toyama, Japan

A balance between pro-inflammatory decidual CD4$^+$ T cells and *FOXP3*$^+$ regulatory T cells (*FOXP3*$^+$ Tregs) is important for maintaining fetomaternal tolerance. Using single-cell RNA-sequencing and T cell receptor repertoire analysis, we determined that diversity and clonality of decidual CD4$^+$ T cell subsets depend on gestational age. Th1/Th2 intermediate and Th1 subsets of CD4$^+$ T cells were clonally expanded in both early and late gestation, whereas *FOXP3*$^+$ Tregs were clonally expanded in late gestation. Th1/Th2 intermediate and *FOXP3*$^+$ Treg subsets showed altered gene expression in preeclampsia (PE) compared to healthy late gestation. The Th1/Th2 intermediate subset exhibited elevated levels of cytotoxicity-related gene expression in PE. Moreover, increased Treg exhaustion was observed in the PE group, and *FOXP3*$^+$ Treg subcluster analysis revealed that the effector Treg like subset drove the Treg exhaustion signatures in PE. The Th1/Th2 intermediate and effector Treg like subsets are possible inflammation-driving subsets in PE.

KEYWORDS

CD4 + T cell, FOXP3, immune tolerance, preeclampsia, pregnancy, regulatory T cell, single-cell RNA-sequencing, T cell receptor

1 Introduction

Extravillous trophoblasts, which express allogeneic fetal antigens, come into direct contact with maternal immune cells but are not rejected because of functional alterations in maternal immune cells. While $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T cells, including Th17, Th1, and Th2, are proinflammatory and contribute to immune recognition of fetal allo-antigens, *FOXP3*$^+$ regulatory T cells (*FOXP3*$^+$ Tregs) provide immune tolerance. A balance between proinflammatory and tolerogenic immune responses is necessary for maintenance of a pregnancy. *FOXP3*$^+$ Tregs are a minor population among all decidual immune cells; however, they are crucial for inducing fetal antigen-specific tolerance in allogeneic pregnancies in mice (1–5) and humans (6, 7).

Preeclampsia (PE) is a pregnancy complication characterized by maternal hypertension, proteinuria, and fetal placental circulation failure that is diagnosed after 20 weeks of gestation (8). Because poor placentation is the basis of PE, especially in early onset PE (diagnosed before 34 weeks), immune maladaptation has been proposed to underlie the development of PE (9, 10), and this relationship is supported by many reports. Epidemiologically, the risk of developing PE is higher in cases with inadequate exposure to paternal antigens (first partner pregnancy, long interpregnancy interval, oocyte donation, etc.), possibly due to inadequate induction of paternal antigen-specific immune tolerance (11–15). In addition, quantitative and functional impairment of Tregs has been reported during PE (6, 16–20). *FOXP3*$^+$ Tregs are a heterogeneous population with multiple subsets. A previous study identified functionally distinct subtypes of *FOXP3*$^+$ Tregs in human peripheral blood, including $FOXP3^{low}CD45RA^+$ naïve Tregs, $FOXP3^{high}CD45RA^-$ effector Tregs, and $FOXP3^{low}CD45RA^-$ non-suppressive T cells (21). Of these, effector Tregs have the strongest suppressive ability, a memory phenotype, and high expression of ICOS, TIGIT, HLA-DR, ENTPD1, and CTLA-4 (21–23). In contrast, $FOXP3^{low}CD45RA^-$ non-suppressive T cells have no suppressive ability and low expression of ICOS and TIGIT and are regarded as activated $FOXP3^+$ effector $CD4^+$ T cells (non-Tregs) (21–23). Decreased frequencies of effector Tregs have been found in the decidua after miscarriages with normal karyotypes (24, 25). Reduced clonal expansion of effector Tregs in the human decidua after PE has also been reported (25). Activation of proinflammatory T cells, including $CD4^+$ Th17 cells (26–29), and inadequate suppression of $CD8^+$ T cells by PD-1 (30) have also been reported in PE.

Although current single-cell RNA sequencing (scRNA-seq)-based and mass-cytometry-based studies have revealed landscapes of highly heterogeneous maternal immune cells and their populational or phenotypic changes according to gestational age and late onset PE (31–37), the characteristics of decidual Tregs and $CD4^+$ T cell subsets and their clonal expansion are not fully described because of their relative rarity.

Therefore, we conducted scRNA-seq and single-cell T cell receptor (TCR) sequencing of decidual $CD4^+$T cells during healthy early gestation, healthy late gestation, and PE-associated gestation. We found that decidual $CD4^+$ T cells and Treg subsets show differences in gene expression and clonality depending on gestational age and the presence or absence of PE. Most interestingly, we observed extensive clonal expansion of *FOXP3*$^+$ effector Tregs during late and PE-associated gestation, as well as increased signatures of Treg exhaustion in the *FOXP3*$^+$ effector Treg like subset in PE.

2 Materials and methods

2.1 Human subjects

This study was approved by the University of Toyama Institutional Review Board (ID: R2016144 and R20161442). Informed consent was obtained from all participants. Four patients who underwent artificial abortion in the first trimester were enrolled at the Ladies' Clinic We! Toyama. Three healthy pregnant women and three patients with PE were enrolled at the University of Toyama. One healthy pregnant woman provided peripheral blood and decidua. All abortions were performed by manual vacuum aspiration. All pregnancies in healthy women and preeclamptic patients were delivered via Caesarean section. PE was defined as gestational hypertension accompanied by proteinuria, maternal organ dysfunction, or uteroplacental dysfunction, at or after 20 weeks of gestation (8). Detailed characteristics of the participants are provided in Supplementary Table 1.

2.2 Cell isolation

First-trimester decidual tissues were isolated from the uterine content obtained by manual vacuum aspiration. The decidua basalis from healthy late gestation and PE deliveries was macroscopically identified and dissected from the maternal surface of the delivered placenta. The samples were processed within 24 h of collection. Decidua was rinsed with phosphate buffered saline (PBS) until the blood was removed, minced with a pair of scissors to produce 1–2 mm pieces, and filtered through 41-μm nylon net filter. The filtered cell suspension was layered with Ficoll Hypaque and centrifuged at 1500 rpm for 30 min with the brakes off. The immune cells at the gradient interface were collected, washed with PBS, and cryopreserved. Peripheral blood mononuclear cells (PBMC), similar to decidual immune cells, were isolated using density gradient centrifugation.

2.3 cDNA library construction using the BD Rhapsody system and sequencing

The cryopreserved mononuclear cells were thawed and incubated with the following antibodies for 20 min: PE anti-CD3, APC-Cy7 anti-CD4, APC anti-CD14, PE-Cy7 anti-CD25, FITC anti-CD37, and BD human Sample Tags (Supplementary Table 2). $CD3^+CD4^+CD14^-CD37^-$cells were sorted into $CD4^+$ T cells using a FACSAria II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). scTCR/Targeted RNA-seq libraries of $CD4^+$ T cells were

constructed using a BD Rhapsody system with BD Rhapsody Targeted mRNA and AbSeq Reagent kit (BD Bioscience, Franklin Lakes, NJ, USA), and a BD Rhapsody T Cell Expression Panel Hs (BD Bioscience, Franklin Lakes, NJ, USA) with human *IL-10* and *NRP1* primer supplements (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer's instructions, except that cDNA denaturation was performed using 0.1M NaOH for 5 min at room temperature. Sequencing was performed using Illumina NovaSeq 6000 and NovaSeq S4 flow cells (200 cycles kit, read 1 67 bp, read 2 155 bp) (Illumina, San Diego, CA, USA) by ImmunoGeneTeqs, Inc. (Chiba, Japan). Details of the key resources are provided in Supplementary Table 2.

2.4 Fastq data preprocessing and generation of the single-cell gene-expression matrix

Fastq data preprocessing was conducted using ImmunoGeneTeqs, Inc. (Chiba, Japan), as follows. After adapter trimming and quality filtering, the base composition of the sequencing data was analyzed using FastQC-v0.11.9. Pair-ended FASTQ files (R1: cell barcode reads; R2: RNA reads) were processed, and the filtered cell barcode reads were annotated using the Python script provided by BD, with minor modifications for compatibility with Python3.8. The associated cDNA reads were mapped to reference RNA (build GRCh38 release-101). Then, the cell barcode information of each read was added to the bowtie2-mapped BAM files, and the read counts of each gene in each cell barcode were determined using mawk. The cells with a total read count above the inflection point were considered valid. To assign each tag to a cell barcode, the read counts of each tag in each valid cell barcode, defined by the cDNA matrix, were extracted from the tag/cell barcode expression matrix. Unassigned cell barcodes were labeled as "not-detected" cells. Then, the sum of the total read counts of each tag was normalized to 10 M reads and the log2 fold change between the first most counted tags and second most counted tags within each cell barcode. Doublet cells (double-positive cells of any pair of Tags (identified by the flowDensity package)) and log2 fold change between the first and second most counted tags < 0.2415 were determined. Finally, the remaining cell barcodes were assigned to the first most-counted tag. The tag expression in each cell barcode was log2(x+1)-transformed and z-scaled by each cell barcode.

TCR data matrices containing VDJ read counts, nucleotide sequences, amino acid sequences of CDR3, and gene usage were provided by ImmunoGeneTeqs Inc. (Chiba, Japan). Cells with paired alpha/beta chains were used for TCR analysis.

2.5 Single-cell data analysis

Distribution-based error correction (DBEC), which included the BD Rhapsody targeted scRNA-seq workflow to reduce background read counts of each gene that were possibly derived from RNA diffusion during the cell lysis step in the BD Rhapsody cartridge, and reverse transcription were conducted by ImmunoGeneTeqs, Inc. (Chiba, Japan).

We created a Seurat object, in which two batches were combined. We calculated metrics (percent mito, percent ribo) and filtered out mitochondrial gene-high cells (> 0.25), doublets and non-hashtag-assigned cells. Because BD Rhapsody-targeted data are non-UMI data (raw count data), we performed global normalization to 1 M tags and scaling using the Seurat ScaleData function by regressing out the library size (total raw read count of each cell) as a confounding factor. We split the combined Seurat objects into a list, with each batch considered an element. We identified variable features individually for each using the FindVariableFeatures function with default parameters. Next, we identified the anchors for each batch and integrated them using the FindIntegrationAnchors function with 50 dimensions. These anchors were then passed to the integrated data function. We used this integrated matrix for the downstream analysis. Dimension reduction was performed using the RunPCA function on an integrated object with the first 50 PCA dimensions. Clusters were visualized using FindNeighbors, FindClusters, and RunUMAP functions with the top 15 dimensions (resolution set to 0.8). Differential markers between the clusters were detected using the FindAllMarker function. Cell types were determined according to previously reported scRNA markers for T cells from peripheral blood, decidua, and cancers (33, 34, 38–40). Module scores of Treg exhaustion-related genes (*TNFRSF18, TNFRSF4, PDCD1, IFNG, LAG3, CCR5, CXCR3, CCR6, HAVCR2, CXCR6, NKG7, SLAMF1, KLRB1, CD2*, and *CXCR1*) were calculated as previously reported (39, 41) using the AddModuleScore function. Differential expression analysis was conducted using the FindMarkers function with default settings. Software and algorithms are listed in Supplementary Table 2. Genes with log fold change > |0.5| and adjusted p-values ≤ 0.05 were regarded statistically significant.

2.6 Enriched Gene Ontology analysis

We uploaded the list of differentially expressed genes (DEGs) identified using the FindMarkers function in Seurat to the Matascape (https://metascape.org/gp/index.html#/main/step1) main page and chose "Express Analysis."

3 Results

3.1 Decidual CD4$^+$ T cells are heterogenous

To assess decidual CD4$^+$ T cell diversity, we conducted a single-cell transcriptome analysis of CD4$^+$ T cells isolated from the decidua basalis of healthy early gestation (6–7 weeks, n = 4), healthy late gestation (37–40 weeks, n = 3), and gestation with early onset PE diagnosed before 34 weeks who delivered between 34 and 37 weeks of gestation (n = 3) (Supplementary Table 1). Women

with autoimmune diseases were excluded from this study. CD4$^+$ T cells from PBMC from one healthy late gestation patient were also included. CD4$^+$ T cells were sorted and subjected to a BD Rhapsody Single-Cell Analysis (Supplementary Figure 1A). Dead cells and doublets were removed from the scRNA-seq data. Dimension reduction and clustering were performed on 24,002 cells that passed quality control (QC) tests.

Thirteen CD4$^+$ T cell clusters were identified based on cluster marker feature genes and previously reported scRNA-seq data for CD4$^+$ T cells (33, 38, 39) (Figures 1A, B, Supplementary Figure 1B, Supplementary Table 3). CD4$^+$ T cells included i) three clusters of *CCR7*- and *SELL*-expressing naïve (0–2-Tn), ii) one *CXCR5*$^+$ follicular T cell like cluster (3-Tfh like), iii) three clusters of memory CD4$^+$ T cells (4–6-Tm), iv) one cluster of Th1/Th2 intermediate cells expressing *GZMK* and *TBX21*, which suggests Th1 type, as well as *GATA3*, which suggests Th2 type (7-Th1/Th2 int); v) one cluster of Th1-type cells expressing high levels of *GZMK, HLA-DR*, and *TBX21* (8-Th1), vi) one cluster of activated CD4$^+$ T cells with high *PRF1* expressing activated T cells (9-*PRF*$^+$ Tct); vii) one cluster of *FOXP3*$^-$ CD4$^+$ T cells expressing *PDCD1, LAG3*, and *IFNG* (10-*PDCD1*$^+$*FOXP3*$^-$ Treg) with a gene expression signature similar to that of FOXP3$^-$PD-1$^+$ Tregs reported previously in decidua (42), viii) one cluster of Th17-type cells with high *RORC* expression (11-Th17); and viii) one cluster of *FOXP3*$^+$ Treg also expressing *TIGIT*, *IL2RA, TNFRSF18* (also known as *GITR*) and *ENTPD1* (also known as *CD39*) (12-*FOXP3*$^+$ Treg) in concordance with prior studies (42) (Figures 1A, B, Supplementary Figure 1B). *CD69* was expressed across all clusters of CD4$^+$ T cells, with the exception of 6-Tm cells (Figure 1B), suggesting their residence in tissues as described previously (33, 35, 43). Thus, decidual CD4$^+$ T cell populations were found to be heterogeneous and include distinct types of activated memory, helper, cytotoxic, and regulatory T cells.

3.2 Decidual CD4$^+$ T cell cluster frequencies differ among healthy early, healthy late and PE pregnancies

Next, CD4$^+$ T cells were separated into early and late gestation and PE and PBMC groups (Figures 1C-E). Decidual CD4$^+$ T cells contained more abundant populations of memory, 7-Th1/Th2 int, 8-Th1, 9-*PRF*$^+$ Tct, 10-*PDCD1*$^+$*FOXP3*$^-$ Treg, 11-Th17, and 12-*FOXP3*$^+$ Treg than PBMC (Figures 1C–E). The frequency of CD4$^+$ T cell subsets did not differ significantly among healthy early, healthy late, and PE groups because of their small sample sizes and inter-patient heterogeneity. However, an increased tendency of 3-Tfh like and 11-Th17 cells was observed in early gestation, whereas late gestation had a modest increase in tendency toward enrichment with the 0-Tn group (Figure 1E). Interestingly, decidual CD4$^+$ T cells from PE samples showed an increased tendency toward 9-*PRF*$^+$Tct cells compared to cells found in late gestation (Figure 1E). The frequency of decidual 12-*FOXP3*$^+$ Treg varied in PE (Figure 1E), as a recent meta-analysis also describes (44). Thus, changes in CD4$^+$ T cell cluster frequencies are related to gestational age and the presence or absence of PE.

3.3 Clonally expanded CD4$^+$ T cells are found in Th1/Th2 int, Th1, *PRF*$^+$ Tct, and *FOXP3*$^+$ Treg clusters

Next, we conducted a TCR analysis to assess clonal expansion within each cluster. Gene expression and TCR repertoire data were combined from 14,020 cells of five subjects (two early gestational decidua, one late gestational decidua, one PE decidua, and one PBMC sample). In total, 13,538 (96.6%) TCR alpha/beta-paired cells were detected. Clonal expansion was primarily observed in the 7-Th1/Th2 int, 8-Th1, 9-*PRF*$^+$Tct, and 12-*FOXP3*$^+$ Treg clusters (Figures 1F, 2A). 10-*PDCD1*$^+$*FOXP3*$^-$ Treg was clonally expanded in one early gestation subject (early-decidua-2) (Figures 1F, 2A). Clonal expansions in 7-Th1/Th2 int and 8-Th1 were observed in early, late gestation and in PE (Figures 1F, 2A). Clonal expansion of 12-*FOXP3*$^+$ Treg was observed in late gestation and PE, but not in early gestation (Figures 1F, 2A), which is consistent with our previous report (25). Inter-cluster overlap of expanded clonotypes (clone size = 2 or more) showed that the majority of expanded clones belonged to one cluster, whereas a few had multiple phenotypes (Figures 2B-E), confirming that TCR usage partly shapes the CD4$^+$ T cell phenotype, as previously described (38, 45–47). Fewer shared clones with Tregs and CD4$^+$ Th subsets have been reported in term decidua (48). We confirmed that clonally expanded cells in the 12-*FOXP3*$^+$ Treg cluster shared fewer overlapping clones with other clusters (Figures 2B–E). In contrast, some clones overlapped among 7-Th1/Th2 int, 8-Th1, 9-*PRF*$^+$ Tct, 11-Th17, and memory T cell subsets (Figures 2B–E).

Collectively, these results indicated that decidual CD4$^+$ T cells are phenotypically and clonally heterogeneous. Naïve, *CXCR5*$^+$ Tfh like, memory, Th1, *PRF*$^+$ Tct, Th17, *FOXP3*$^-$*PD-1*$^+$ Tregs, and *FOXP3*$^+$ Treg clusters observed here were consistent with previously reported clusters confirmed by gene expression and flowcytometry (33, 38, 39, 42); however, 7-Th1/Th2 was not identified in previous reports. Despite inter-subject heterogeneity, clonal expansion was predominantly observed in 7-Th1/Th2 int, 8-Th1, 9-*PRF*$^+$Tct, and 12-*FOXP3*$^+$ Treg, but not in naïve clusters in the donors analyzed. The 8-Th1, 9-*PRF*$^+$ Tct, 11-Th17, and 7-Th1/Th2 clusters shared some clones, suggesting that they also share some differentiation processes. However, the major expanded clones were unique to each cluster (Figures 2B–E), supporting the validity of the presence of Th1/Th2 clusters in the decidua. The distinct TCR repertoire between 12-*FOXP3*$^+$ Treg and CD4$^+$ T cell subsets suggests that decidual *FOXP3*$^+$ Tregs have clonal origins different from those of decidual CD4$^+$ Th subsets.

3.4 Decidual CD4$^+$ effector, memory, and Tregs have decreased signatures of lymphocyte activation in late gestation compared to early gestation

Distinct decidual T-cell frequencies and transcriptomic signatures in early compared to late gestation have been reported previously (33, 35). However, the gestational age-dependent transcriptomic differences in CD4$^+$ T cells are not well

10.3389/fimmu.2024.1401738

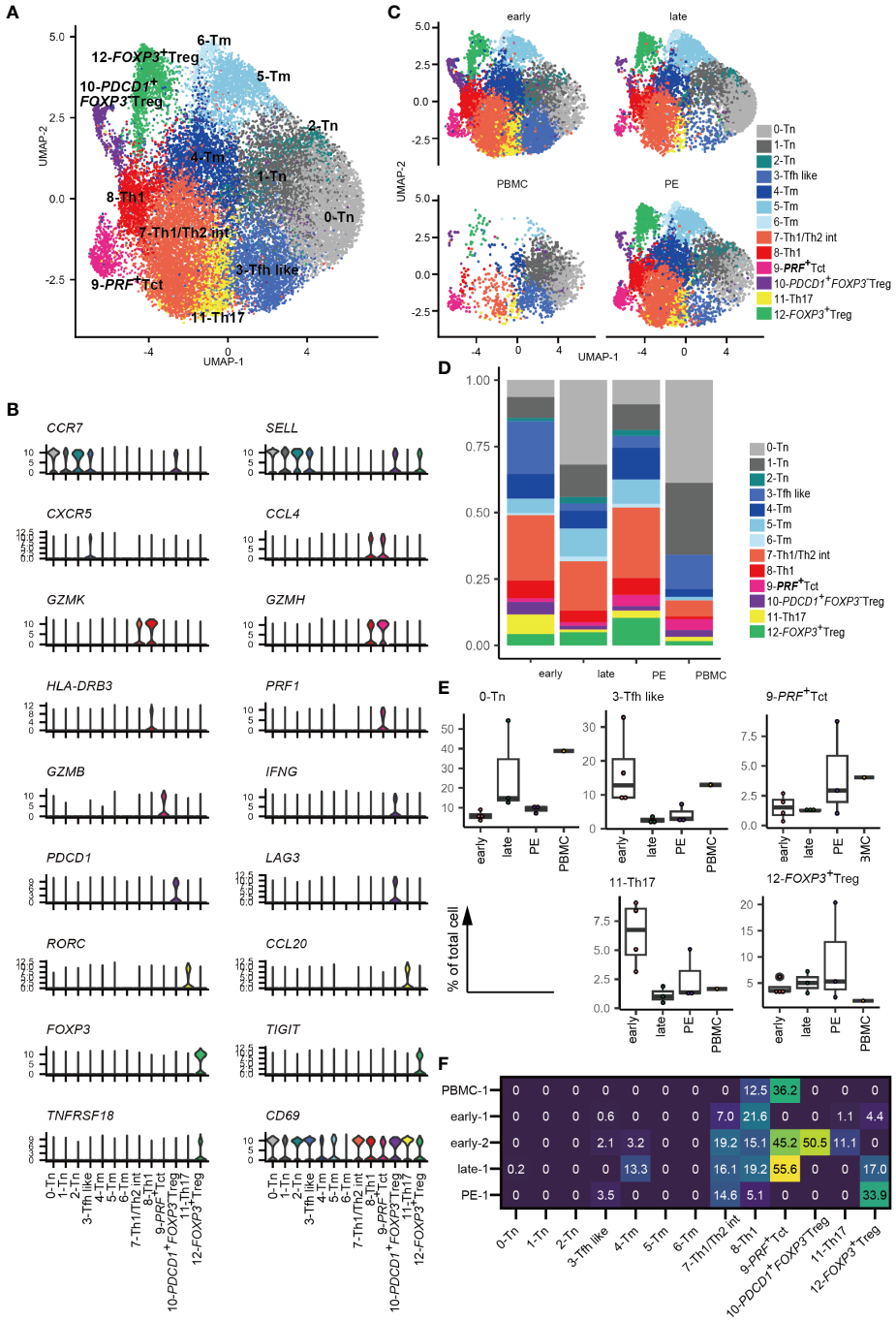


FIGURE 1
Landscape of decidual CD4$^+$ T cells. **(A)** UMAP plot of 24,002 cells from 11 samples showing CD4$^+$ T cell subsets. Thirteen distinct clusters were annotated using gene expression markers. **(B)** Violin plots represent the normalized expression levels (y-axis) of key cluster-defining genes in CD4$^+$ T cells. **(C)** UMAP plots of CD4$^+$ T cell subsets based on sample origin: healthy early gestation decidua (n = 4, 8472 cells) (upper left), healthy late gestation decidua (n = 3, 6572 cells) (upper right), healthy late gestation PBMC (n = 1, 2628 cells) (lower left), and PE decidua delivered at term (n = 3, 6330 cells) (lower right). **(D)** Stacked bar graph comparing the distribution of CD4$^+$ T-cell clusters based on sample origin. **(E)** Floating box plots showing the abundance of cells in 0-Tn, 3-Tfh like, 9-*PRF*$^+$Tct, 11-Th17, and 12-*FOXP3*$^+$ Treg clusters. **(F)** Heatmap showing percentages of expanded clones (size = 2 or more cells) in each cluster. The columns indicate CD4$^+$ T cell clusters. Rows indicate sample origins.

understood. An analysis of DEGs between early and late gestation was performed for each CD4$^+$ T cell cluster. Enriched GOs from the DEGs were analyzed using Metascape (49). Most CD4$^+$ T cell clusters had more downregulated genes in late than early gestation, and DEGs were particularly prevalent in the 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^+$ Treg clusters (Figure 3A, Supplementary Figures 2A–C). Focusing on the GOs of downregulated genes in healthy late gestation compared to early gestation in these three clusters, similar GOs were shared across the clusters (Figure 3B). In particular, GOs related to migration, such as

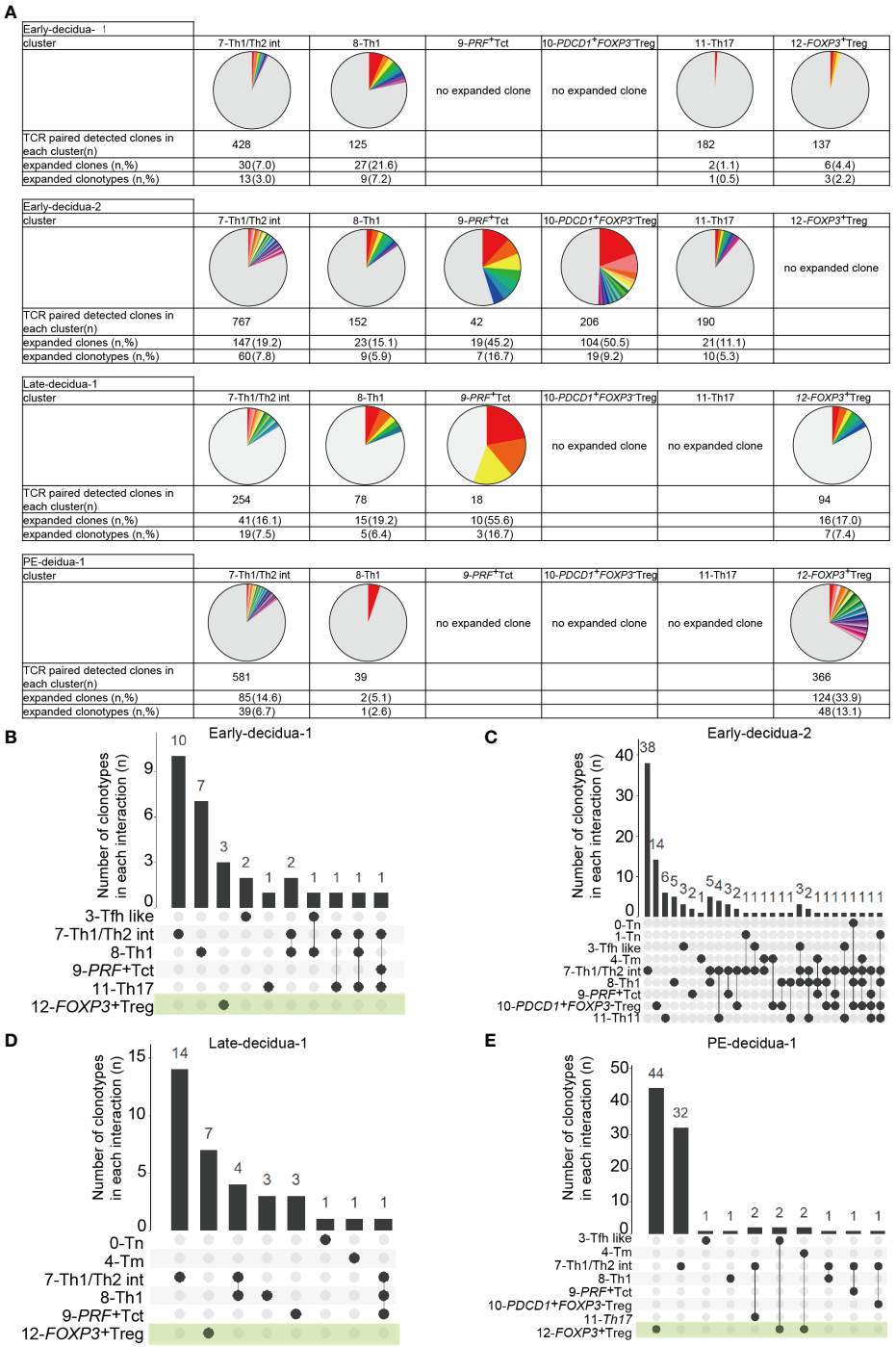


FIGURE 2
Distributions of expanded clonotypes in each CD4+ T cell cluster. **(A)** Pie charts show the ratio of expanded clones (n ≥ 2 clones) in each cluster. Gray parts represent unique clonotypes (n = 1 clones). Colored parts represent expanded clonotypes. The same color does not mean the same clonotype. **(B–E)** UpSetR graphs showing inter-cluster overlap of clonotypes that have clone sizes of 2 or more for each subject. Histogram bar (top) shows number of clonotypes shared between individual clusters (bottom left). Each dot (bottom) represents a cluster in which clonotypes exist. Connected lines represent clusters that share clonotypes.

leukocyte migration and regulation of leukocyte migration, were shared among the 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^+$ Treg clusters (Figure 3B). In addition, GOs related to activation, such as leukocyte activation, T cell activation, and adaptive immune response, were downregulated in 7-Th1/Th2 int and 12-*FOXP3*$^+$ Treg in late gestation (Figure 3B). Genes involved in leukocyte activation, such as *NKG7* and *PRF1*, and those involved in adaptive immune responses, such as *TRAC* and *CD3G*, were downregulated in 7-Th1/Th2 int in late gestation (Figure 3B, Supplementary Figure 2B, Supplementary Table 4). In 12-*FOXP3*$^+$ Treg, *LGALS1* and *CTLA4*, which are related to Treg function, were upregulated in late gestation, whereas more genes related to T cell activation and

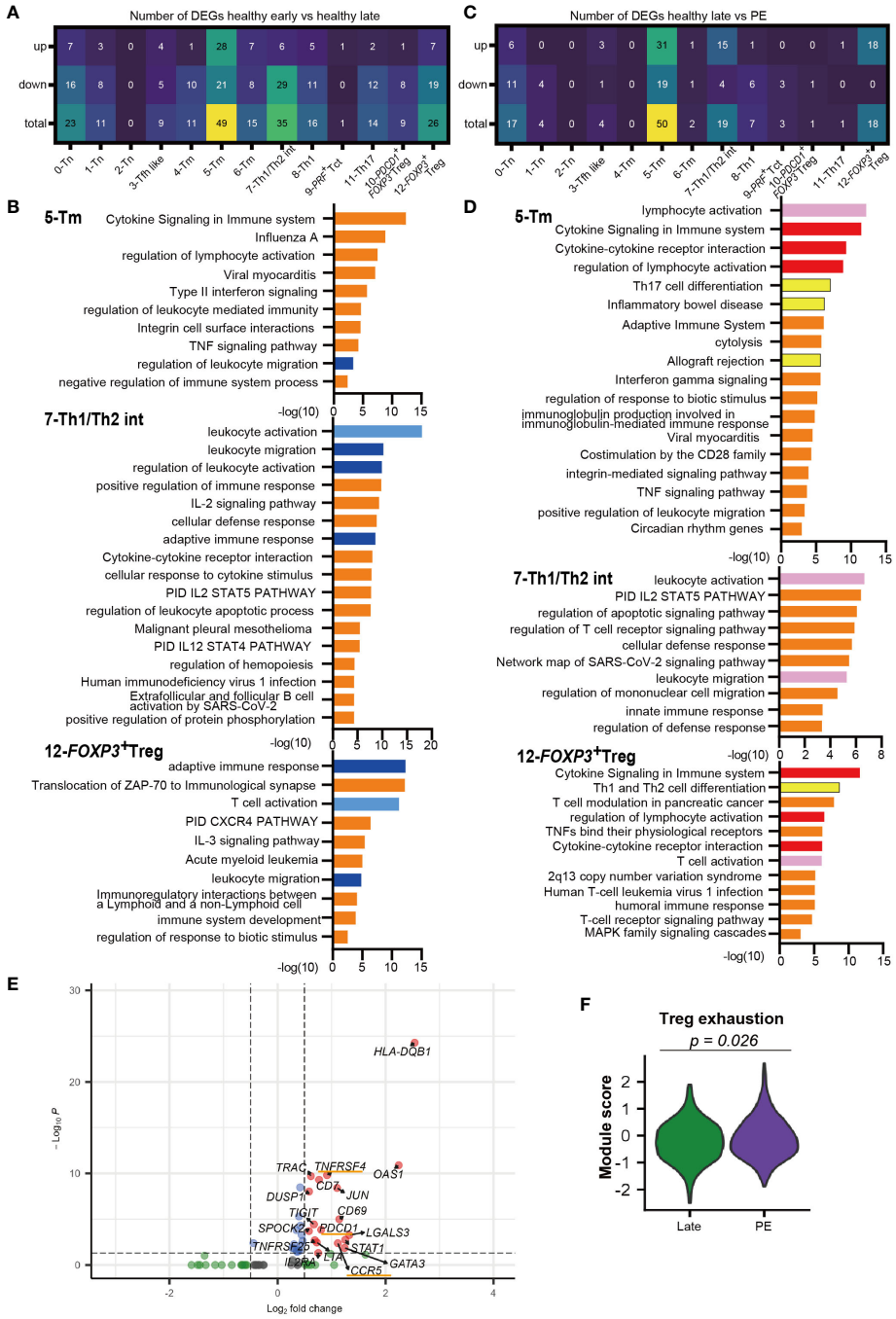


FIGURE 3
Different transcriptomic signatures between healthy early vs. healthy late and healthy late vs. PE decidua. **(A)** Number of DEGs that are upregulated or downregulated in healthy late gestation compared to healthy early gestation decidua. DEG: adjusted p-value < 0.05, |log2 fold change| > 0.5. **(B)** GOs of downregulated genes in 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^{+}$ Treg clusters identified by Metascape in healthy late decidua compared to healthy early decidua. Shared GOs between clusters and similar GOs are marked with blue and sky blue, respectively. **(C)** Number of DEGs that are upregulated or downregulated in PE compared to healthy late gestation decidua. DEG: adjusted p-value < 0.05, |log2 fold change| > 0.5. **(D)** GOs of upregulated genes in 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^{+}$ Treg clusters identified by Metascape in PE compared to healthy late decidua. Shared GOs between clusters, similar GOs, and unique key GOs are marked with red, pink, and yellow, respectively. **(E)** Volcano plot representing DEGs in the 12-*FOXP3*$^{+}$ Treg cluster shared between healthy late gestation and PE. **(F)** Violon plot showing the module score of Treg exhaustion in the 12-*FOXP3*$^{+}$ Treg cluster from term and PE (Mann–Whitney U test).

adaptive immune response, such as HLA-class II molecules, were downregulated in late gestation (Figure 3B, Supplementary Figure 2C, Supplementary Table 4).

In summary, this study revealed significant differences in gene expression of $CD4^+$ T cells between healthy early and late decidua. Specifically, 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^+$ Treg in healthy late gestation had substantially downregulated gene sets associated with leukocyte activation and migration compared to those in early gestation. Interestingly, 12-*FOXP3*$^+$ Treg showed downregulation of genes involved in cell activation and immune responses; however, they showed upregulation of genes related to Treg function in late gestation.

3.5 Decidual $CD4^+$ effector, memory, and Tregs have increased proinflammatory signatures in PE-associated compared to healthy late gestation

Next, we compared DEGs between the healthy late gestation and PE groups to determine transcriptomic changes in T cell clusters. DEG analysis showed that genes upregulated in PE were mainly observed in clusters 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^+$ Treg, which were consistent with the clusters that had the most DEGs in the early and late comparisons (Figures 3C, E, Supplementary Figures 2D, E). Next, GO analysis of upregulated genes with PE in the 5-Tm, 7-Th1/Th2 int, and 12-*FOXP3*$^+$ Treg clusters revealed that activation-related GOs (lymphocyte activation, leukocyte activation, and T cell activation) were shared among the three clusters (Figure 3D). The cytokine signaling and regulation of lymphocyte activation pathways were upregulated in 5-Tm and 12-*FOXP3*$^+$ Treg in PE (Figure 3D). In 7-Th1/Th2 int, leukocyte activation pathways, including *NKG2* and *PRF1*, were activated in PE (Figure 3D, Supplementary Figure 2E). In 5-Tm, GOs including Th17 differentiation pathway, inflammatory bowel disease, and allograft rejection, represented by *IL12RB1*, *IRF4*, *LAT*, and *IL22*, were observed in PE (Figure 3D, Supplementary Figure 2D, Supplementary Table 4). Previous studies have shown excessive Th17 cells in PE (26–29); however, in our study, the Th17 cluster did not show upregulated genes with PE. In addition, neither 5-Tm nor 11-Th17 cells showed clonal expansion in PE (Figure 1F), suggesting that a pro-inflammatory environment, and not antigen stimulation, may contribute to increased Th17 signatures in decidual $CD4^+$ T cells.

Interestingly, gene sets associated with Th1 and Th2 differentiation, including *GATA3* and *STAT1*, were upregulated in 12-*FOXP3*$^+$ Treg from the PE group (Figures 3D, E). *STAT1* overexpression (39) and *GATA3* overexpression in *FOXP3*$^+$ Tregs (50, 51) have been reported in autoimmune diseases. Furthermore, *PDCD1*, *CCR5*, and *TNFRSF4* were upregulated only in 12-*FOXP3*$^+$ Treg in the PE group (Figure 3E). Previous reports have shown that these genes are upregulated in exhausted Tregs with reduced suppressive function in autoimmune disease (39, 41). Another report showed that PD-1^{high} exhausted Tregs were significantly increased in the peripheral blood of patients with PE (52). Notably, the module score of Treg exhaustion-related genes (39, 41) was significantly higher in PE than in healthy late gestation (Figure 3F).

Taken together, 7-Th1/Th2 int and 5-Tm were activated and showed a proinflammatory signature, whereas 12-*FOXP3*$^+$ Treg showed an exhausted signature in PE.

3.6 *FOXP3*$^+$ Treg sub-clusters contain Naïve Treg, *FOXP3*$^+$ effector Treg-like, and *TIGIT* low Treg populations

Next, we investigated the *FOXP3*$^+$ Treg sub-cluster by further analyzing all 1354 cells from 11 samples (Figure 4A). Although *CD45RA* was not detected, five *FOXP3*$^+$ Treg sub-clusters were identified that showed similarity in marker gene expression with previously identified $FOXP3^{low}CD45RA^+$ naïve Treg, $FOXP3^{high}CD45RA^-$ effector Treg, and $FOXP3^{low}CD45RA^-$T cells (21–23, 38–40, 53). *CCR7*, *SELL*, and *TCF7* were highly expressed in Treg0 Naïve cluster, similar to naïve Tregs, as previously described. Treg1 *TIGIT* low cluster had low levels of *TIGIT* and *ICOS*, similar to $FOXP3^{low}CD45RA^-$ T cells. Treg4 effector like cluster had high levels of *ICOS*, *TIGIT*, *ENTPD1*, and *CTLA-4*, similar to effector Tregs (Figure 4B, Supplementary Figure 3A). The Treg4 effector like cluster expressed exhaustion-related genes such as *HAVCR2*, *LAG3*, and *PDCD1*; however, they also showed the highest expression of soluble suppressors such as *PRF1* and *LGALS1* (Figure 4B, Supplementary Figure 3A), supporting Treg4 effector like cluster as an effector and Treg-like properties. The effector Treg like subset, naïve Treg subset, and $FOXP3^{low}CD45RA^-$ T cell subset in decidua were previously reported by flowcytometry based studies (24, 25, 35). In addition, expanded clones were predominantly observed in Treg4 effector like cluster in the healthy late (36.4%) and PE (27.4%) (Figure 4C), consistent with our previous report (25). Treg2 and Treg3 had intermediate characteristics of Treg1 Naive and Treg4 effector like subsets in terms of Treg-specific marker genes; however, Treg3 showed higher expression of HLA-class II molecules than Treg2 (Figure 4B, Supplementary Figure 3A). Some scRNA-seq-based studies have identified naïve Treg-like subsets, effector Treg-like subsets, and various other undefined subsets (38–40, 53). The functions of these Treg subsets should be defined in future studies. The Treg0 Naïve cluster was abundant in PBMC, whereas Treg2, Treg3, and Treg4 effector like clusters were abundant in the decidua (Supplementary Figures 3B–G). Cells in the Treg 4 effector like subset were abundant during late gestation (Supplementary Figure 3G). Thus, decidual Tregs are heterogeneous, and changes in cluster frequencies depend on gestational age.

3.7 Treg4 effector like subcluster drives alternative Treg functional signature in PE

Next, we investigated whether a specific *FOXP3*$^+$ Treg cluster drove the Treg exhaustion signature in PE samples (Figure 3F) by performing DEG analysis of Tregs in late healthy and PE samples. The number of upregulated genes in PE was highest in the Treg4 effector like subset (Figure 4D), and DEG analysis of Treg4 effector like cells showed that Treg exhaustion-related genes (*PDCD1*,

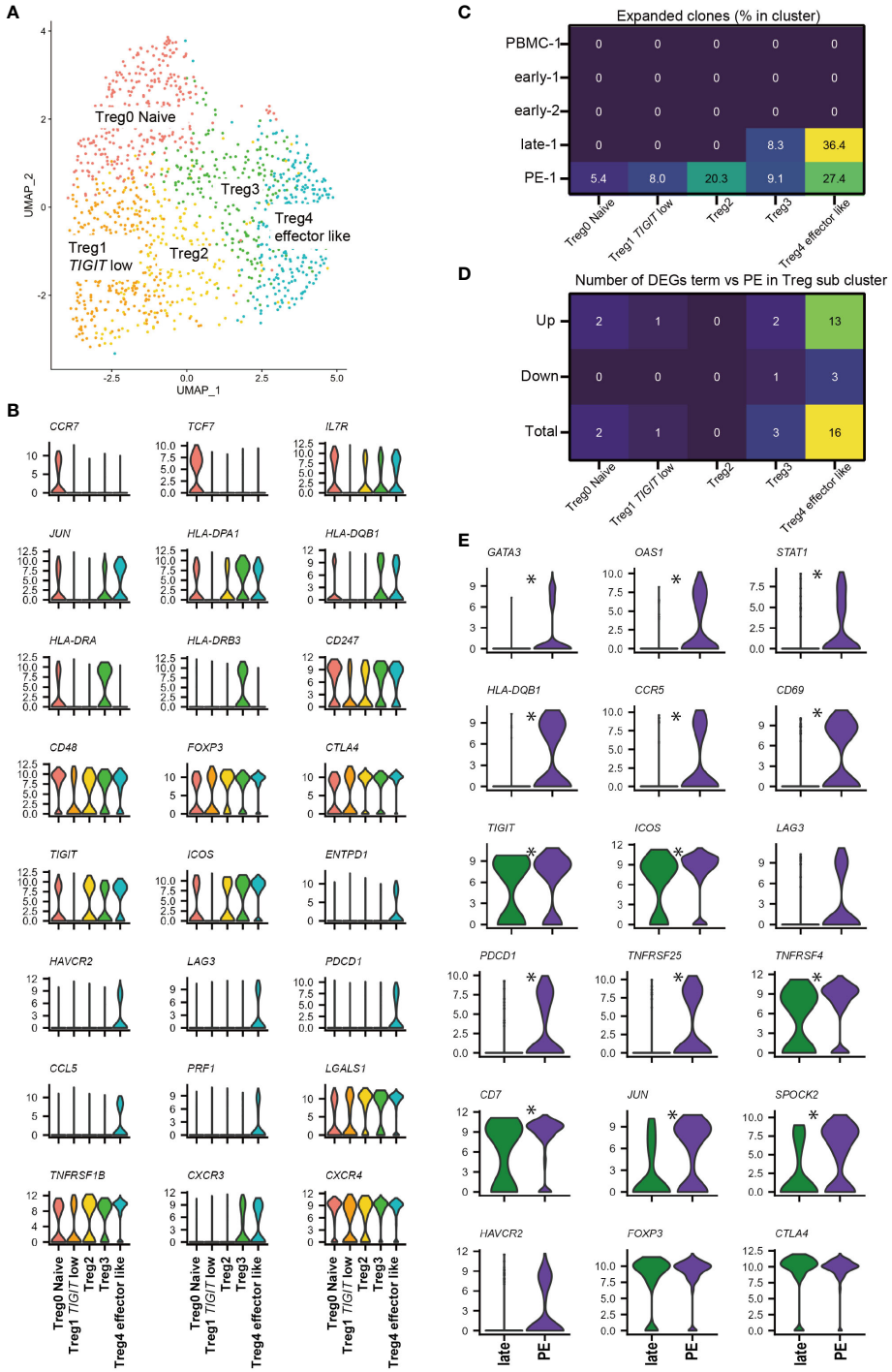


FIGURE 4
FOXP3$^+$ Treg sub-cluster analysis and alternative Treg functional signature in PE. **(A)** UMAP plot of 1354 cells from 11 samples showing Treg subsets. Five distinct sub-clusters were annotated based on unique gene-expression markers. **(B)** Violin plots representing the normalized expression level (y-axis) of key cluster-defining genes in the *FOXP3*$^+$ Treg sub-clusters. **(C)** Heatmap showing percentages of expanded clones (size = 2 or more cells) in each cluster. Columns indicate *FOXP3*$^+$ Treg sub-clusters. Rows indicate sample origins. **(D)** Heatmap showing the number of DEGs that are upregulated or downregulated in PE compared to healthy late gestation decidua in each Treg sub-cluster. DEG: adjusted p-value < 0.05, |log2 fold change| > 0.2. **(E)** Violin plots showing selected genes in Treg4 effector like cluster compared with healthy late and PE. * represents differentially expressed genes (adjusted p-value < 0.05, |log2 fold change| > 0.5) in the whole 12-*FOXP3*$^+$ Treg cluster or Treg4 effector like cluster subset.

TNFRSF25, *TNFRSF4*, *CCR5*, *LAG3*, and *HAVCR2*) (39, 41) were more highly expressed in PE than in late healthy pregnancy (Figure 4E). Increased expression of *STAT1* and *GATA3* in PE samples was also observed in Treg4 effector like cluster (Figure 4E). Based on the results of our TCR clonality and DEG analyses, the Treg4 effector like clusters may reflect antigen stimulation, driving the Treg exhaustion signature in PE.

4 Discussion

At the fetomaternal interface, a delicate balance between proinflammatory $CD8^+$ CTLs and $CD4^+$ T cells, which can respond to fetal antigens, and Tregs, which impart immune tolerance, is required. From the viewpoint of T cell immunity in PE, an imbalance between Th17 and Tregs (26–29), insufficient effector Treg expansion (25), Treg dysfunction (18), and insufficient suppression of CTLs (30) have been reported. In this study, we showed 1) the diversity of decidual $CD4^+$ T cell subsets and clonality dependence on gestational age, 2) altered gene expression in Th1/Th2 int, Treg, and memory $CD4^+$ T cells in PE, and 3) increased signatures of Treg exhaustion in PE compared to that in healthy late gestation pregnancies.

Decidual $CD4^+$ T cells are a heterogeneous population consisting of naïve, memory, effector (Th1/Th2 int, Th1, PRF^+ Tct, and Th17), $PDCD1^+FOXP3^-$ Treg, and $FOXP3^+$ Treg. All the clusters other than Th1/Th2 int were consistent with previously reported characteristics of CD4 T cell subclusters (33, 38, 39, 42). Newly identified Th1/Th2 int cluster expressed Th1 and Th2 signature genes simultaneously. Clonal expansion was mainly observed in Th1/Th2 int, Th1, PRF^+ Tct, $PDCD1^+FOXP3^-$ Treg, and Treg cells, suggesting that these T cell types are involved in antigen-specific responses. The fewer shared clones between Tregs and other $CD4^+$ T cell subsets suggest distinct clonal origins for these populations. By contrast, the majority of expanded clonotypes were unique to each Th1/Th2 int, Th1, PRF^+ Tct, $PDCD1^+FOXP3^-$ Treg, and Th17 subsets, some clones were shared between these clusters. This result supports the theory that effector T cell subsets are shaped by TCR sequences, and that each subset is a continuum phenotype (38, 47). We could not validate the Th1/Th2 int cluster by flowcytometry; however, this subset had a distinctive TCR repertoire, supporting that it has a unique phenotype that is different from that of other helper $CD4^+$ T cell subsets. $PDCD1^+FOXP3^-$ Tregs express exhaustion-associated molecules and *IFNG*. This unique signature is similar to that of $FOXP3^-$ $PD1^{HI}$ Treg as previously reported (42).

The Th1/Th2 int, $FOXP3^+$Treg, and Tm subsets had more DEGs than the other $CD4^+$ T cell types when healthy early- and late-gestation samples were compared. In the Th1/Th2 int subset, activation- and migration-related genes are downregulated in late gestation samples. Because the Th1/Th2 int subset had clonally expanded populations, it is reasonable to expect that this subset would be suppressed in late gestation. Although $FOXP3^+$ Tregs showed downregulation of genes related to T cell activation and migration, *CTLA-4* and *LGALS1* were upregulated in late gestation samples. In addition, clonal expansion of Tregs appeared late rather than early in gestation, which is consistent with results of our previous study (25). These results suggested that the $FOXP3^+$ Treg subset underwent antigen-responsive functional changes leading up to late gestation.

Interestingly, the Th1/Th2 int subset, which had downregulated activation-related genes in late gestation compared to early gestation, showed elevated expression of T cell activation-related genes in PE, whereas $FOXP3^+$ Tregs overexpressed the Treg exhaustion-related genes *STAT1* and *GATA3* in PE. Altered gene expression in $FOXP3^+$ Tregs from patients with PE was driven by a clonally expanded Treg4 effector like cluster. In peripheral blood from PE patients, $PD\text{-}1^{high}$exhausted Treg cells were increased (52). Furthermore, elevation of exhausted Tregs with impaired suppressive abilities have been reported in systemic lupus erythematosus (SLE), inflammatory bowel disease, and malignant tumors (39, 41). In SLE, elevated levels of *STAT1* and other exhaustion related molecules in Tregs are associated with disease severity (39, 54). In addition, a relationship between excessive type 1 interferon (IFN) signaling and Treg exhaustion has been suggested in SLE (39). Our data did not show elevated excessive type 1 IFN signaling pathway in Tregs of PE; however, overexpression of *STAT1* and Treg exhaustion-related genes in PE was consistent with that in SLE. *GATA3*-overexpressing Tregs have been observed in inflamed intestines, which contribute to the accumulation of Tregs in inflamed tissues (50, 51). The DEGs of Tregs in PE overlapped with those in SLE even though samples from patients with autoimmune diseases were excluded in this study. Pregnancies complicated with SLE are at a high risk of developing PE (55–59).Vice versa, a differential DNA accessibility analysis of $CD4^+$ T cells from patients with SLE included eclampsia as a disease-related ontology (39), suggesting common underlying $CD4^+$ T cell abnormalities in SLE and PE.

A major limitation of this study was its small sample size. In addition, the newly identified subset has not been validated by flow cytometry since there are some molecules with mismatched protein expression and gene expression patterns. A validation cohort study using paired scRNAseq and flowcytometry is desirable. In addition, functional analysis of Tregs in PE will provide further insights into the mechanisms underlying Treg exhaustion.

In summary, we identified clonally expanded Th1/Th2 int and $FOXP3^+$ effector Treg subsets with altered PE gene expression. The Th1/Th2 int subset is activated, whereas the Treg subset is exhausted in PE. Further studies characterizing functionally altered $CD4^+$ T-cell subsets and Tregs will accelerate the development of immunological treatments for PE.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The DNA Data Bank of Japan (DDBJ)/PRJDB17285.

Ethics statement

The studies involving humans were approved by University of Toyama Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

ST: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal Analysis, Conceptualization. SSh: Writing – review & editing, Investigation, Formal Analysis. TT: Writing – review & editing. TS: Writing – review & editing, Funding acquisition. KM: Writing – review & editing, Resources, Investigation. AY-U: Writing – review & editing, Investigation. KR: Writing – review & editing. MT: Writing – review & editing. AS: Writing – review & editing, Resources. SSa: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. AN: Writing – review & editing, Supervision, Methodology, Funding acquisition.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by JSPS KAKENHI Grant Numbers 17K11221 (TS), 19K18690 (ST), 21K16764 (ST), 22KK0287(ST), 19K09750(AN), and 22H03223 (AN), AMED under Grant Number JP18gk0110018 (SSa), 42th Japan Medical Women's Association research grant (ST).

Acknowledgments

We would like to thank ImmunoGeneTeqs, Inc. (Chiba, Japan) for scTCR/targeted RNA sequence library construction, sequencing, and Fastq data preprocessing. We would like to thank Editage (www.editage.com) for the English language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1401738/full#supplementary-material

References

1. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol*. (2004) 5:266–71. doi: 10.1038/ni1037

2. Gomez-Lopez N, Arenas-Hernandez M, Romero R, Miller D, Garcia-Flores V, Leng Y, et al. Regulatory T cells play a role in a subset of idiopathic preterm labor/birth and adverse neonatal outcomes. *Cell Rep*. (2020) 32:107874. doi: 10.1016/j.celrep.2020.107874

3. Kahn DA, Baltimore D. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. *Proc Natl Acad Sci USA*. (2010) 107:9299–304. doi: 10.1073/pnas.1003909107

4. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature*. (2012) 490:102–6. doi: 10.1038/nature11462

5. Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, et al. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. *J Reprod Immunol*. (2010) 85:121–9. doi: 10.1016/j.jri.2010.02.006

6. Sasaki Y. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Mol Hum Reprod*. (2004) 10:347–53. doi: 10.1093/molehr/gah044

7. Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-v.d.Voort-Maarschalk M, Roelen DL, et al. Fetal–maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J Reprod Immunol*. (2009) 82:148–57. doi: 10.1016/j.jri.2009.05.003

8. Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, et al. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. *Pregnancy Hypertension*. (2018) 13:291–310. doi: 10.1016/j.preghy.2018.05.004

9. Robillard P-Y, Dekker G, Scioscia M, Saito S. Progress in the understanding of the pathophysiology of immunologic maladaptation related to early-onset preeclampsia and metabolic syndrome related to late-onset preeclampsia. *Am J Obstet Gynecol*. (2022) 226:S867–75. doi: 10.1016/j.ajog.2021.11.019

10. Saito S, Tsuda S, Nakashima A. T cell immunity and the etiology and pathogenesis of preeclampsia. *J Reprod Immunol*. (2023) 159:104125. doi: 10.1016/j.jri.2023.104125

11. Klonoff-Cohen HS, Savitz DA, Cefalo RC, McCann MF. An epidemiologic study of contraception and preeclampsia. *JAMA*. (1989) 262:3143–7. doi: 10.1001/jama.262.22.3143

12. Koelman CA, Coumans ABC, Nijman HW, Doxiadis IIN, Dekker GA, Claas FHJ. Correlation between oral sex and a low incidence of preeclampsia: a role for soluble HLA in seminal fluid? *J Reprod Immunol*. (2000) 46:155–66. doi: 10.1016/S0165-0378(99)00062-5

13. Robillard P-Y, Hulsey TC, Alexander GR, Keenan A, de Caunes F, Papiernik E. Paternity patterns and risk of preeclampsia in the last pregnancy in multiparae. *J Reprod Immunol*. (1993) 24:1–12. doi: 10.1016/0165-0378(93)90032-D

14. Salha O, Sharma V, Dada T, Nugent D, Rutherford AJ, Tomlinson AJ, et al. The influence of donated gametes on the incidence of hypertensive disorders of pregnancy. *Hum Reprod*. (1999) 14:2268–73. doi: 10.1093/humrep/14.9.2268

15. Skj R. The interval between pregnancies and the risk of preeclampsia. *New Engl J Med*. (2002) 356:33–8. doi: 10.1056/NEJMoa011379

16. Darmochwal-Kolarz D, Kludka-Sternik M, Tabarkiewicz J, Kolarz B, Rolinski J, Leszczynska-Gorzelak B, et al. The predominance of Th17 lymphocytes and decreased number and function of Treg cells in preeclampsia. *J Reprod Immunol*. (2012) 93:75–81. doi: 10.1016/j.jri.2012.01.006

17. Hsu P, Santner-Nanan B, Dahlstrom JE, Fadia M, Chandra A, Peek M, et al. Altered decidual DC-SIGN+ Antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. *Am J Pathol*. (2012) 181:2149–60. doi: 10.1016/j.ajpath.2012.08.032

18. Nguyen TA, Kahn DA, Loewendorf AI. Maternal—Fetal rejection reactions are unconstrained in preeclamptic women. *PloS One*. (2017) 12:e0188250. doi: 10.1371/journal.pone.0188250

19. Sasaki Y, Darmochwal-Kolarz D, Suzuki D, Sakai M, Ito M, Shima T, et al. Proportion of peripheral blood and decidual CD4+ CD25bright regulatory T cells in pre-eclampsia. *Clin Exp Immunol*. (2007) 149:139–45. doi: 10.1111/j.1365-2249.2007.03397.x

20. Toldi G, Švec P, Vásárhelyi B, Mészáros G, Rigó J, Tulassay T, et al. Decreased number of FoxP3+ regulatory T cells in preeclampsia. *Acta Obstet Gynecol Scand*. (2008) 87:1229–33. doi: 10.1080/00016340802389470

21. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the foxP3 transcription factor. *Immunity*. (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019

22. Cuadrado E, Van Den Biggelaar M, De Kivit S, Chen Y, Slot M, Doubal I, et al. Proteomic analyses of human regulatory T cells reveal adaptations in signaling pathways that protect cellular identity. *Immunity*. (2018) 48:1046–1059.e6. doi: 10.1016/j.immuni.2018.04.008

23. Miyara M, Chader D, Sage E, Sugiyama D, Nishikawa H, Bouvry D, et al. Sialyl Lewis x (CD15s) identifies highly differentiated and most suppressive FOXP3 ^{high} regulatory T cells in humans. *Proc Natl Acad Sci USA*. (2015) 112:7225–30. doi: 10.1073/pnas.1508224112

24. Inada K, Shima T, Ito M, Ushijima A, Saito S. Helios-positive functional regulatory T cells are decreased in decidua of miscarriage cases with normal fetal chromosomal content. *J Reprod Immunol*. (2015) 107:10–9. doi: 10.1016/j.jri.2014.09.053

25. Tsuda S, Zhang X, Hamana H, Shima T, Ushijima A, Tsuda K, et al. Clonally expanded decidual effector regulatory T cells increase in late gestation of normal pregnancy, but not in preeclampsia, in humans. *Front Immunol*. (2018) 9:1934. doi: 10.3389/fimmu.2018.01934

26. Cornelius DC, Amaral LM, Wallace K, Campbell N, Thomas AJ, Scott J, et al. Reduced uterine perfusion pressure T-helper 17 cells cause pathophysiology associated with preeclampsia during pregnancy. *Am J Physiology-Regulatory Integr Comp Physiol*. (2016) 311:R1192–9. doi: 10.1152/ajpregu.00117.2016

27. Cornelius DC, Amaral LM, Harmon A, Wallace K, Thomas AJ, Campbell N, et al. An increased population of regulatory T cells improves the pathophysiology of placental ischemia in a rat model of preeclampsia. *Am J Physiology-Regulatory Integr Comp Physiol*. (2015) 309:R884–91. doi: 10.1152/ajpregu.00154.2015

28. Pourakbari R, Parhizkar F, Soltani-Zangbar MS, Samadi P, Zamani M, Aghebati-Maleki L, et al. Preeclampsia-derived exosomes imbalance the activity of th17 and treg in PBMCs from healthy pregnant women. *Reprod Sci*. (2022) 30(4):1186–97. doi: 10.1007/s43032-022-01059-x

29. Santner-Nanan B, Peek MJ, Khanam R, Richarts L, Zhu E, Fazekas de St Groth B, et al. Systemic increase in the ratio between foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. *J Immunol*. (2009) 183:7023–30. doi: 10.4049/jimmunol.0901154

30. Morita K, Tsuda S, Kobayashi E, Hamana H, Tsuda K, Shima T, et al. Analysis of TCR repertoire and PD-1 expression in decidual and peripheral CD8+ T cells reveals distinct immune mechanisms in miscarriage and preeclampsia. *Front Immunol*. (2020) 11:1082. doi: 10.3389/fimmu.2020.01082

31. Arutyunyan A, Roberts K, Troulé K, Wong FCK, Sheridan MA, Kats I, et al. Spatial multiomics map of trophoblast development in early pregnancy. *Nature*. (2023) 616:143–51. doi: 10.1038/s41586-023-05869-0

32. Pique-Regi R, Romero R, Tarca AL, Sendler ED, Xu Y, Garcia-Flores V, et al. Single cell transcriptional signatures of the human placenta in term and preterm parturition. *eLife*. (2019) 8:e52004. doi: 10.7554/eLife.52004

33. Sureshchandra S, Doratt BM, True H, Mendoza N, Rincon M, Marshall NE, et al. Multimodal profiling of term human decidua demonstrates immune adaptations with pregravid obesity. *Cell Rep*. (2023) 42:112769. doi: 10.1016/j.celrep.2023.112769

34. Sureshchandra S, Zulu MZ, Doratt BM, Jankeel A, Tifrea D, Edwards R, et al. Single-cell RNA sequencing reveals immunological rewiring at the maternal-fetal interface following asymptomatic/mild SARS-CoV-2 infection. *Cell Rep*. (2022) 39:110938. doi: 10.1016/j.celrep.2022.110938

35. van der Zwan A, van Unen V, Beyrend G, Laban S, van der Keur C, Kapsenberg HJM, et al. Visualizing dynamic changes at the maternal-fetal interface throughout human pregnancy by mass cytometry. *Front Immunol*. (2020) 11:571300. doi: 10.3389/fimmu.2020.571300

36. Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, et al. Single-cell reconstruction of the early maternal–fetal interface in humans. *Nature*. (2018) 563:347–53. doi: 10.1038/s41586-018-0698-6

37. Yang J, Gong L, Liu Q, Zhao H, Wang Z, Li X, et al. Single-cell RNA-seq reveals developmental deficiencies in both the placentation and the decidualization in women with late-onset preeclampsia. *Front Immunol*. (2023) 14:1142273. doi: 10.3389/fimmu.2023.1142273

38. Azizi E, Carr AJ, Plitas G, Cornish AE, Konopacki C, Prabhakaran S, et al. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell*. (2018) 174:1293–1308.e36. doi: 10.1016/j.cell.2018.05.060

39. Guo C, Liu Q, Zong D, Zhang W, Zuo Z, Yu Q, et al. Single-cell transcriptome profiling and chromatin accessibility reveal an exhausted regulatory CD4+ T cell subset in systemic lupus erythematosus. *Cell Rep*. (2022) 41:111606. doi: 10.1016/j.celrep.2022.111606

40. Zemmour D, Zilionis R, Kiner E, Klein AM, Mathis D, Benoist C. Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nat Immunol*. (2018) 19:291–301. doi: 10.1038/s41590-018-0051-0

41. Lowther DE, Goods BA, Lucca LE, Lerner BA, Raddassi K, Van Dijk D, et al. PD-1 marks dysfunctional regulatory T cells in Malignant gliomas. *JCI Insight*. (2016) 1:e85935. doi: 10.1172/jci.insight.85935

42. Salvany-Celades M, van der Zwan A, Benner M, Setrajcic-Dragos V, Bougleux Gomes HA, Iyer V, et al. Three types of functional regulatory T cells control T cell responses at the human maternal-fetal interface. *Cell Rep*. (2019) 27:2537–2547.e5. doi: 10.1016/j.celrep.2019.04.109

43. Saito S, Nishikawa K, Morii T, Narita N, Enomoto M. Expression of activation antigens CD69, HLA-DR, interleukin-2 receptor-alpha (IL-2Ra) and IL-2Rfi on T cells of human decidua at an early stage of pregnancy. *Immunology*. (1992) 75(4):710–2

44. Green S, Politis M, Rallis KS, Saenz De Villaverde Cortabarria A, Efthymiou A, Mureanu N, et al. Regulatory T cells in pregnancy adverse outcomes: A systematic review and meta-analysis. *Front Immunol*. (2021) 12:737862. doi: 10.3389/fimmu.2021.737862

45. Huang H, Wang C, Rubelt F, Scriba TJ, Davis MM. Analyzing the Mycobacterium tuberculosis immune response by T-cell receptor clustering with GLIPH2 and genome-wide antigen screening. *Nat Biotechnol*. (2020) 38:1194–202. doi: 10.1038/s41587-020-0505-4

46. Li H, van der Leun AM, Yofe I, Lubling Y, Gelbard-Solodkin D, van Akkooi ACJ, et al. Dysfunctional CD8 T cells form a proliferative, dynamically regulated compartment within human melanoma. *Cell*. (2019) 176:775–789.e18. doi: 10.1016/j.cell.2018.11.043

47. Schattgen SA, Guion K, Crawford JC, Souquette A, Barrio AM, Stubbington MJT, et al. Integrating T cell receptor sequences and transcriptional profiles by clonotype neighbor graph analysis (CoNGA). *Nat Biotechnol*. (2022) 40:54–63. doi: 10.1038/s41587-021-00989-2

48. Chasman DA, Welch Schwartz R, Vazquez J, Chavarria M, Jenkins ET, Lopez GE, et al. Proteogenomic and V(D)J analysis of human decidual T cells highlights unique transcriptional programming and clonal distribution. *J Immunol*. (2023) 211:154–62. doi: 10.4049/jimmunol.2200061

49. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun*. (2019) 10:1523. doi: 10.1038/s41467-019-09234-6

50. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol*. (2016) 16:295–309. doi: 10.1038/nri.2016.36

51. Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, et al. GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. *J Clin Invest*. (2011) 121:4503–15. doi: 10.1172/JCI57456

52. Toldi G, Vásárhelyi ZE, Rigó J, Orbán C, Tamássy Z, Bajnok A, et al. Prevalence of regulatory T-cell subtypes in preeclampsia. *Am J Reprod Immunol*. (2015) 74:110–5. doi: 10.1111/aji.12380

53. Luo Y, Xu C, Wang B, Niu Q, Su X, Bai Y, et al. Single-cell transcriptomic analysis reveals disparate effector differentiation pathways in human Treg compartment. *Nat Commun*. (2021) 12:3913. doi: 10.1038/s41467-021-24213-6

54. Goropevšek A, Holcar M, Avčin T. The role of STAT signaling pathways in the pathogenesis of systemic lupus erythematosus. *Clinic Rev Allerg Immunol*. (2017) 52:164–81. doi: 10.1007/s12016-016-8550-y

55. Bramham K, Hunt BJ, Bewley S, Germain S, Calatayud I, Khamashta MA, et al. Pregnancy outcomes in systemic lupus erythematosus with and without previous nephritis. *J Rheumatol*. (2011) 38:1906–13. doi: 10.3899/jrheum.100997

56. Clowse MEB, Jamison M, Myers E, James AH. A national study of the complications of lupus in pregnancy. *Am J Obstet Gynecol*. (2008) 199:127.e1–6. doi: 10.1016/j.ajog.2008.03.012

57. Gladman DD, Tandon A, Ibañez D, Urowitz MB. The effect of lupus nephritis on pregnancy outcome and fetal and maternal complications. *J Rheumatol*. (2010) 37:754–8. doi: 10.3899/jrheum.090872

58. Moroni G, Doria A, Giglio E, Imbasciati E, Tani C, Zen M, et al. Maternal outcome in pregnant women with lupus nephritis. A prospective multicenter study. *J Autoimmun*. (2016) 74:194–200. doi: 10.1016/j.jaut.2016.06.012

59. Tsuda S, Sameshima A, Sekine M, Kawaguchi H, Fujita D, Makino S, et al. Pre-conception status, obstetric outcome and use of medications during pregnancy of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) in Japan: Multi-center retrospective descriptive study. *Modern Rheumatol*. (2020) 30:852–61. doi: 10.1080/14397595.2019.1661592

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 23 May 2024
DOI 10.3389/fimmu.2024.1409378

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Monica Cappelletti,
University of California, Los Angeles, United States
Meredith Anne Kelleher,
Oregon Health & Science University, United States

*CORRESPONDENCE
Alison J. Eastman
alison.eastman@vumc.org

RECEIVED 29 March 2024
ACCEPTED 10 May 2024
PUBLISHED 23 May 2024

CITATION
Gaddy JA, Moore RE, Lochner JS, Rogers LM, Noble KN, Giri A, Aronoff DM, Cliffel D and Eastman AJ (2024) Palmitate and group B *Streptococcus* synergistically and differentially induce IL-1β from human gestational membranes. *Front. Immunol.* 15:1409378. doi: 10.3389/fimmu.2024.1409378

COPYRIGHT
© 2024 Gaddy, Moore, Lochner, Rogers, Noble, Giri, Aronoff, Cliffel and Eastman. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Palmitate and group B *Streptococcus* synergistically and differentially induce IL-1β from human gestational membranes

Jennifer A. Gaddy[1,2], Rebecca E. Moore[1,3], Jonathan S. Lochner[4,5], Lisa M. Rogers[6], Kristen N. Noble[5], Ayush Giri[1,7], David M. Aronoff[6], David Cliffel[8] and Alison J. Eastman[7]*

[1]Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, United States, [2]Tennessee Valley Healthcare Systems, Department of Veterans Affairs, Nashville, TN, United States, [3]Publications Division, American Chemical Society, Washington, DC, United States, [4]Department of Microbiology, Immunology and Tropical Medicine, George Washington University, Washington, DC, United States, [5]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, United States, [6]Department Internal Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [7]Department of Obstetrics and Gynecology, Vanderbilt University Medical Center, Nashville, TN, United States, [8]Department of Chemistry, Vanderbilt University, Nashville, TN, United States

Introduction: Rupture of the gestational membranes often precedes major pregnancy complications, including preterm labor and preterm birth. One major cause of inflammation in the gestational membranes, chorioamnionitis (CAM) is often a result of bacterial infection. The commensal bacterium *Streptococcus agalactiae*, or Group B *Streptococcus* (GBS) is a leading infectious cause of CAM. Obesity is on the rise worldwide and roughly 1 in 4 pregnancy complications is related to obesity, and individuals with obesity are also more likely to be colonized by GBS. The gestational membranes are comprised of several distinct cell layers which are, from outermost to innermost: maternally-derived decidual stromal cells (DSCs), fetal cytotrophoblasts (CTBs), fetal mesenchymal cells, and fetal amnion epithelial cells (AECs). In addition, the gestational membranes have several immune cell populations; macrophages are the most common phagocyte. Here we characterize the effects of palmitate, the most common long-chain saturated fatty acid, on the inflammatory response of each layer of the gestational membranes when infected with GBS, using human cell lines and primary human tissue.

Results: Palmitate itself slightly but significantly augments GBS proliferation. Palmitate and GBS co-stimulation synergized to induce many inflammatory proteins and cytokines, particularly IL-1β and matrix metalloproteinase 9 from DSCs, CTBs, and macrophages, but not from AECs. Many of these findings are recapitulated when treating cells with palmitate and a TLR2 or TLR4 agonist, suggesting broad applicability of palmitate-pathogen synergy. Co-culture of macrophages with DSCs or CTBs, upon co-stimulation with GBS and palmitate, resulted in increased inflammatory responses, contrary to previous work in the absence of palmitate. In whole gestational membrane biopsies, the amnion layer appeared to dampen immune responses from the DSC and CTB layers (the choriodecidua) to GBS and palmitate co-stimulation. Addition of the

monounsaturated fatty acid oleate, the most abundant monounsaturated fatty acid in circulation, dampened the proinflammatory effect of palmitate.

Discussion: These studies reveal a complex interplay between the immunological response of the distinct layers of the gestational membrane to GBS infection and that such responses can be altered by exposure to long-chain saturated fatty acids. These data provide insight into how metabolic syndromes such as obesity might contribute to an increased risk for GBS disease during pregnancy.

KEYWORDS

pregnancy, gestational membranes, group B *Streptococcus*, obesity, palmitate, preterm prelabor rupture of membranes, preterm birth, inflammation

Introduction

The onset of labor and/or childbirth prior to term is often preceded by the rupture of extraplacental, gestational ("fetal") membranes (1–3). Preterm premature rupture of membranes (PPROM) is commonly associated with histological inflammation, a condition termed chorioamnionitis (CAM). Acute, neutrophilic CAM can be caused by infectious or non-infectious stimuli, but a significant infectious cause of CAM is the bacterium Group B *Streptococcus* (GBS) (4–9). Further consequences of GBS-associated CAM include maternal and fetal sepsis and stillbirth (10–16).

The gestational membranes differentiate along with the trophectoderm during early human pregnancy. Both these tissues contain multiple cell types derived from both the fetus and the mother. The placenta is largely made up of cytotrophoblasts (CTBs), a fetal cell type which differentiates into several distinct forms. The gestational membranes are comprised of an outermost layer, the decidua, dominated by maternal decidual stromal cells (DSCs); a central chorion layer of fetal CTBs abutting the decidua; an inner amnion comprised of fetal mesenchymal cells, and a single layer of fetal amnion epithelial cells (AECs) lining the innermost (fetal-facing) aspect of the membranes. The amnion has ample extracellular matrix and basement membrane for structural stability. Macrophages are the principle resident phagocyte within uninflamed gestational membranes and there are cells of the adaptive immune system there as well (17). The precise cause of membrane rupture during CAM remains to be determined, but it likely involves matrix metalloproteinases (MMPs) produced by structural and immune cells of the membranes, and possibly the bacteria themselves (18–20). Additionally, several pro-inflammatory cytokines such as IL-6, IL-1β, IL-8, and TNF-α have been associated with CAM and preterm birth (21, 22).

The interplay of saturated fatty acids, pregnancy, obesity, and inflammation is complex. Saturated fatty acids are associated with inflammation [reviewed in (23)]. In normal weight individuals consuming a western diet, the monounsaturated fatty acid oleate and the LCSFA palmitate comprise (on average) 31% and 27% of fatty acids in the blood stream, respectively. The composition of these fatty acids are changed during obesity, with palmitate being the most abundant fatty acid in circulation at 33%, representing approximately a 20% increase than in individuals of normal-weight (24, 25). Thus, palmitate and oleate are often used to model the fatty acid component of obesity *in vitro*. Furthermore, palmitate specifically has been associated with inflammation, adverse pregnancy outcomes, and in several other pregnancy-associated conditions, including gestational diabetes mellitus and preeclampsia (26–30). Pregnancy progressively increases the circulating concentrations of fatty acids to increase energy availability, among other effects (31). While we would expect the serum levels of palmitate to increase during pregnancies complicated with obesity similarly to non-pregnant conditions, the concentration of serum palmitate in obese and normal weight pregnancies is also impacted by fetal sex (32). For instance, the fatty acid profiles of maternal plasma in obese pregnant persons differs from normal weight controls in distinct ways depending on fetal sex: obese pregnancies with female fetuses have higher maternal plasma oleate levels relative to controls, while obese pregnancies with male fetuses have lower levels of oleate than controls (32). There are also differences in unsaturated fatty acids between male and female placentas (33).

An estimated 1 in 4 pregnancy complications can be tied to obesity (34, 35), and this number is expected to rise as obesity rates increase. During non-pregnant settings, individuals with obesity are at an increased risk of rectovaginal colonization with GBS (36–38) and thus at greater risk of GBS-related pregnancy complications including CAM, preterm prelabor rupture of membranes, and fetal sepsis (39, 40). The precise cause of greater GBS colonization in obese pregnant people is unknown; however, the contribution of LCSFAs to inflammation has been well established (41).

In this study, we sought to determine the impact of the LCSFA palmitate on GBS-induced inflammation in different compartments of the gestational membranes. We were particularly interested in the potential for synergy between GBS and palmitate in the

induction of proinflammatory cytokines, chemokines, and MMP production throughout the gestational membranes, since these peptides play significant roles in regulating the cellular inflammatory response to infection and the integrity of the extracellular matrix. We found that the DSC and CTB cell types displayed synergy in IL-1β secretion when treated with GBS and palmitate together. In contrast to previous work in the absence of palmitate that showed suppression of macrophage activation by both DSCs and CTBs (42), neither cell type inhibited activation of primary placental macrophages or macrophages of the cell line THP-1 in response to infection when palmitate was present. The AEC layer was largely nonresponsive to GBS and palmitate co-incubation and may actively inhibit the inflammation in the choriodecidua when the membranes are intact. The differences of each structural cell type of the gestational membrane in response to GBS and palmitate suggest complex regulation and that paracrine signaling between layers of the gestational membrane will be necessary.

Materials and methods

Cell lines and reagents

These studies used a telomerase-immortalized human endometrial stromal cell (THESC) uterine stromal cell line (obtained from ATCC, Manassas, VA, USA) differentiated into decidual stromal cells (DSC), as described below, the Jeg3 cytotrophoblast cell line (CTB, a kind gift from Dr. Carolyn Coyne), and the Tohoku Hospital Pediatrics-1 (THP-1) human monocytic leukemia-derived cell line (ATCC). DSCs were cultured in DMEM/F12 without phenol red (Invitrogen, Carlsbad, CA, USA) supplemented with 10% charcoal-stripped fetal bovine serum (csFBS, HyClone, Logan, UT, USA) and 1% antibiotic antimycotic solution (A/A) (Gibco, Waltham, Massachusetts, USA). We induced decidualization in the THESC line over 7 days with 0.5 mM 8-Br-cAMP,10 nM estradiol, and 1μM medroxyprogesterone acetate (Sigma-Aldrich, St. Louis, MS, USA) administered every 2 days to generate DSCs (43, 44). Induction of prolactin and IGFBP1 secretion, indicative of decidualization, was confirmed by ELISA (Alpha diagnostic international, San Antonio, TX, USA). CTBs were cultured in MEM without phenol red (Invitrogen) supplemented with 10% csFBS and 1% A/A. THP-1 cells were cultured in RPMI 1640 (Invitrogen) with 10% csFBS and 1% A/A. THP-1 cells were differentiated to adherent macrophage-like cells using phorbol myristate acetate (PMA, 5 ng/mL; Sigma, St. Louis, MO, USA) overnight, washing off nonadherent cells, and removing macrophages by incubating adherent cells using Cell Dissociation Buffer Enzyme-Free PBS-based (Gibco) for 5 minutes at 37°C and subsequent cell scraping.

Palmitate and oleate were obtained from Nu-Chek Prep (Elysian, MN, USA), dissolved in ethanol as in (45) and used at a final concentration of 0.4 mM without an added carrier. This concentration was selected based on circulating levels of palmitate in obese and/or diabetic individuals (39, 40) and to align with the palmitate doses employed in previous *in vitro* palmitate studies from a variety of groups (45–47). TLR4 agonist lipopolysaccharide (LPS, ultrapure from *E. coli* K12 strain) and TLR2 agonist PAM3CSK4 (PAM) were obtained from *In vivo*Gen (San Diego, CA, USA) and used at a final concentration of 1 μg/mL. The clinical GBS isolate GB00112 (GB112) was obtained from Dr. Shannon Manning (Michigan State University, East Lansing, MI, USA) (48). GBS was cultured in Todd-Hewitt broth (THB) overnight at 37°C, then pelleted and resuspended in PBS. GBS was used at a multiplicity of infection (MOI) of 10 for DSC, CTB, and AEC cells, and at an MOI of 100 for THP-1 cells. For experiments using whole or separated (amnion physically separated from choriodecidua) human gestational membrane punches, 1 x 10^7 CFU of GBS was added to wells and thoroughly mixed.

Human primary cell isolations

Human placental CTB isolation

These studies were conducted in accordance with the Vanderbilt University Institutional Review Board (IRB#181998) and Declaration of Helsinki. Pregnant people (aged 21-40 years) meeting our recruitment criteria receiving elective (scheduled) non-laboring but full-term Cesarean sections at Vanderbilt University Medical Center were approached and consented for donation of their placenta and gestational membranes. Exclusion criteria were: clinical evidence of CAM, immunocompromised conditions, collagen vascular disease, multi-fetal pregnancy, patients younger than 21 years of age or older than 40, evidence of bacterial vaginosis, cervical cerclage, third trimester bleeding, preeclampsia, diabetes (gestational or preexisting), SARS-CoV2 infection, or major medical conditions (chronic renal disease, sarcoidosis, hepatitis, HIV, etc). Demographics and known variables of patients are included in Supplementary Table 1.

Human placental macrophage isolation

Human placental macrophages from the villous core were isolated as previously described (49). Briefly, placental cotyledon tissue, separated from the maternal decidua basalis, was vigorously washed with PBS to remove circulating blood. The tissue was minced into small pieces and weighed to determine final grams collected. Tissue fragments were placed into 250 mL sterile bottles with sterile digestion solution containing 150 μg/mL deoxyribonuclease, 1 mg/mL collagenase, and 1 mg/mL hyaluronidase at 10 mL per gram of tissue. Placenta was digested for 1 h at 37°C, shaking at 180 RPM. Digested tissue was filtered through a 280 μm metal sieve, followed by 180 and 80 μm nylon screens. Cells were centrifuged at 1500 RPM and resuspended in 25% Percoll diluted in cold RPMI media containing 10% FBS and 1% A/A (referred hereafter as RPMI +/+) and overlaid onto 50% Percoll, plus 2 mL of PBS on top of the density gradient. CD14+ macrophages were isolated by positive selection after red blood cell (RBC) lysis (solution from Invitrogen) using the magnetic MACS® large cell separation column system according to the manufacturer's instructions.

Isolated CD14+ placental macrophages were plated and rested overnight in RPMI+/+ at 37 °C with 5% CO2 before experimentation. Purity of placental macrophage preparations was determined to be 95.89 +/- 0.6986% based on CD68 positivity.

Human gestational membrane amnion epithelial cell isolation

Gestational membrane was physically separated into amnion and choriodecidua by peeling the layers from each other. Choriodecidua was discarded and amnion was cut into approximately 2-inch squares and added to a beaker containing 100mL warmed 0.25% Trypsin. Amnion was incubated in a shaking incubator at 200RPM at 37°C for 60 minutes. Amnion-trypsin solution was strained through a metal screen, flow-through containing AECs saved, amnion squares returned to beaker, and another 100 mL 0.25% trypsin was added. The beaker with amnion and trypsin was returned to shaking incubator for 30 minutes. Flow-through containing AECs was strained through 70 µm strainer and divided into 50-mL conical tubes resuspended in complete media. After 30 minute incubation, amnion-trypsin solution was filtered, newborn calf serum added to neutralize trypsin, flow-through was further strained through 70 µm screens, and spun to pellet AECs. Pellets were pooled, washed in PBS, spun down again to pellet, and subjected to red blood cell lysis as per manufacturer's protocol. Cells were pelleted after RBC lysis step, resuspended in complete media, counted, and plated in 24-well dishes at a concentration of 1 x 10^6/mL, 1 mL per well. Wells were washed on subsequent days and cells monitored until they formed a connected, confluent epithelial cell monolayer.

Human gestational membrane punch biopsy preparation

Human gestational membranes were washed in warm, sterile PBS to remove blood and clots. Membranes were spread over a sterile silicone pad and 12 mm punch biopsies were obtained. For experiments using separated amnion and choriodecidua, amnion was physically pulled from choriodecidua, then amnion and choriodecidual membrane layers were spread individually over the silicone pad and 12 mm biopsy punches made. Tissue punches were added to wells of 24-well plates with 1 mL complete DMEM/F12 media and rested overnight before infection.

Fatty acid treatment and infection

Media was aspirated from wells, cells or tissue rinsed with sterile PBS, and then palmitate, oleate, vehicle control (ethanol), and/or GBS was added to selected wells at the same time in antibiotic-free media (RPMI 1640 or DMEM/F12, depending on cell type) with 10% csFBS. Cultures sat in antibiotic-free media with or without GBS and fatty acids for 1 hour. Antibiotic/antimycotic was added to wells to a concentration of 1x (from 100X stock), and then cultures were incubated for an additional 24 hours. Media was then harvested for ELISA.

Enzyme-linked immunosorbent assay

ELISA analysis was performed on supernatants from cultures as per manufacturer's protocol for each analyte (IL-1β, IL-8, TNFα, CCL2, CCL5, G-CSF, GM-CSF, and MMP9) (R&D systems, Minneapolis, MN, USA).

Statistical analysis

All experiments were performed in multiple, independent biological replicates as noted in each figure. Data points from independent experiments are presented along with means and analyzed with GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Data were analyzed by 2-way ANOVA with multiple comparisons. Differences were considered statistically significant for $p < 0.05$.

Results

Palmitate-supplemented media promotes GBS proliferation

Prior to beginning experiments, we assessed whether palmitate affected GBS growth. After 24 hours, THB media supplemented with palmitate resulted in greater proliferation of GBS (Figure 1A) as estimated by spectrophotometry (OD_{600}). To control for the potential GBS dosing differences between palmitate and non-palmitate groups, we assessed cytokine production at multiplicity of infections (MOIs) of 1, 10, and 100. Our standard MOI is 10, thus the addition of palmitate has the potential to increase this MOI. When we assayed supernatants of DSCs, CTBs, and THP-1 macrophages infected with different MOIs of GBS, we found no differences between the MOIs of 10 and 100 (Figures 1B–D), and thus have reason to believe that differences in cytokine production in the presence or absence of palmitate throughout the manuscript resulted from the presence of palmitate and not to changes in GBS MOI.

Palmitate and GBS co-stimulation induces greater cytokine response in DSCs, CTBs, and macrophages than either treatment individually

To determine the effect of palmitate and GBS dual stimulation on the choriodecidua, we induced decidualization in DSCs as described in methods. DSC monocultures were then infected with GBS, treated with palmitate, neither, or both. In our initial cytokine screen, we found that IL-1β (Figure 1E) and CCL2 (Supplementary Figure S1A) were both induced by GBS infection and by palmitate treatment, and the combination of palmitate and GBS synergized, producing more than the simple summation of the IL-1β produced by each stimulus alone. MMP9 (Figure 1F), CCL5, and GM-CSF (Supplementary Figures S1B, C) were all induced by palmitate and

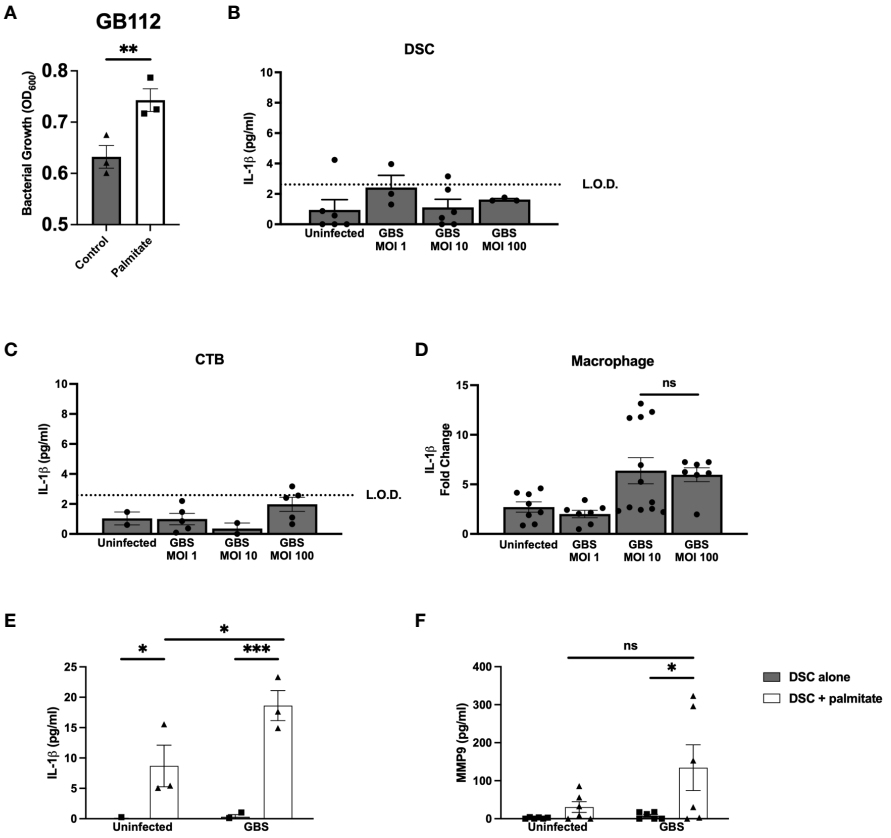


FIGURE 1
Long chain saturated fatty acid palmitate induces Group B *Streptococcus* growth, synergizes with GBS infection to induce IL-1β and MMP9 from human decidual stromal cells. **(A)** OD_{600} reading of GB112 grown for 24 hours in THB broth with and without added palmitate. IL-1β secretion measured by ELISA from the uterine stromal cell line THESC decidualized over 7 days with estrogen, progesterone, and cAMP (referred to as DSCs) **(B)**, the CTB cell line HTR-8 **(C)**, and the macrophage cell line THP.1 **(D)** infected with GBS for 24 hours at MOIs of 1, 10, and 100. **(E)** IL-1β and **(F)** MMP9 secretion measured by ELISA from uninfected or GBS-infected DSCs with or without added palmitate for 24 hours. For all experiments in Figure 1, N = 3 or more separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's T-test **(A–D)**, or 2-way ANOVA **(E, F)**. ns, not significant; L.O.D., limit of detection.

GBS combined, but not by either stimulus alone, also consistent with a synergistic interaction. G-CSF was induced by palmitate both during GBS infection and when uninfected (Supplementary Figure S1D). Neither palmitate nor GBS significantly induced IL-8 or TNFα secretion, although there was a trend for increased production in the presence of palmitate in both cytokines (Supplementary Figures S1E, F).

When treated with PAM or LPS to assess the applicability of these findings to other pathogens, we observed similar, if not more dramatic, results in DSCs (Supplementary Figure S2). Palmitate synergized with both PAM and LPS induced greater IL-1β, IL-8, CCL5, G-CSF, and CCL2 secretion, while MMP9, GM-CSF, and TNFα levels did not change significantly between treatments (there were non-significant trends towards increases) (Supplementary Figure S2).

Moving inwards to cell types of the chorion, we used the Jeg3 cell line of CTBs as a model of chorionic trophoblasts, to assess the impact of palmitate and GBS dual stimulation on the cytokine response in CTBs alone. Narrowing the field of proteins measured to focus on MMP9 and IL-1β, we found that palmitate stimulation alone did not induce IL-1β secretion as in the DSCs (Figure 2A), but GBS infection did induce IL-1β production both with and without palmitate, and palmitate stimulation further increased GBS-induced IL-1β production in CTBs (Figure 2A). Similar to the DSCs, palmitate induced MMP9 production in the presence or absence of infection (Figure 2B). Stimulation of CTBs with palmitate and TLR ligands PAM or LPS synergistically induced greater TNFα, MMP9, and GM-CSF production (Supplementary Figure S3).

We modeled the macrophage response to GBS and palmitate stimulation using the macrophage-like cell line THP-1 differentiated with PMA. Similar to both the DSCs and CTBs, GBS and palmitate stimulation increased IL-1β cytokine production significantly more than either stimulation alone (Figure 3A). MMP9 was induced by GBS stimulation with no significant increases in the presence of palmitate (Figure 3B). Macrophage responses to the combination of PAM or LPS varied depending on the analyte: while GM-CSF was induced synergistically when co-stimulated with PAM or LPS and palmitate, MMP9 and TNFα varied: PAM treatment resulted in additive effects for TNFα while LPS did not, and LPS resulted in additive effects for MMP9 while PAM did not (Supplementary Figure S4).

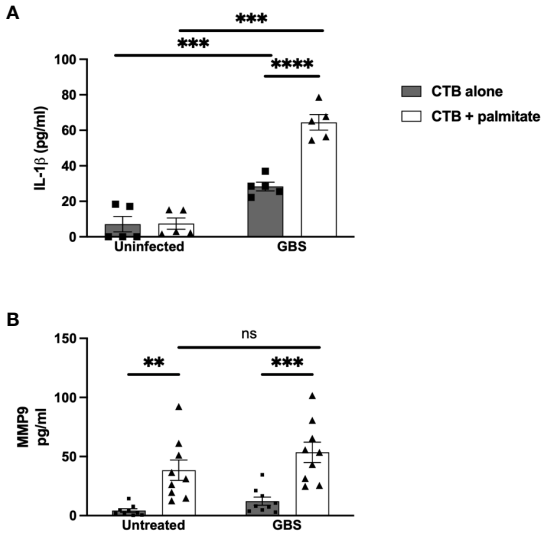


FIGURE 2
LCFA palmitate induces MMP9 secretion and synergizes with GBS to induce IL-1β secretion from human cytotrophoblasts. **(A)** IL-1β and **(B)** MMP9 secretion measured by ELISA from cytotrophoblast cell line HTR8 uninfected or GBS-infected with or without added palmitate for 24 hours. N = 3 or more separate experiments with averaged independent replicates. **p < 0.01, ***p < 0.001, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

Palmitate and GBS co-stimulation modulates cell-cell interactions

DSCs and CTBs can suppress macrophage cytokine production (42, 50). To assess whether this happens in the context of IL-1β or MMP9 in the presence of GBS and/or palmitate, we combined immune and structural cells (decidualized THESC cells (DSCs) or CTBs (Jeg3 cell line)) with THP-1 macrophages at a ratio of 10 structural cells to 1 macrophage (10:1). We found that IL-1β was still significantly induced by GBS and palmitate during co-culture (Figures 4A, B), and not suppressed. However, neither co-culture system appeared to augment IL-1β production over what macrophages alone produced (Figure 3). When using primary CTBs with primary placental macrophages instead of Jeg3 CTBs and THP-1 macrophages, we found that palmitate and GBS also induced IL-1β synergistically, but that the combination of CTBs with primary placental macrophages was not synergistic (Figure 4C). In the co-culture system, MMP9 was induced significantly in DSC-macrophage co-culture only during GBS and palmitate treatment together (Figure 4D). GBS and palmitate together increased MMP9 expression over baseline in CTB-macrophage co-culture (Figure 4E). In primary placental CTB and macrophage co-cultures, MMP9 was not induced by any treatment and in fact trends towards suppression with GBS and palmitate treatment (Figure 4F).

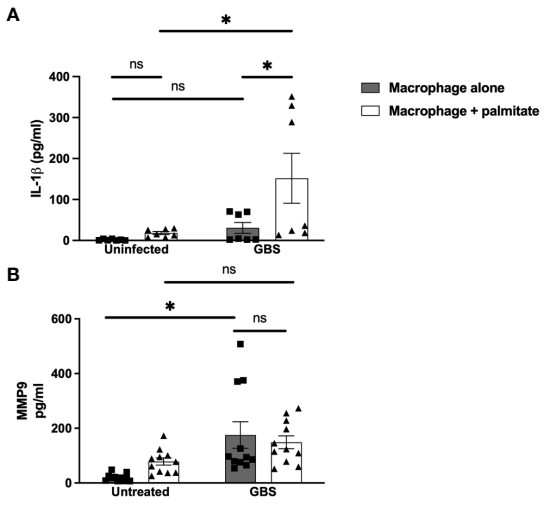


FIGURE 3
LCFA palmitate synergizes with GBS to induce IL-1β secretion from macrophages but not MMP9. **(A)** IL-1β and **(B)** MMP9 secretion measured by ELISA from PMA-stimulated human monocyte cell line THP-1 uninfected or GBS-infected with or without added palmitate for 24 hours. N = 5 or more separate experiments with averaged independent replicates. *p < 0.05 by 2-way ANOVA. ns, not significant.

Whole and separated human gestational membrane punches exhibit a range of responses to GBS and palmitate co-stimulation

We next assessed the IL-1β and MMP9 responses to GBS and palmitate in whole human gestational membrane biopsy specimens obtained from term, nonlaboring c-sections. Interestingly, we found that GBS induced IL-1β expression, but palmitate addition to GBS did not synergistically induce IL-1β (Figure 5A). To tease apart this finding, we matched the data from each untreated membrane punch to its own corresponding palmitate-stimulated membrane punch (e.g. punched from the same patient's membranes) in both infected and uninfected conditions. During uninfected conditions, there was very little induction of IL-1β by palmitate, and palmitate-stimulated IL-1β levels were on a spectrum ranging from suppression to induction (Figure 5A). During GBS stimulation, there was a clear divide in punches from the same membranes with and without palmitate: roughly equal numbers of punches had their IL-1β induced as had IL-1β suppressed by palmitate during GBS infection (Supplementary Figure S5A). Organizing the data by maternal body-mass index (BMI), fetal sex, age, or GBS colonization status was unable to resolve the discrepancy (data not shown).

To resolve the discrepancy in IL-1β production from whole membrane punch responses to GBS and palmitate compared to isolated cell lines and co-cultures, we next separated amnion from choriodecidua and repeated the experiment quantifying IL-1β from culture supernatants (Figures 5B, C; Supplementary Figure S5C). In the choriodecidua, GBS and palmitate synergized to induce IL-1β as seen in the cell lines. Conversely, the amnion did not show significant IL-1β induction under any condition and in fact, palmitate treatment trended non-significantly towards suppression of IL-1β (Figure 5C).

MMP9 production was induced 5-fold with GBS stimulation alone in whole membrane punches, which was significantly greater than the 2.5-fold increase with GBS and palmitate together

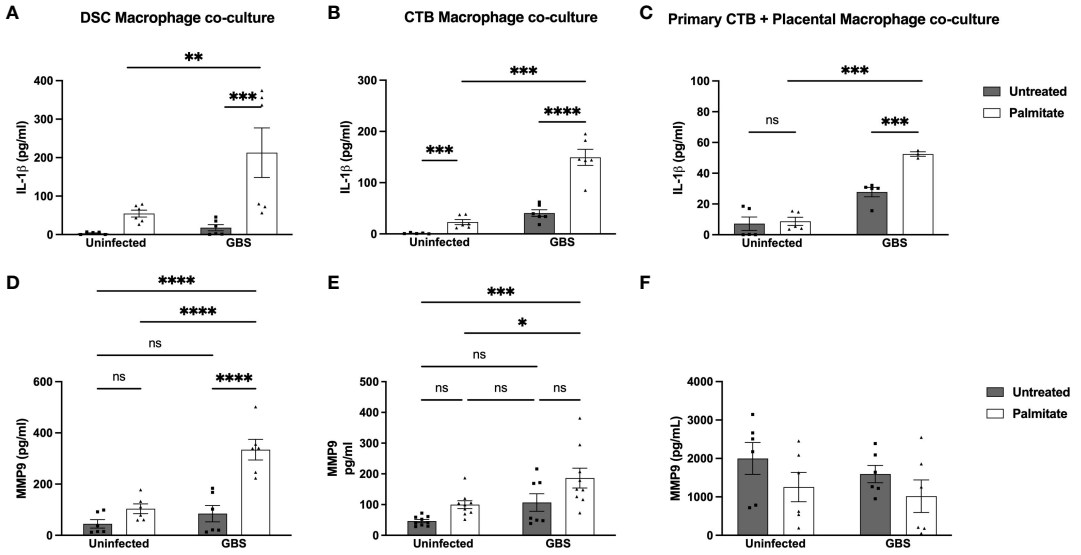


FIGURE 4
Palmitate and GBS synergize to induce IL-1β secretion from DSC-Mφ and CTB-Mφ co-cultures, while MMP9 secretion is more complicated. **(A–C)** IL-1β and **(D–F)** MMP secreted protein by ELISA in co-cultures of cell line and primary structural and immune cells. **(A, D)** ELISA data of co-cultures of DSCs with THP-1 Mφ over 24hrs of palmitate treatment and GBS infection. **(B, E)** Co-cultures of HTR8 CTBs and THP-1 Mφs over 24hrs of palmitate treatment and GBS infection. **(C, F)** primary placental CTBs co-cultured with primary placental Mφ over 24hrs of palmitate treatment and GBS infection. N = minimum of 4 separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

(Figure 5D). The response of paired samples showed a consistent pattern of each patient's MMP9 secreted from membrane punches decreasing with palmitate addition (Supplementary Figure S5B). We next assessed the contribution of the choriodecidua versus the amnion in this response and found that no conditions alone or together induced MMP9 from either the choriodecidua or the amnion (Figures 5E, F; Supplementary Figure S5D). This was distinct from the results obtained with cell lines (Figures 1, 2).

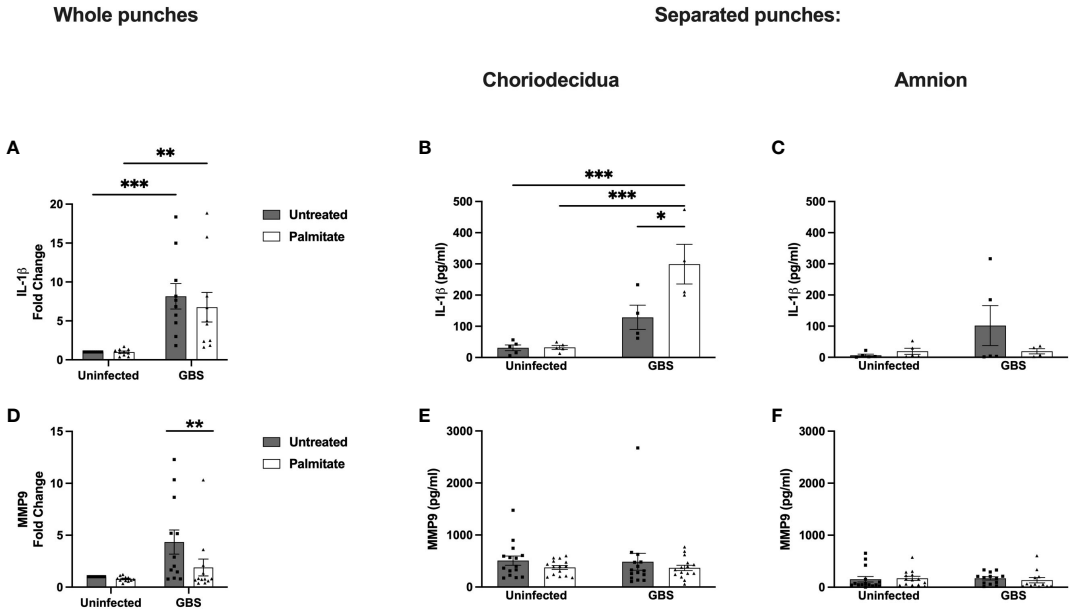


FIGURE 5
Whole and separated human extraplacental membrane punches have differential IL-1β and MMP9 responses to palmitate and GBS. **(A–C)** IL-1β and **(D–F)** MMP9 secretion from whole and separated human extraplacental membrane punches after 24 hours of culture with GBS and palmitate. **(A, D)** Whole membrane punches show increase in IL-1β and MMP9 during GBS infection that is not tied to palmitate treatment. **(B)** GBS and palmitate synergize in the choriodecidua to induce IL-1β, while there is no induction of MMP9 secretion **(E)** from any treatment. **(C, F)** Amnion punches do not induce IL-1β or MMP9 significantly under any treatments. N = minimum of 3 separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ***p < 0.001 by 2-way ANOVA.

10.3389/fimmu.2024.1409378

AECs are largely nonresponsive to individual or co-treatment with GBS or palmitate

We next determined the response of primary amnion epithelial cells (AECs) obtained from human placenta to GBS and/or palmitate. The AECs did not induce IL-1β over the limit of detection in response to GBS or palmitate alone or in combination (Figure 6A). However, there was a nonsignificant trend towards suppressed MMP9 in all treatment groups relative to controls, similar to the nonsignificant MMP9 trend seen in primary CTB-Mφ co-cultures (Figure 6B).

Addition of the unsaturated fatty acid oleate blocks palmitate and GBS-associated IL-1β induction

There are reports in the literature that unsaturated fatty acids can alleviate palmitate-induced changes to inflammation (51). We thus added the unsaturated fatty acid oleate to DSC, CTB, and THP-1 macrophage cultures with and without GBS infection and palmitate treatment and measured IL-1β secretion. We did not measure MMP9 with oleate due to the inconsistent induction in the presence of palmitate across the cell lines used. In DSCs, palmitate and GBS still synergistically induced IL-1β, oleate did not induce IL-1β, and the addition of oleate to palmitate was sufficient to significantly suppress palmitate-induced IL-1β secretion (Figure 7A). In CTBs, palmitate and GBS synergistically induced IL-1β, while the addition of oleate did not. The addition of oleate to palmitate during GBS infection was unclear: oleate and palmitate during GBS infection did not differ significantly from oleate and GBS stimulation or palmitate and GBS stimulation, while oleate/GBS and palmitate/GBS did differ significantly (Figure 7B). Finally, THP-1 macrophages stimulated with palmitate and GBS synergistically induced IL-1β, while oleate and GBS did not induce IL-1β, and, similarly to the DSCs, the addition of oleate to palmitate during GBS infection suppressed IL-1β production (Figure 7C).

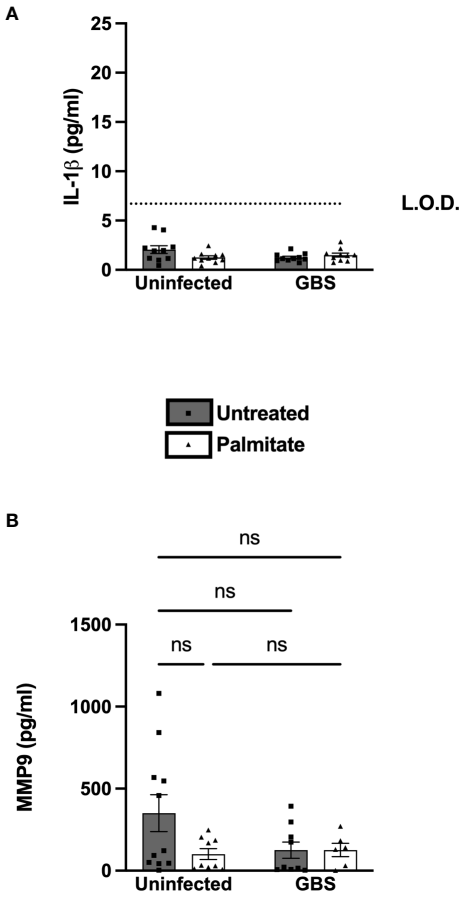


FIGURE 6
Primary human amnion epithelial cells (AECs) do not induce IL-1β or MMP9 under any treatments. **(A)** IL-1β and **(B)** MMP9 secretion measured by ELISA from uninfected or GBS-infected AECs with or without added palmitate for 24 hours. For all experiments, N = 4 or more separate experiments with averaged independent replicates. ns, not significant 2-way ANOVA. L.O.D., limit of detection.

Discussion

In this manuscript, we have shown *in vitro* and ex vivo that distinct layers of the gestational membranes respond differently to the saturated fat palmitate in the presence or absence of the common vaginal commensal bacterium GBS. We demonstrate that palmitate enhances GBS proliferation (Figure 1), which may partially explain the increased complications due to GBS in people with obesity during pregnancy, due simply to the increased proliferation of GBS (52, 53). While independent layers in the choriodecidua respond to GBS and palmitate stimulation with synergy in cytokine and MMP induction, the amnion epithelium does not contribute to this phenotype and is largely unaffected by GBS and palmitate stimulation, both in isolated primary amnion epithelial cells and in gestational membrane punch biopsies, where the amnion has been separated from the choriodecidua. This was not surprising, as the broad dampening of inflammation by the amnion is well-established (54–56). Furthermore, we have shown that macrophages (either cell line or primary placental macrophages) co-cultured with structural cells of the choriodecidua in the presence of palmitate and GBS are not suppressed by the structural cells as can be seen in the absence of palmitate in previous work (42, 50). Different patterns of analyte induction in the presence or absence of GBS-palmitate synergy may be due to signaling and transcription factors controlling the particular analytes in a particular cell type; for instance, while IL-1β is reliably induced synergistically from GBS and palmitate in combination, MMP9 synergy is less reliable. IL-1β is controlled at many levels and processed from a pro-form to an active form, while MMP9 is largely controlled at the transcriptional level with the association of coactivators distinct from IL-1β (57). Synergy between palmitate and GBS stimulation likely depends entirely on the signaling pathways to, and regulation of, each particular analyte. Sensitization deriving from one stimulus then augmenting other stimuli has been shown during pregnancy before (58–62), although

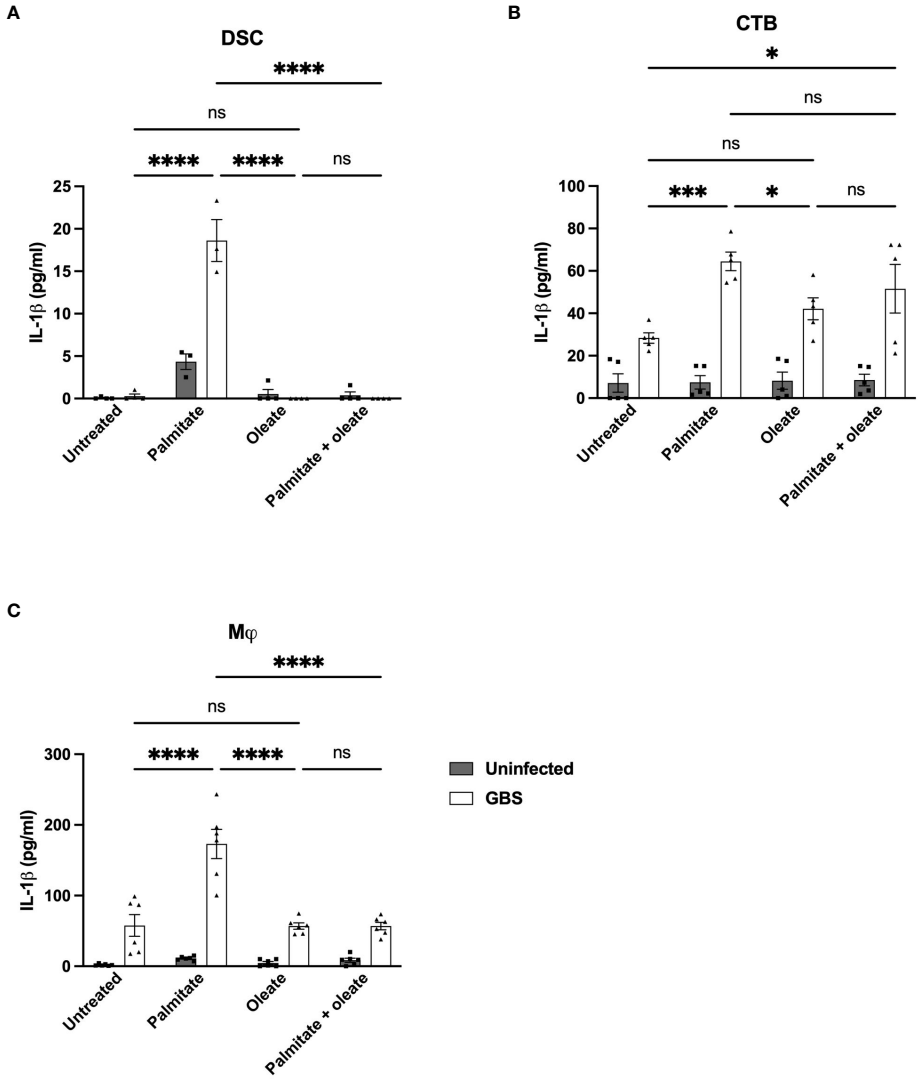


FIGURE 7
Oleate ameliorates the synergistic induction of IL-1β from palmitate and GBS dual stimulation in DSC, CTB, and Mj cell lines. IL-1β secretion measured by ELISA from **(A)** DSC, **(B)** CTB, and **(C)** THP-1 Mφ when infected with GBS and treated with palmitate and/or oleate. N = minimum of 4 separate experiments with averaged independent replicates. *p < 0.05, ***p < 0.001, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

this has largely been shown for the interplay between viral stimulation or viral PAMPs and subsequent stimulation.

This synergistic production of cytokines and MMPs is not specific to live GBS, as shown by the ability of a TLR4 agonist (LPS) to induce synergy in cytokine expression when administered with palmitate (Supplementary Figure S2). Because we observed cytokine augmentation when palmitate was combined with live GBS [a gram-positive bacterium) as well as a purified TLR4 agonist (LPS, a gram-negative pathogen-associated molecular pattern (PAMP)], this suggests broad applicability of our findings to different bacterial species and strains, both gram positive and gram negative. Further, it suggests that palmitate-induced alterations to GBS proliferation and metabolism are not the primary cause of increased inflammation in the gestational membranes, since the synergistic inflammation occurs with isolated PAMPs as well. We observed a relative inability of TLR2 agonist PAM3CSK4 to induce cytokines or MMPs on its own or in combination with palmitate when applied to DSCs. This lack of stimulation was recapitulated in CTBs as well (Supplementary Figure S3), with the exception of GM-CSF, where PAM3CSK4 was able to induce GM-CSF alone and synergize with palmitate. However, PAM3CSK4 was able to induce several cytokines alone (IL-1β, CCL5, GM-CSF), but not MMP9, and synergize with palmitate in THP-1 macrophages similar to live GBS and purified LPS (Supplementary Figure S4). Additional experimentation needs to be carried out to address the discrepancy between TLR2 activation and palmitate synergy in structural cells versus immune cells (such as macrophages) to determine the mechanism (s) at play, such as specific pathway activation or suppression, or receptor expression. Additional approaches could include using Pam2Cys or lipoteichotic acid (LTA), which utilize a different heterodimer of TLR2; while PAM3CSK4 utilizes a TLR1-TLR2

heterodimer, LTA and Pam2Cys utilize a TLR2-TLR6 heterodimer. The discrepancy between PAM3CSK4 and GBS-induced activation of cytokine and MMP may have to do with the activation of the TLR1/2 versus TLR2/6 heterodimers (63).

There are many varied fatty acids in the bloodstream, and one limitation of this study is the use of palmitate in isolation, or in combination with a single unsaturated fatty acid (oleate), instead of looking at the effects of a cocktail of saturated and unsaturated fatty acids in varying proportions on the cell types found within the gestational membrane. We acknowledge this shortcoming as a necessary oversimplification for the purposes of *in vitro* experimentation. Palmitate was chosen as it is the most abundant saturated fatty acid in circulation during obesity and can be the dominant circulating fatty acid. Furthermore, it is the most common saturated fatty acid used for *in vitro* experiments of obesity in the literature, and as such there is a large body of knowledge surrounding its mechanisms, making it an ideal candidate to study our models presented here in a reductionist manner. Continuing this line of inquiry using distinct mixtures of fatty acids, and the use of our previously-published metabolic cocktail (glucose, insulin, palmitate) (64) will more accurately reflect the milieu seen *in vivo*. However, defining basic patterns of synergy between infectious stimuli and a fatty acid using a reductionist approach gives us a base from which to determine the relative contributions of different fatty acids and sugars to the inflammatory state of the gestational membranes. The mechanism behind palmitate-induced inflammation remains unclear. There is much discrepancy in published work as to whether palmitate can signal through TLR4 (65–71) or not (72). As such, the mechanism behind synergy of GBS and palmitate is also unclear, but it is likely that palmitate stimulates a pathway or receptor independent of TLR4 signaling because palmitate treatment augments the inflammatory response to TLR4 agonists in macrophages, CTBs, and LPS (Supplementary Figures S2-S4). This includes the induction of: IL-1β, IL-8, CCL2, CCL5, and G-CSF in DSCs; TNFα, MMP9, and GM-CSF in CTBs; and MMP9 and GM-CSF in macrophages.

The condition of "diabesity", a combination of type-2 diabetes and obesity, may more accurately reflect the reality of which pregnant individuals experience a greater rate of pregnancy complications than BMI alone. The inability of BMI to assess body fat distribution (abdominal versus distributed) or muscle mass adds heterogeneity into the system. Additionally, metabolic syndrome, a classification that encompasses the risk of developing type-2 diabetes and cardiovascular disease, can be found in individuals with any BMI (73). Access to patient electronic medical records containing bloodwork results such as circulating hemoglobin A1C or lipids, or a waist-to-hip ratio, could more accurately stratify our human gestational membrane punch data to reveal patterns that maternal BMI does not.

We expected to see an increase in inflammatory mediators upon co-stimulation of whole human gestational membrane punches with palmitate and GBS. However, for the analytes we selected for these experiments, we were unable to find consistent patterns or statistical significance between treatments. We had 11 patients and 24 membrane punches for each patient allowing for biological and technical replicates for each patient. As shown in Supplementary Table 1, the BMI of most patients was between the overweight and morbidly obese categories by BMI. However, 1) there was not a direct relationship between BMI and magnitude of cytokine induction (data not shown), and 2) palmitate and GBS did synergize in IL-1β production from the membrane punches of roughly half the patients, while the other half of patients' membrane punches showed no synergy or decreased production upon palmitate stimulation. We were similarly unable to show a direct correlation between BMI and these cytokine induction patterns. Sorting by fetal sex, maternal GBS status, ethnicity, or gestational age also did not uncover any correlation between magnitude or direction of cytokine induction. It is very possible that with a larger N value of patient samples, the impact of particular patient variables could be observed. Recent studies have found that male placentas from pregnancies of obese people had significantly increased levels of fetal placental macrophages, which could easily have an impact on inflammatory responses in the gestational membranes (74). The gestational membrane punch biopsies we used, even when separated, likely contained up to 13% immune cells (75). And fetal sex also has an impact on how many and what kind of circulating maternal fatty acids accumulate in the placenta (32, 33), which could add another confounder to data from human gestational membranes: the impact of increased saturated fatty acids (already pro-inflammatory) and unsaturated fatty acids (which may be anti-inflammatory) already present in the membrane punches.

Additionally, a better understanding of the metabolic conditions that drive the molecular underpinnings of infection and inflammation during pregnancy can inform immunological interventions to ameliorate the risk of adverse perinatal outcomes. IL-1β is a tantalizing target because this molecule has been implicated as an important biomarker associated with chorioamnionitis, PPROM, and preterm labor (21). IL-1β is a pro-inflammatory cytokine that perturbs maternal-fetal tolerance (76). Our work demonstrates that palmitate and GBS infection promote IL-1β production, underscoring the role both metabolic dysfunction and infection could play in pregnancy homeostasis. There is an association between absolute levels of IL-1β and pregnancy complications: as levels of IL-1β increase, the likelihood of preterm deliveries increases (77). Accordingly, there is likely biological relevance for the synergy of GBS and palmitate: it is not just presence or absence of IL-1β that can result in pregnancy complications, but the greater the IL-1β levels, the greater the likelihood of pregnancy complications, and thus the combination of palmitate and GBS inducing more IL-1β in than either one alone would predispose pregnant people with both stimuli to a greater likelihood of pregnancy complications.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Vanderbilt University Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JG: Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. RM: Investigation, Writing – original draft, Writing – review & editing. JL: Investigation, Writing – review & editing. LR: Investigation, Writing – review & editing. KN: Investigation, Writing – review & editing. AG: Formal analysis, Methodology, Writing – review & editing. DA: Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. DC: Funding acquisition, Resources, Supervision, Writing – review & editing. AE: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Institutes of Health (NIH) 1F32HD100087-01A1(AE), R01HD102752 (DC), R01HD090061 (JG), R01AI134036 (JG and DA), and Department of Veterans Affairs Merit Award I01BX005352 Office of Research (to JG).

Acknowledgments

AE would like to acknowledge Nadja for assistance in focus.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1409378/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Cytokine induction by GBS, palmitate, or both in DSCs infected with GBS and/or treated with palmitate. ELISA analysis of secreted cytokines from DSCs. **(A)** CCL2, **(B)**, CCL5, **(C)** GM-CSF, **(D)** G-CSF, **(E)** IL-8, and **(F)** TNFα each followed different patterns of induction by 24 hour infection and/or treatment with GBS and/or palmitate. N = minimum of 3 separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

SUPPLEMENTARY FIGURE 2
Secreted inflammatory protein induction by TLR2 and 4 agonists in conjunction with palmitate in DSCs. **(A)** IL-1β, **(B)** MMP9, **(C)** IL-8, **(D)** CCL2, **(E)** CCL5, **(F)** GM-CSF, **(G)** G-CSF, **(H)** TNFα each follow different patterns of induction by 24hr stimulation with TLR2 and 4 ligands PAM3CSK4 (PAM) and LPS, respectively. N = minimum of 3 separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

SUPPLEMENTARY FIGURE 3
Secreted inflammatory protein induction by TLR2 (PAM) and 4 (LPS) agonists in conjunction with palmitate in CTBs. **(A)** TNFα, **(B)** MMP9, and **(C)** GM-CSF. N = minimum of 3 separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

SUPPLEMENTARY FIGURE 4
Secreted inflammatory protein induction by TLR2 (PAM) and 4 (LPS) agonists in conjunction with palmitate in THP-1 macrophages. **(A)** TNFα, **(B)** MMP9, and **(C)** GM-CSF. N = minimum of 3 separate experiments with averaged independent replicates. *p < 0.05, **p < 0.01, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

SUPPLEMENTARY FIGURE 5
Matched sample and side-by-side comparison of IL-1β and MMP9 secretion from membrane punches from human extraplacental membrane.**(A)** IL-1β and **(B)** MMP9 ELISAs of 24hr supernatants from individual patient's membrane punches matched with line between treatments with GBS and palmitate. **(C)** IL-1β and **(D)** MMP9 ELISA of CHD and AMN for side-by-side comparison of protein secretion between adjacent extraplacental membrane layers. **(A, B)** N = 11 independent experiments with averaged independent replicates; **(C, D)** N = 5 independent experiments with averaged independent replicates. * p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by 2-way ANOVA. ns, not significant.

References

1. Galinsky R, Polglase GR, Hooper SB, Black MJ, Moss TJ. The consequences of chorioamnionitis: preterm birth and effects on development. *J Pregnancy*. (2013) 2013:412831. doi: 10.1155/2013/412831

2. Goldenberg RL, McClure EM, Saleem S, Reddy UM. Infection-related stillbirths. *Lancet*. (2010) 375:1482–90. doi: 10.1016/S0140-6736(09)61712-8

3. Meinert M, Malmstrom A, Petersen AC, Eriksen GV, Uldbjerg N. Chorioamniontis in preterm delivery is associated with degradation of decorin and biglycan and depletion of hyaluronan in fetal membranes. *Placenta*. (2014) 35:546–51. doi: 10.1016/j.placenta.2014.05.004

4. Anderson BL, Simhan HN, Simons KM, Wiesenfeld HC. Untreated asymptomatic group B streptococcal bacteriuria early in pregnancy and chorioamnionitis at delivery. *Am J Obstetrics Gynecology*. (2007) 196:524.e1–5. doi: 10.1016/j.ajog.2007.01.006

5. Horvath B, Lakatos F, Toth C, Bodecs T, Bodis J. Silent chorioamnionitis and associated pregnancy outcomes: a review of clinical data gathered over a 16-year period. *J Perinat Med*. (2014) 42:441–7. doi: 10.1515/jpm-2013-0186

6. Madhi SA, Briner C, Maswime S, Mose S, Mlandu P, Chawana R, et al. Causes of stillbirths among women from South Africa: a prospective, observational study. *Lancet Glob Health*. (2019) 7:e503–12. doi: 10.1016/S2214-109X(18)30541-2

7. Nan C, Dangor Z, Cutland CL, Edwards MS, Madhi SA, Cunnington MC, et al. *Streptococcus*-related stillbirth: a systematic review. *BJOG*. (2015) 122:1437–45. doi: 10.1111/1471-0528.13527

8. Seale AC, Blencowe H, Bianchi-Jassir F, Embleton N, Bassat Q, Ordi J, et al. Stillbirth with group B *streptococcus* disease worldwide: systematic review and meta-analyses. *Clin Infect Dis*. (2017) 65:S125–32. doi: 10.1093/cid/cix585

9. Wortham JM, Hansen NI, Schrag SJ, Hale E, Van Meurs K, Sanchez PJ, et al. Chorioamnionitis and culture-confirmed, early-onset neonatal infections. *Pediatrics*. (2016) 137:1–11. doi: 10.1542/peds.2015-2323

10. Armistead B, Oler E, Adams Waldorf K, Rajagopal L. The double life of group B streptococcus: asymptomatic colonizer and potent pathogen. *J Mol Biol*. (2019) 431:2914–31. doi: 10.1016/j.jmb.2019.01.035

11. Dutta S, Reddy R, Sheikh S, Kalra J, Ray P, Narang A. Intrapartum antibiotics and risk factors for early onset sepsis. *Arch Dis Child Fetal Neonatal Ed*. (2010) 95:F99–103. doi: 10.1136/adc.2009.163220

12. Johansson Gudjonsdottir M, Elfvin A, Hentz E, Adlerberth I, Tessin I, Trollfors B. Changes in incidence and etiology of early-onset neonatal infections 1997-2017 - a retrospective cohort study in western Sweden. *BMC Pediatr*. (2019) 19:490. doi: 10.1186/s12887-019-1866-z

13. Singh T, Barnes EH, Isaacs D, Australian Study Group for Neonatal I. Early-onset neonatal infections in Australia and New Zealand, 2002-2012. *Arch Dis Child Fetal Neonatal*. (2019) 104:F248–52. doi: 10.1136/archdischild-2017-314671

14. Stan CM, Boulvain M, Bovier PA, Auckenthaler R, Berner M, Irion O. Choosing a strategy to prevent neonatal early-onset group B streptococcal sepsis: economic evaluation. *BJOG*. (2001) 108:840–7. doi: 10.1111/j.1471-0528.2001.00201.x

15. Villamor E, Norman M, Johansson S, Cnattingius S. Maternal obesity and risk of early-onset neonatal bacterial sepsis: Nationwide cohort and sibling-controlled studies. *Clin Infect Dis*. (2021) 73(9):e2656–64. doi: 10.1093/cid/ciaa783

16. Hall J, Adams NH, Bartlett L, Seale AC, Lamagni T, Bianchi-Jassir F, et al. Maternal disease with group B streptococcus and serotype distribution worldwide: systematic review and meta-analyses. *Clin Infect Dis*. (2017) 65:S112–24. doi: 10.1093/cid/cix660

17. Pique-Regi R, Romero R, Tarca AL, Sendler ED, Xu Y, Garcia-Flores V, et al. Single cell transcriptional signatures of the human placenta in term and preterm parturition. *Elife*. (2019) 8:e52004. doi: 10.7554/eLife.52004

18. Athayde N, Edwin SS, Romero R, Gomez R, Maymon E, Pacora P, et al. A role for matrix metalloproteinase-9 in spontaneous rupture of the fetal membranes. *Am J Obstetrics Gynecology*. (1998) 179:1248–53. doi: 10.1016/s0002-9378(98)70141-3

19. McLaren J, Taylor DJ, Bell SC. Increased concentration of pro–matrix metalloproteinase 9 in term fetal membranes overlying the cervix before labor: Implications for membrane remodeling and rupture. *Am J Obstetrics Gynecology*. (2000) 182:409–16. doi: 10.1016/s0002-9378(00)70232-8

20. Xu P, Alfaidy N, Challis JRG. Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in human placenta and fetal membranes in relation to preterm and term labor. *J Clin Endocrinol Metab*. (2002) 87:1353–61. doi: 10.1210/jcem.87.3.8320

21. Jain VG, Willis KA, Jobe A, Ambalavanan N. Chorioamnionitis and neonatal outcomes. *Pediatr Res*. (2022) 91:289–96. doi: 10.1038/s41390-021-01633-0

22. Balciuniene G, Gulbiniene V, Dumalakiene I, Viliene R, Bartkeviciene D, Pilypiene I, et al. Prognostic markers for chorioamnionitis: IL-6, TNF-alpha, and MMP-8 in vaginally obtained amniotic fluid. *J Clin Med*. (2021) 10(5):1136. doi: 10.3390/jcm10051136

23. Fritsche KL. The science of fatty acids and inflammation. *Adv Nutr*. (2015) 6:293S–301S. doi: 10.3945/an.114.006940

24. Staiger H, Staiger K, Stefan N, Wahl HG, Machicao F, Kellerer M, et al. Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells. *Diabetes*. (2004) 53:3209–16. doi: 10.2337/diabetes.53.12.3209

25. Staaf J, Ubhayasekera SJKA, Sargsyan E, Chowdhury A, Kristinsson H, Manell H, et al. Initial hyperinsulinemia and subsequent β-cell dysfunction is associated with elevated palmitate levels. *Pediatr Res*. (2016) 80:267–74. doi: 10.1038/pr.2016.80

26. Min Y, Ghebremeskel K, Lowy C, Thomas B, Crawford MA. Adverse effect of obesity on red cell membrane arachidonic and docosahexaenoic acids in gestational diabetes. *Diabetologia*. (2004) 47:75–81. doi: 10.1007/s00125-003-1275-5

27. Tan B, Ma Y, Zhang L, Li N, Zhang J. The application of metabolomics analysis in the research of gestational diabetes mellitus and preeclampsia. *J Obstet Gynaecol Res*. (2020) 46:1310–8. doi: 10.1111/jog.14304

28. Luo M, Guo J, Lu W, Fang X, Zhang R, Tang M, et al. The mediating role of maternal metabolites between lipids and adverse pregnancy outcomes of gestational diabetes mellitus. *Front Med (Lausanne)*. (2022) 9:925602. doi: 10.3389/fmed.2022.925602

29. Ha SY, Qiu XM, Lai ZZ, Yang HL, Wang Y, Ruan LY, et al. Excess palmitate induces decidual stromal cell apoptosis *via* the TLR4/JNK/NF-kB pathways and possibly through glutamine oxidation. *Mol Hum Reprod*. (2020) 26:88–100. doi: 10.1093/molehr/gaaa004

30. Villa PM, Laivuori H, Kajantie E, Kaaja R. Free fatty acid profiles in preeclampsia. *Prostaglandins Leukot Essent Fatty Acids*. (2009) 81:17–21. doi: 10.1016/j.plefa.2009.05.002

31. Haggarty P. Fatty acid supply to the human fetus. *Annu Rev Nutr*. (2010) 30:237–55. doi: 10.1146/annurev.nutr.012809.104742

32. Brass E, Hanson E, O'Tierney-Ginn PF. Placental oleic acid uptake is lower in male offspring of obese women. *Placenta*. (2013) 34:503–9. doi: 10.1016/j.placenta.2013.03.009

33. Powell TL, Barner K, Madi L, Armstrong M, Manke J, Uhlson C, et al. Sex-specific responses in placental fatty acid oxidation, esterification and transfer capacity to maternal obesity. *Biochim Biophys Acta (BBA) - Mol Cell Biol Lipids*. (2021) 1866 (3):158861. doi: 10.1016/j.bbalip.2020.158861

34. Imhoff-Kunsch B, Briggs V, Goldenberg T, Ramakrishnan U. Effect of n-3 long-chain polyunsaturated fatty acid intake during pregnancy on maternal, infant, and child health outcomes: a systematic review. *Paediatr Perinat Epidemiol*. (2012) 26 Suppl:1, 91–107. doi: 10.1111/j.1365-3016.2012.01292.x

35. Davidson BC, Cantrill RC. Fatty acid nomenclature. A short review. *S Afr Med J*. (1985) 67:633–4. doi: 10.1002/chin.198542383

36. Vidakovic AJ, Jaddoe VW, Gishti O, Felix JF, Williams MA, Hofman A, et al. Body mass index, gestational weight gain and fatty acid concentrations during pregnancy: the Generation R Study. *Eur J Epidemiol*. (2015) 30:1175–85. doi: 10.1007/s10654-015-0106-6

37. Tomedi LE, Chang CC, Newby PK, Evans RW, Luther JF, Wisner KL, et al. Pre-pregnancy obesity and maternal nutritional biomarker status during pregnancy: a factor analysis. *Public Health Nutr*. (2013) 16:1414–8. doi: 10.1017/S1368980013000736

38. Micallef M, Munro I, Phang M, Garg M. Plasma n-3 Polyunsaturated Fatty Acids are negatively associated with obesity. *Br J Nutr*. (2009) 102:1370–4. doi: 10.1017/S0007114509382173

39. Trico D, Mengozzi A, Nesti L, Hatunic M, Gabriel Sanchez R, Konrad T, et al. Circulating palmitoleic acid is an independent determinant of insulin sensitivity, beta cell function and glucose tolerance in non-diabetic individuals: a longitudinal analysis. *Diabetologia*. (2020) 63:206–18. doi: 10.1007/s00125-019-05013-6

40. Persson XM, Blachnio-Zabielska AU, Jensen MD. Rapid measurement of plasma free fatty acid concentration and isotopic enrichment using LC/MS. *J Lipid Res*. (2010) 51:2761–5. doi: 10.1194/jlr.M008011

41. Haghiac M, Yang XH, Presley L, Smith S, Dettelback S, Minium J, et al. Dietary omega-3 fatty acid supplementation reduces inflammation in obese pregnant women: A randomized double-blind controlled clinical trial. *PloS One*. (2015) 10:e0137309. doi: 10.1371/journal.pone.0137309

42. Eastman AJ, Vrana EN, Grimaldo MT, Jones AD, Rogers LM, Alcendor DJ, et al. Cytotrophoblasts suppress macrophage-mediated inflammation through a contact-dependent mechanism. *Am J Reprod Immunol*. (2021) 85:e13352. doi: 10.1111/aji.13352

43. Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E, et al. A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology*. (2004) 145:2291–6. doi: 10.1210/en.2003-1606

44. Salamonsen LA, Dimitriadis E, Jones RL, Nie G. Complex regulation of decidualization: A role for cytokines and proteases—A review. *Placenta*. (2003) 24: S76–85. doi: 10.1053/plac.2002.0928

45. Rogers LM, Serezani CH, Eastman AJ, Hasty AH, Englund-Ogge L, Jacobsson B, et al. Palmitate induces apoptotic cell death and inflammasome activation in human placental macrophages. *Placenta*. (2020) 90:45–51. doi: 10.1016/j.placenta.2019.12.009

46. Bunn RC, Cockrell GE, Ou Y, Thrailkill KM, Lumpkin CK Jr., Fowlkes JL. Palmitate and insulin synergistically induce IL-6 expression in human monocytes. *Cardiovasc Diabetol*. (2010) 9:73. doi: 10.1186/1475-2840-9-73

47. Kratz M, Coats BR, Hisert KB, Hagman D, Mutskov V, Peris E, et al. Metabolic dysfunction drives a mechanistically distinct proinflammatory phenotype in adipose tissue macrophages. *Cell Metab*. (2014) 20:614–25. doi: 10.1016/j.cmet.2014.08.010

48. Spaetgens R, DeBella K, Ma D, Robertson S, Mucenski M, Davies HD. Perinatal antibiotic usage and changes in colonization and resistance rates of group B streptococcus and other pathogens. *Obstet Gynecol.* (2002) 100:525–33. doi: 10.1016/s0029-7844(02)02068-9

49. Tetz LM, Aronoff DM, Loch-Caruso R. Mono-ethylhexyl phthalate stimulates prostaglandin secretion in human placental macrophages and THP-1 cells. *Reprod Biol Endocrinol.* (2015) 13:56. doi: 10.1186/s12958-015-0046-8

50. Rogers LM, Anders AP, Doster RS, Gill EA, Gnecco JS, Holley JM, et al. Decidual stromal cell-derived PGE_2 regulates macrophage responses to microbial threat. *Am J Reprod Immunol.* (2018) 80:e13032. doi: 10.1111/aji.13032

51. Hong YJ, Ahn HJ, Shin J, Lee JH, Kim JH, Park HW, et al. Unsaturated fatty acids protect trophoblast cells from saturated fatty acid-induced autophagy defects. *J Reprod Immunol.* (2018) 125:56–63. doi: 10.1016/j.jri.2017.12.001

52. Hakansson S, Kallen K. High maternal body mass index increases the risk of neonatal early onset group B streptococcal disease. *Acta Paediatr.* (2008) 97:1386–9. doi: 10.1111/j.1651-2227.2008.00940.x

53. Pitts SI, Maruthur NM, Langley GE, Pondo T, Shutt KA, Hollick R, et al. Obesity, diabetes, and the risk of invasive group B streptococcal disease in nonpregnant adults in the United States. *Open Forum Infect Dis.* (2018) 5:ofy030. doi: 10.1093/ofid/ofy030

54. Zhang Q, Lai D. Application of human amniotic epithelial cells in regenerative medicine: a systematic review. *Stem Cell Res Ther.* (2020) 11(1):439. doi: 10.1186/s13287-020-01951-w

55. Mamede AC, Carvalho MJ, Abrantes AM, Laranjo M, Maia CJ, Botelho MF. Amniotic membrane: from structure and functions to clinical applications. *Cell Tissue Res.* (2012) 349:447–58. doi: 10.1007/s00441-012-1424-6

56. Alipour R, Motedayyen H, Sereshki N, Rafiee M, Alsahebfosul F, Pourazar A. Human amniotic epithelial cells affect the functions of neutrophils. *Int J Stem Cells.* (2020) 13:212–20. doi: 10.15283/ijsc19155

57. Zhao X, Benveniste EN. Transcriptional activation of human matrix metalloproteinase-9 gene expression by multiple co-activators. *J Mol Biol.* (2008) 383:945–56. doi: 10.1016/j.jmb.2008.08.071

58. Rasheed ZBM, Lee YS, Kim SH, Rai RK, Ruano CSM, Anucha E, et al. Differential response of gestational tissues to TLR3 viral priming prior to exposure to bacterial TLR2 and TLR2/6 agonists. *Front Immunol.* (2020) 11:1899. doi: 10.3389/fimmu.2020.01899

59. Racicot K, Aldo P, El-Guindy A, Kwon JY, Romero R, Mor G. Cutting edge: fetal/placental type I IFN can affect maternal survival and fetal viral load during viral infection. *J Immunol.* (2017) 198:3029–32. doi: 10.4049/jimmunol.1601824

60. Racicot K, Kwon JY, Aldo P, Silasi M, Mor G. Understanding the complexity of the immune system during pregnancy. *Am J Reprod Immunol.* (2014) 72:107–16. doi: 10.1111/aji.12289

61. Cardenas I, Mor G, Aldo P, Lang SM, Stabach P, Sharp A, et al. Placental viral infection sensitizes to endotoxin-induced pre-term labor: a double hit hypothesis. *Am J Reprod Immunol.* (2011) 65:110–7. doi: 10.1111/aji.2010.65.issue-2

62. Cardenas I, Means RE, Aldo P, Koga K, Lang SM, Booth CJ, et al. Viral infection of the placenta leads to fetal inflammation and sensitization to bacterial products predisposing to preterm labor. *J Immunol.* (2010) 185:1248–57. doi: 10.4049/jimmunol.1000289

63. Asplin IR, Carl DJ, Way SS, Jones AL. Role of Toll-like receptor 2 in innate resistance to Group B Streptococcus. *Microbial Pathogenesis.* (2008) 44:43–51. doi: 10.1016/j.micpath.2007.08.001

64. Rogers LM, Serezani CH, Eastman AJ, Hasty AH, Englund-Ögge L, Jacobsson B, et al. Palmitate induces apoptotic cell death and inflammasome activation in human placental macrophages. *Placenta.* (2019) 90:45–51. doi: 10.1101/799718

65. Huang S, Rutkowsky JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, et al. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *J Lipid Res.* (2012) 53:2002–13. doi: 10.1194/jlr.D029546

66. Kato H, Haishima Y, Iida T, Tanaka A, Tanamoto K. Chemical structure of lipid A isolated from Flavobacterium meningosepticum lipopolysaccharide. *J Bacteriol.* (1998) 180:3891–9. doi: 10.1128/JB.180.15.3891-3899.1998

67. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. *Diabetes.* (2008) 57:2595–602. doi: 10.2337/db08-0038

68. Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, et al. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology.* (2009) 126:233–45. doi: 10.1111/j.1365-2567.2008.02892.x

69. Wong SW, Kwon MJ, Choi AM, Kim HP, Nakahira K, Hwang DH. Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. *J Biol Chem.* (2009) 284:27384–92. doi: 10.1074/jbc.M109.044065

70. Zhao H, Shao D, Jiang C, Shi J, Li Q, Huang Q, et al. Biological activity of lipopeptides from Bacillus. *Appl Microbiol Biotechnol.* (2017) 101:5951–60. doi: 10.1007/s00253-017-8396-0

71. Nicholas DA, Zhang K, Hung C, Glasgow S, Aruni AW, Unternaehrer J, et al. Palmitic acid is a toll-like receptor 4 ligand that induces human dendritic cell secretion of IL-1beta. *PloS One.* (2017) 12:e0176793. doi: 10.1371/journal.pone.0176793

72. Lancaster GI, Langley KG, Berglund NA, Kammoun HL, Reibe S, Estevez E, et al. Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipid-induced inflammation by reprogramming macrophage metabolism. *Cell Metab.* (2018) 27:1096–1110.e5. doi: 10.1016/j.cmet.2018.03.014

73. Han TS, Lean MEJ. Metabolic syndrome. *Medicine.* (2015) 43:80–7. doi: 10.1016/j.mpmed.2014.11.006

74. Shook LL, James KE, Roberts DJ, Powe CE, Perlis RH, Thornburg KL, et al. Sex-specific impact of maternal obesity on fetal placental macrophages and cord blood triglycerides. *Placenta.* (2023) 140:100–8. doi: 10.1016/j.placenta.2023.08.001

75. Gnecco JS, Anders AP, Cliffel D, Pensabene V, Rogers LM, Osteen K, et al. Instrumenting a fetal membrane on a chip as emerging technology for preterm birth research. *Curr Pharm Des.* (2017) 23:6115–24. doi: 10.2174/1381612823666170825142649

76. Gomez-Lopez N, Galaz J, Miller D, Farias-Jofre M, Liu Z, Arenas-Hernandez M, et al. The immunobiology of preterm labor and birth: intra-amniotic inflammation or breakdown of maternal-fetal homeostasis. *Reproduction.* (2022) 164:R11–45. doi: 10.1530/REP-22-0046

77. Puchner K, Iavazzo C, Gourgiotis D, Boutsikou M, Baka S, Hassiakos D, et al. Mid-trimester amniotic fluid interleukins (IL-1beta, IL-10 and IL-18) as possible predictors of preterm delivery. *In Vivo.* (2011) 25:141–8.

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 04 June 2024
DOI 10.3389/fimmu.2024.1416162

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Yohei Sato,
Jikei University School of Medicine, Japan
Andrea Ivonne Loewendorf,
ImmunoVation, United States

*CORRESPONDENCE
Suhas G. Kallapur
skallapur@mednet.ucla.edu

†These authors have contributed equally to this work

RECEIVED 11 April 2024
ACCEPTED 20 May 2024
PUBLISHED 04 June 2024

CITATION
Presicce P, Roland C, Senthamaraikannan P, Cappelletti M, Hammons M, Miller LA, Jobe AH, Chougnet CA, DeFranco E and Kallapur SG (2024) IL-1 and TNF mediates IL-6 signaling at the maternal-fetal interface during intrauterine inflammation. *Front. Immunol.* 15:1416162. doi: 10.3389/fimmu.2024.1416162

COPYRIGHT
© 2024 Presicce, Roland, Senthamaraikannan, Cappelletti, Hammons, Miller, Jobe, Chougnet, DeFranco and Kallapur. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

IL-1 and TNF mediates IL-6 signaling at the maternal-fetal interface during intrauterine inflammation

Pietro Presicce[1†], Cynthia Roland[2†], Paranthaman Senthamaraikannan[3], Monica Cappelletti[4], McKensie Hammons[1], Lisa A. Miller[5], Alan H. Jobe[3], Claire A. Chougnet[6], Emily DeFranco[2] and Suhas G. Kallapur[1*]

[1]Divisions of Neonatology and Developmental Biology, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, CA, United States, [2]Department of Obstetrics/Gynecology, Maternal-Fetal Medicine, University of Cincinnati, Cincinnati, OH, United States, [3]Division of Neonatology/Pulmonary Biology, Cincinnati Children's Hospital Research Foundation, and the University of Cincinnati College of Medicine, Cincinnati, OH, United States, [4]Division of Immunogenetics, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, CA, United States, [5]Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, Davis, CA, United States, [6]Immunobiology, Cincinnati Children's Hospital Research Foundation, and the University of Cincinnati College of Medicine, Cincinnati, OH, United States

Introduction: IL6 signaling plays an important role in triggering labor and IL6 is an established biomarker of intrauterine infection/inflammation (IUI) driven preterm labor (PTL). The biology of IL6 during IUI at the maternal-fetal interface was investigated in samples from human subjects and non-human primates (NHP).

Methods: Pregnant women with histologic chorioamnionitis diagnosed by placenta histology were recruited (n=28 term, n=43 for preterm pregnancies from 26-36 completed weeks of gestation). IUI was induced in Rhesus macaque by intraamniotic injection of lipopolysachharide (LPS, n=23). IL1 signaling was blocked using Anakinra (human IL-1 receptor antagonist, n=13), and Tumor necrosis factor (TNF) signaling was blocked by anti TNF-antibody (Adalimumab n=14). The blockers were given before LPS. All animals including controls (intraamniotic injection of saline n=27), were delivered 16h after LPS/saline exposure at about 80% gestation.

Results: IUI induced a robust expression of *IL6* mRNAs in the fetal membranes (chorion-amnion-decidua tissue) both in humans (term and preterm) and NHP. The major sources of *IL6* mRNA expression were the amnion mesenchymal cells (AMC) and decidua stroma cells. Additionally, during IUI in the NHP, *ADAM17* (a protease that cleaves membrane bound IL6 receptor (IL6R) to release a soluble form) and *IL6R* mRNA increased in the fetal membranes, and the ratio of IL6 and soluble forms of IL6R, gp130 increased in the amniotic fluid signifying upregulation of IL6 trans-signaling. Both IL1 and TNF blockade suppressed LPS-induced *IL6* mRNAs in the AMC and variably decreased elements of IL6 trans-signaling.

10.3389/fimmu.2024.1416162

Discussion: These data suggest that IL1 and TNF blockers may be useful anti-inflammatory agents via suppression of IL6 signaling at the maternal-fetal interface.

KEYWORDS

chorioamnionitis, amnion, inflammation, innate immunity, reproductive immunology

Introduction

Intrauterine infection or inflammation (IUI) is a major cause of preterm labor/preterm births (PTL/PTB) (1). In addition to causing prematurity, IUI also causes fetal inflammation with injury to fetal lung, gastrointestinal tract and brain (2–4), further compounding morbidity in the preterm neonate. To date, the most validated marker of IUI is interleukin 6 (IL6) levels in the amniotic fluid (AF), cervical fluid, and cord blood (5–10). IL6 also appears to be involved in the inflammatory cascade leading to PTL because clinical studies show a dose response of AF IL6 with the incidence of PTL, and inhibition of IL6 signaling decreased IUI induced PTB in mice (11–13). We recently reported that in IUI models of Rhesus macaques, a higher expression of IL6 signaling pathways in the fetal membranes is needed to induce PTL (14). Thus, clinical and experimental data strongly implicate IL6 signaling pathway in the causation of PTL/PTB.

IL6 is a pleitropic cytokine expressed by multiple cell types (15), and is known to have both pro- and anti-inflammatory signaling properties in multiple tissues (16). IL6 signaling occurs via three distinct mechanisms. Classic signaling requires a membrane-bound IL6 receptor which forms a complex with the ubiqutoiusly expressed gp130 protein. This complex then activates the JAK-STAT pathway, particularly STAT3. This classic signaling is thought to be regenerative, homeostatic, or anti-inflammatory in nature. In contrast, IL6 trans-signaling occurs when IL6 binds to a soluble IL6 receptor (sIL6R) which then allows IL6 signaling via gp130 in cells not expressing the membrane-bound IL6R and is pro-inflammatory (15, 17). A recently identified third form of signaling called cluster signaling involves preformed complexes of membrane-bound IL6–mIL6R on one cell activating gp130 subunits on target cells (18). Soluble glycoprotein130 (sgp130) by binding to circulating IL6 inhibits IL6 trans-signaling and sgp130 levels decrease in pregnancies complicated by IUI (19, 20). Thus, the ratios of IL6/sIL6R and IL6/sgp130 have often been used as an indicator of IL6 trans-signaling (20–23).

IL1 and TNF are potent cytokines that regulate and orchestrate multiple aspects of immune response. IL1 expression is regulated very tightly with both transcriptional and translational level of control involving the inflammasome complex, which regulates caspase-1 that ultimately cleaves pro-IL1β to active IL1β (24). In clinical trials for cardio-vascular diseases, IL1 inhibitors decrease IL6 signaling (25). We reported that in a preterm Rhesus macaque model of IUI induced by intraamniotic injection of LPS neutrophil infiltration of the fetal membranes, neutrophil activation, and pro-inflammatory mediators in the intrauterine compartments are significantly decreased by inhibition of IL1 or TNF signaling (26, 27). We therefore hypothesized that IL6 signaling during IUI is regulated by IL1 and TNF signaling. IL6 signaling in fetal membranes was compared in women with IUI delivering at preterm and term gestation vs. gestation matched no IUI subjects. Next, we inhibited IL1 or TNF signaling in the Rhesus macaques IUI model with recombinant human IL-1 receptor antagonist (Anakinra) or Adalimumab (anti-TNF antibody). We focused on the fetal membranes as a target tissue for evaluation of IL6 signaling and cell type expression of IL6 since this tissue has the most differential gene expression during IUI (28).

Materials and methods

Human samples

Twenty-eight pregnant women with term pregnancies (>37 weeks of gestation) and forty-three pregnant women with preterm pregnancies from 26^0 to 36^6 weeks of gestation were recruited. Maternal and neonatal demographic characteristics of the cohorts are shown in Supplementary Table 1. IUI was diagnosed by placenta pathology of histologic chorioamnionitis (Chorio) based on Redline's criteria (29). Cohorts were developed based on Chorio positive term and preterm samples. Some preterm samples were used in previous studies (26). The numbers of samples for each experiment are shown in the corresponding figure.

Animals

Time-mated female Rhesus macaques received 1 ml saline solution (n=27) or 1 mg LPS (n=23, MilliporeSigma) in 1 ml saline solution by ultrasound guided intra-amniotic (IA) injection. LPS induced inflammation was blocked by rhIL-1RA (n=13; Anakinra, Sobi) given to the pregnant monkey intra-amntiotically (IA) (50 mg) + maternal subcutaneous (SC) (100 mg) 1 and 3 hours before LPS respectively, or Tumor necrosis factor (TNF) blocker Adalimumab (n=14; Humira, AbbVie Inc. North Chicago, IL) given IA (40 mg) +

SC (40 mg) 1 and 3 h before LPS respectively, as previously described (26, 27, 30). Dams were surgically delivered 16 hours later since our previous results demonstrate that most inflammatory markers were higher at 16 hours compared with longer exposures (Supplementary Figure 1) (14, 27, 30). Some animals were used in previous studies (26, 27, 30, 31). Maternal and neonatal demographic characteristics of the cohorts are shown in Supplementary Table 2. There were no spontaneous deaths or preterm labor in the animals. It was not always possible to obtain all the tissues/fluids from each animal. The numbers of animals for each experiment are shown in the corresponding figure. Sample integrity during varying periods of freezer preservation for different samples was verified and assays with all samples for a particular experiment were run at the same time.

Extra-placental membranes isolation

Human and Rhesus extra-placental chorioamnion-decidua (CAMD) fetal membranes were collected within 30 minutes of delivery, dissected away from the placenta, and prepared as previously described (26, 27, 32). To study the contribution of each layer within the fetal membranes, amnion, chorion, and decidua parietalis were physically separated within 30 minutes of delivery and snap-frozen for RNA studies.

In situ RNA hybridization and immunofluorescence

In situ RNA hybridization and Immunofluorescence dual staining was performed using RNAscope technology (Advanced Cell Diagnostics, Hayward, California) following the manufacturer's protocol. Briefly, formalin fixed paraffin embedded Rhesus and human chorioamnion-decidua (CAMD) was cut into 5 µm sections and mounted on Poly L-Lysine adhesive coated glass slides (Newcomersupply, Middleton, WI). After de-paraffinization for 1h at 60°C, slides were treated for 10 min with Pretreatment 1 solution at room temperature. Subsequently the slides were treated at boiling with Pretreatment 2 solution for 15 min, followed by Pretreatment 3 (protease) for 30 min at 40°C. Following pretreatments, the sections were hybridized with IL-6 probes (RNA scope LS2.5 probe - MMS-497321 and HS-310371, ACD) for 2 hours at 42°C followed by RNA scope amplification (RNAscope 2.5 HD reagent kit, cat.# 322350) and FAST RED chromogenic substrate was used for visualization of staining. As positive and negative controls, RNAscope 2.5 probe-Ppib and RNAscope 2.5 probe_dapB were used. After *In situ* hybridization step, the slides were subjected to immunofluorescence staining to co-localize different proteins by incubation with either anti-human vimentin (Cat.# SC7557; dilution 1:200, Santa Cruz), Pancytokeratin (Cat.#SC81714; dilution 1:100, Santa Cruz), CD45 (CD45–2B11; Cat# 14–9457-82; dilution 1:50, Thermofisher) in 10% normal horse serum/0.2% Tween 20 at 4°C overnight. Staining was visualized using fluorescently labeled secondary antibodies (AF488; 1:200 dilution; Invitrogen) for 1 hour at room temperature. Nuclear counterstain was achieved using ProLong Gold antifade with DAPI. Stained slides were imaged on Leica microscopy. Images were collected using a Zeiss Axioplan 2 microscope and AxioVision 4 software (Zeiss).

IL6, soluble IL6 receptor (sIL6R) and soluble gp130 (sgp130) ELISA

sIL-6R and sgp130 levels in rhesus amniotic fluid were determined by Rhesus macaque IL-6R ELISA kit (cat.# - ELK-IL6sR; RayBio, Norcross, GA) and Human Soluble gp130 Quantikine ELISA kit (cat.# - DGP00; R&D Systems, Minneapolis, MN) following manufacturer's instructions.

RNA Isolation, cDNA generation and quantitative RT-PCR

Total RNA was extracted from snap frozen rhesus tissues after homogenizing in TRIzol. RNA concentration and quality was measured by Nanodrop Spectrophotometer. Reverse transcription of RNA was performed using Verso cDNA synthesis kit (Thermo Fisher Scientific, Grand Island, NY) following manufacturer's protocol. Quantitative RT-PCR (qPCR) was carried out in a StepOnePlus Real Time PCR system (Applied Biosystems) following standard cycling conditions. qPCR assays were performed with Rhesus- and human-specific TaqMan gene expression primers (Thermo-Fisher Scientific, Grand Island, NY). The Eukaryotic 18S mRNA was an endogenous control for normalization of the target RNAs and a sample from an IA saline animal or human preterm chorio negative sample was used as a calibrator. The values were expressed relative to the average value of the control group. For quantification, average of 5 randomly selected HPF fields were plotted as the representative value for the sample. Counts were performed in a blinded manner.

Statistical analyses

GraphPad Prism software (version 9.0) was used to graph and analyze statistical significance. Values were expressed as means ± SEM. Two-tailed Mann-Whitney *U* tests (for non-normally distributed continuous variables), 2-tailed Student *t* test (for Gaussian distributed data points), and Fisher's exact test (for categorical variables) were used to determine differences between groups. Results were considered significant for $P \leq 0.05$.

Results

IUI induced *IL6* gene expression in term and preterm choarioamnion-decidua samples from human subjects

Expression of genes in the IL6 signaling pathway were assessed in our IUI cohort in both term and preterm gestation subjects (see Supplementary Table 1 for demographic and clinical details). First,

the expression levels were assessed in the extraplacental fetal membranes comprising the amnion, chorion, and decidua parietalis. Consistent with previous reports, *IL6* expression increased ~2.5 fold in both term and preterm samples (Figure 1A). The expression of *ADAM17* (encoding a protease that releases membrane bound IL6 receptor to a soluble form (sIL6R)), *IL6R*, and the downstream transcription factor *STAT3* did not change after IUI (Figure 1B). In the term but not preterm samples, there was a decrease in *IL6ST* (*gp130*) expression (Figure 1B). Next, the three different layers of fetal membranes were physically separated to understand the tissue specificity of induction of IL6 pathway. In term samples, IUI induced a significant increase of *IL6* mRNA in all the three layers compared to no IUI samples (Figure 2A). In the preterm compared to no IUI samples, there was a trend toward increased expression of *IL6* in the amnion (p=0.09; Figure 2B). In preterm chorion but not decidua samples, IUI induced an increase in *IL6* expression (Figure 2B), Of note, term samples show a higher magnitude of fold increase compared to preterm samples (compare Figures 2A, B).

Decidua stromal cells and amniotic mesenchymal cells are the major source of *IL-6* during IUI

To understand which cells express *IL6*, immuno-colocalization studies were done using established markers Vimentin (mesenchymal cells) and Pancytokeratin (trophoblastic cells) (33, 34). In term subjects, *IL6* expression increased significantly in the decidua stroma cells (DSC) with trends in amnion mesenchymal cells

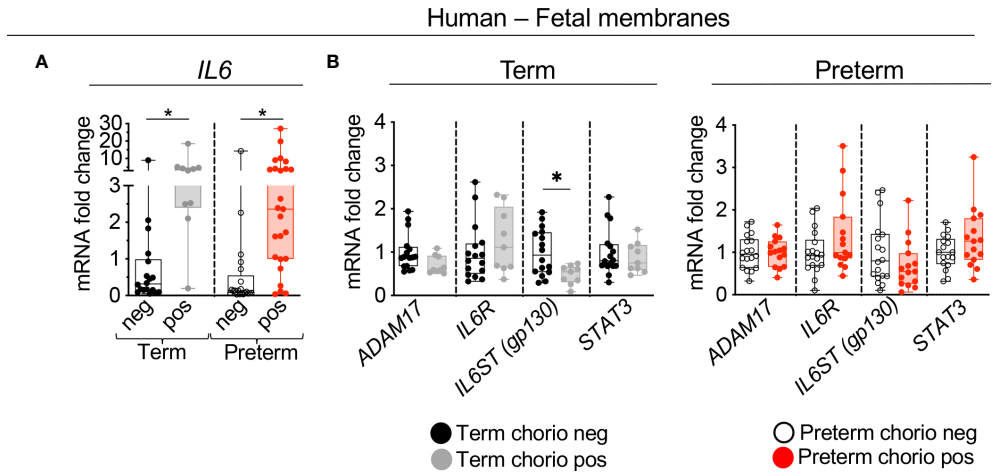


FIGURE 1
Chorioamnionitis increased the expression of *IL6* mRNA in human term and preterm fetal membranes. Human extraplacental chorioamnion-decidua (CAMD, fetal membranes) samples from women that delivered at term or preterm were obtained within 30 minutes of delivery and chorioamnionitis was diagnosed by placenta histology. **(A)** Chorioamnionitis (chorio) induces a significant increase of *IL6* mRNA expression in both preterm and term samples. **(B)** Chorio induces a significant decrease of *IL6ST* (*gp130*) in term CAMD samples compared to term without chorio (Term chorio neg) samples. (Term chorio neg n=18; Term chorio pos n=9–10; Preterm chorio neg n=18–21; Preterm chorio pos n=15–25). Expression of gene mRNA was measured by quantitative PCR (Taqman probes) and average mRNA values are fold increases over the average value for no chorio after internally normalizing to the housekeeping 18S RNA. Data are mean ± SEM, *p < 0.05 vs. samples without chorio (Mann–Whitney U-test).

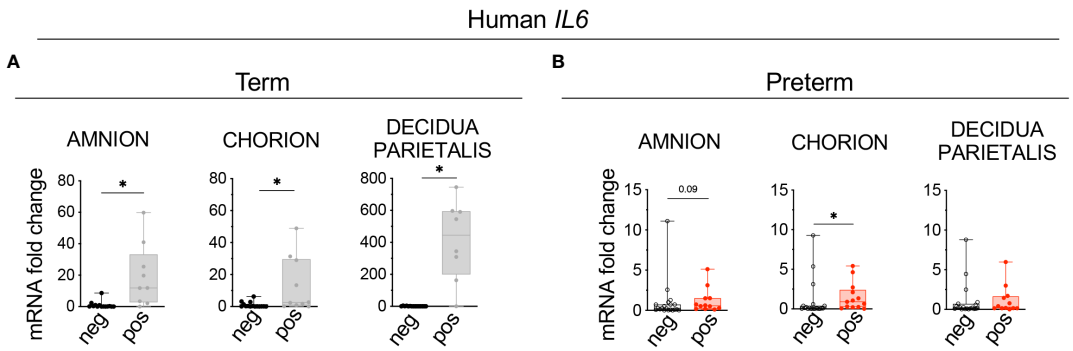


FIGURE 2
Chorio increased significantly the expression of *IL6* mRNA in each of the three tissues (amnion, chorion, decidua) of term samples and in the chorion of preterm samples. Amnion, chorion, and decidua parietalis were physically separated. **(A)** Chorio induces a significant increase of *IL6* mRNA expression in the three layers of term samples (Term neg n=15; Term pos n=10). **(B)** Similar results were observed in the chorion of preterm samples, (Preterm chorio neg n=18–19; Preterm chorio pos n=12–13). Data are mean ± SEM, *p < 0.05 vs. samples without chorio (Mann–Whitney U-test).

10.3389/fimmu.2024.1416162

(AMC) and extra villous trophoblast (EVT) in the chorion (Figures 3A, C). In the preterm subjects both DSC and AMC expression of *IL6* increased significantly with trend toward an increase in the EVT (Figures 3B, C). In the term population DSC was the major cell type expressing *IL6*, while in the preterm both DSC and AMC were dominant cell types expressing *IL6*. To understand contributions from immune cells, *IL6* mRNA detection was combined with immune-colocalization with CD45. Regardless of the presence of inflammation, in both term and preterm human fetal membranes, there were very few $CD45^+$ immune cells expressing *IL6*, and the majority of the cells expressing *IL6* were the non-immune $CD45^-$ cells (Supplementary Figures 2A, B). Similar results

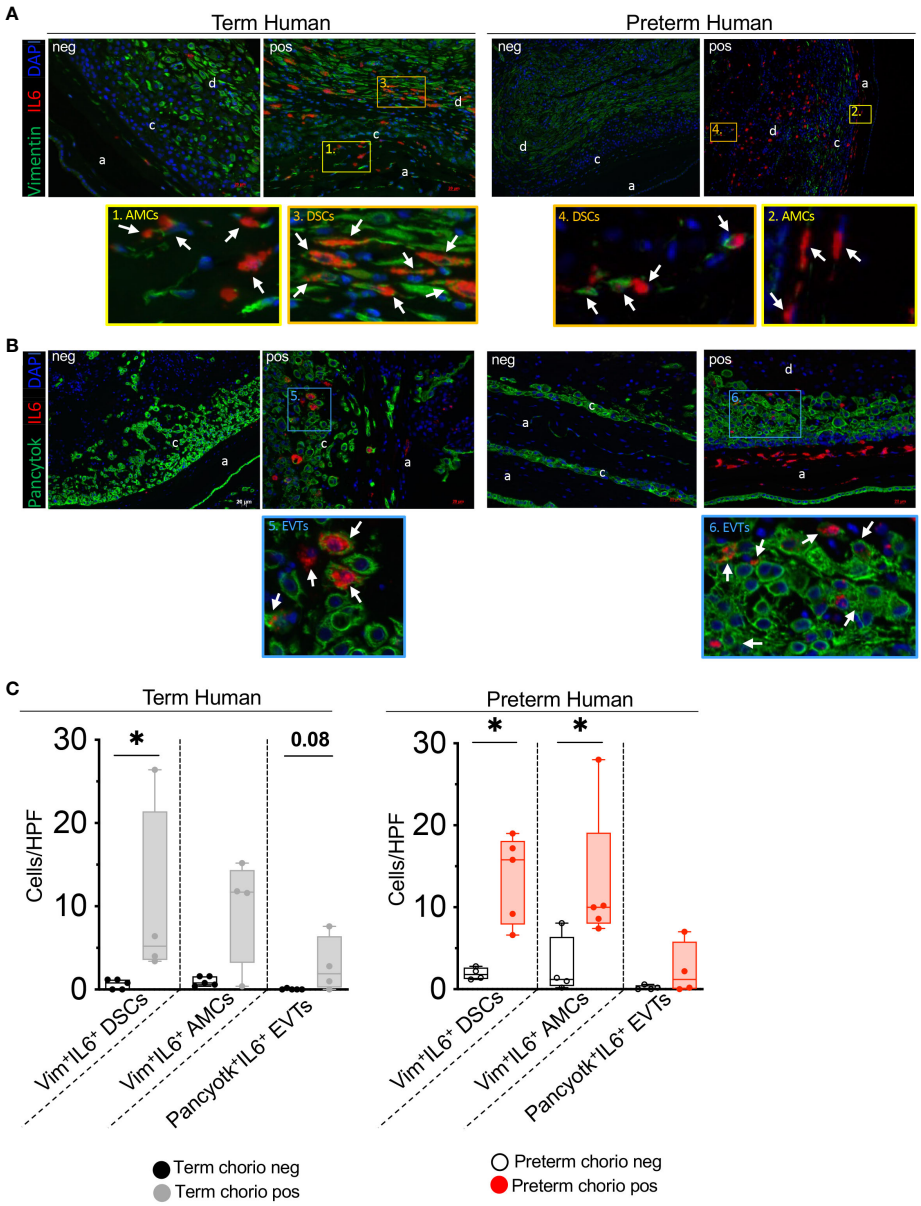


FIGURE 3
Chorio significantly increased the number of human $IL6^+$ stroma cells in the decidua parietalis and $IL6^+$ mesenchymal cells in the amnion. Fixed fetal membranes (chorion-amnion-decidua parietalis) paraffin embedded sections were stained by immuno-colocalization. **(A)** Representative images showing *IL6* mRNA identified by RNAscope *in situ* hybridization and Vimentin (Vim) colocalization by immunofluorescence. *IL6* is shown in red and Vim in green. White arrows in the magnified yellow inset (#1-term, #2-preterm) indicate Vim^+IL6^+ Amniotic Mesenchymal Cells (AMCs), while white arrows in the magnified orange inset (#3-term, #4-preterm) indicate Vim^+IL6^+ Decidual Stromal Cells (DSCs). **(B)** Representative images showing *IL6* mRNA identified by RNAscope *in situ* hybridization and Pan-cytokeratin (Pancytok) colocalization by immunofluorescence. *IL6* is shown in red and Pancytok in green. DAPI indicates nuclear staining (blue) in all images. White arrows in the magnified blue inset indicate $Pancytok^+IL6^+$ Extra Villous Trophoblast (EVTs) in both Term pos (inset #5) and Preterm pos (inset #6) subjects. (a, amnion; c, chorion, and d, decidua) **(C)** Quantification of Vim^+ DSC, AMC, and $Pancytok^+$ EVT cells expressing *IL6* in amnion, chorion, and decidua. Average of 5 randomly selected HPF fields were plotted as the representative value for the sample. Counts were performed in a blinded manner. Data are mean ± SEM. HPF, high-power field; (Term neg n=5; Term pos n=4), (Preterm chorio neg n=4; Preterm chorio pos n=5). *p < 0.05 (Mann–Whitney U-test).

were observed in the Rhesus macaque (Supplementary Figures 3A, B).

IL-1ra and TNF-blockade decrease the expression of components of IL6 signaling pathway in Rhesus IUI induced by LPS

To understand mechanisms of IL6 regulation, experiments were done in our previously established preterm Rhesus macaque model of IUI induced by intraamniotic injection of LPS, where the timing of IUI is known and efficacy of the inhibitors was established (26, 27). Similar to human subjects, *IL6* expression increased in the Rhesus chorion-amnion-decidua parietalis tissue compared to saline controls (Figure 4A). Unlike the humans, *ADAM17*, *IL6R* and *STAT3* expression increased after LPS exposure (Figure 4B). There was no change in *IL6ST* (*gp130*) expression. Both IL1 and TNF inhibition decreased *IL6* mRNA expression in the fetal membranes (Figure 4A). IL1 but not TNF inhibition decreased *ADAM17* expression, but neither inhibitors significantly reduced *IL6R* and *STAT3* expression (Figure 4B).

Next, we assessed the contribution of amnion, chorion, and decidua parietalis separately as in human subjects. Rhesus IUI induced by LPS increased *IL6* mRNA expression in the amnon, chorion, and decidua parietalis with the highest fold increase in the amnion (Figure 5). Both IL1 and TNF inhibition significantly decreased LPS-induced *IL6* expression in the amnion (Figure 5). TNF-blockade but not IL1-blockade decreased significantly *IL6* expression in the chorion (Figure 5). On the contrary, the inhibitors did not decrease *IL6* mRNA expression in the decidua parietalis (Figure 5). To quantify trans-signaling, we measured sIL6R and sgp130 levels in the Rhesus amniotic fluid (AF). We previously reported that LPS-exposure increased significantly IL6 levels in AF and these were decreased by both IL1- and TNF-inhibition (26, 27). However, LPS did not significantly alter soluble IL6 receptor (sIL6R) (Figure 6A) and sgp130 protein levels in the AF (Figure 6B). The ratios of IL6/sIL6R and IL6/sgp130 increased significantly upon LPS exposure compared to saline controls (Figure 6C). IL1-, but not TNF-inhibitor non-significantly decreased the LPS induced IL6/sIL6R (p=0.06) and IL6/sgp130 (p=0.09) ratios (Figure 6C).

Spatial expression of *IL6* in the Rhesus macaque

LPS-exposure significantly increased the number of *IL6*$^+$Vimentin$^+$ amniotic mesenchymal cells (AMCs) (Figures 7A, B). Both IL1 and TNF inhibition significantly decreased the numbers of LPS induced AMC *IL6* expression (Figures 7A, B). In the decidua, LPS nearly significantly increased the number of *IL6*$^+$Vimentin$^+$ decidua stromal cells (DSCs) compared to saline controls (p=0.06; Figures 7A, B), but IL-1ra and TNF-blockade did not affect the number of LPS induced *IL6*$^+$DSCs (Figures 7A, B). In the chorion, the number of *IL6*$^+$Pan-Cytokeratin$^+$ extra villous trophoblasts (EVTs) did not increase upon LPS-exposure (Figures 7C, D).

Discussion

Clinical and experimental evidence strongly demonstrate that IL6 signaling is activated in multiple compartments in maternal and fetal tissues during IUI leading to preterm labor (5–10). While human studies are informative, causal relationship cannot be established since the findings are mainly associative. We therefore

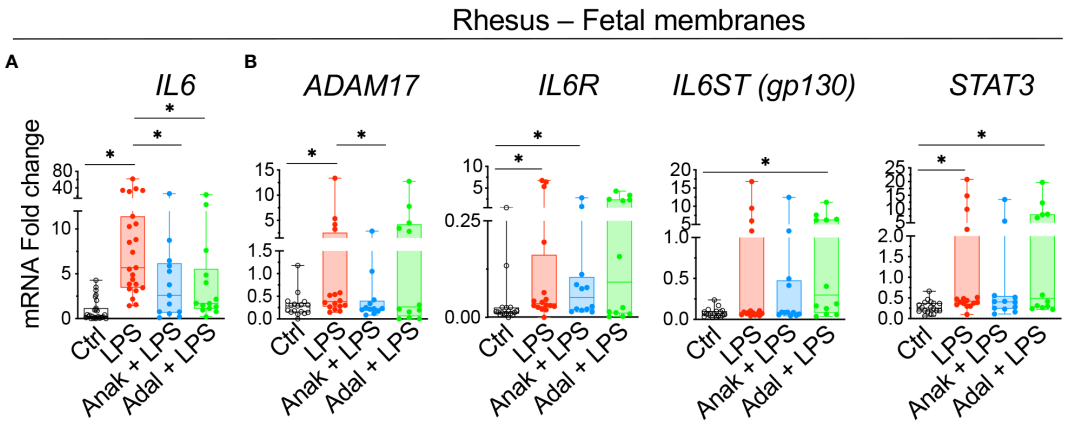


FIGURE 4
Chorioamnionitis increased the expression of *IL6* mRNA in rhesus preterm fetal membranes in a IL1- and TNF-dependent fashion. Chorioamnionitis was induced by intraamniotic (IA) injection of LPS. Controls (Ctrl) received intraamniotic saline. Rhesus extraplacental chorioamnion-decidua (fetal membranes) samples were obtained at delivery 16h after IA LPS/saline and chorioamnionitis (chorio) was diagnosed by placenta histology. mRNAs for molecules in *IL6* signaling were measured. **(A)** Chorio induces a significant increase of *IL6* mRNA expression. Both inhibitors Anakinra (Anak) and Adalimumab (Adal) significantly decreased *IL6* mRNA expression compared to LPS-exposed animals. (Ctrl n=27; LPS n=23; Anak+LPS n=13; Adal +LPS n=14). **(B)** Chorio induces a significant increase of *ADAM17*, *IL6R*, and *STAT3* mRNAs in LPS-exposed samples compared to ctrl samples. Anakinra but not Adalimumab decreased LPS-induced *ADAM17* mRNA. (Ctrl n=18; LPS n=16; Anak+LPS n=12; Adal+LPS n=12). Average mRNA values are fold increases over the average value for no chorio (dotted line) after internally normalizing to the housekeeping 18S RNA. Data are mean ± SEM, *p < 0.05 (Mann–Whitney U-test).

10.3389/fimmu.2024.1416162

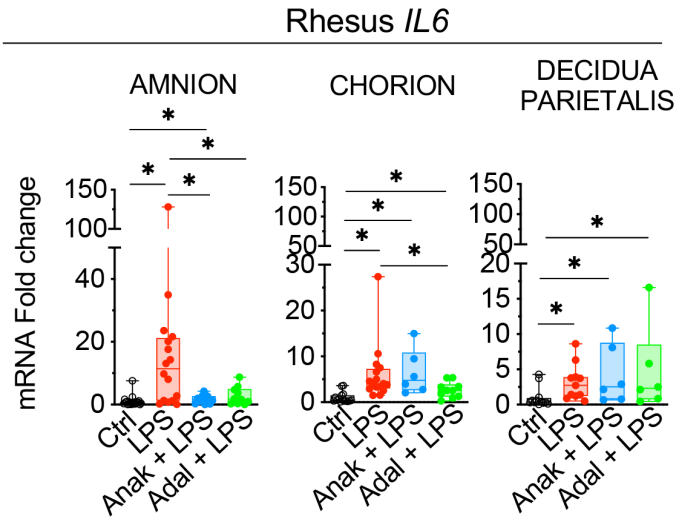


FIGURE 5
Partial decrease of LPS-induced *IL6* mRNAs in fetal membranes by Anakinra and Adalimumab. Amnion, chorion, and decidua parietalis were physically separated. LPS-exposure induced a significant increase of *IL6* mRNA expression in the three tissue layers. Anakinra decreased LPS-induced *IL6* mRNA expression in amnion, while Adalimumab decreased its expression in both amnion and chorion. The inhibitors did not have efficacy in decidua parietalis. Data are mean ± SEM, *p < 0.05 (Mann–Whitney U-test).

paired human studies with a previously validated non-human primate (NHP) model of IUI (26, 27). Main findings of the study are that during IUI the major producers of IL6 are the decidua stroma cells and amnion mesenchymal cells in both preterm Rhesus macaque model of LPS-induced IUI and in preterm human subjects with chorioamnionitis. IL6 trans-signaling, a mechanism known to amplify IL6 signaling (35), is upregulated during IUI. Interesingly, amnion mesenchymal cell but not decidua production of IL6 is inhibited by IL1- or TNF- inhibitors (Figure 8). Although 5–12% of term infants are exposed to IUI (36), there is not much known. This study sheds light on IUI in term infants.

Although IL6 can be secreted by a number of different cell-types we found that the main cell types producing IL6 in both human and Rhesus macaque during IUI are the amnion mesenchymal cell (AMC) and decidua stroma cells (DSC). There were subtle differences in cell-type specific IL6 expression during IUI based on gestational age. In term infants, DSC was a more prominent source of IL6, while in the preterm AMC and DSC were both equally involved (Figure 3). DSC expression of IL6 during chorioamnionitis was previously reported (37). In the preterm Rhesus macaque, AMC was a more prominent source of IL6 expression during IUI (Figure 7). In both the Rhesus macaque and humans, extravillous trophoblast expression of IL6 was

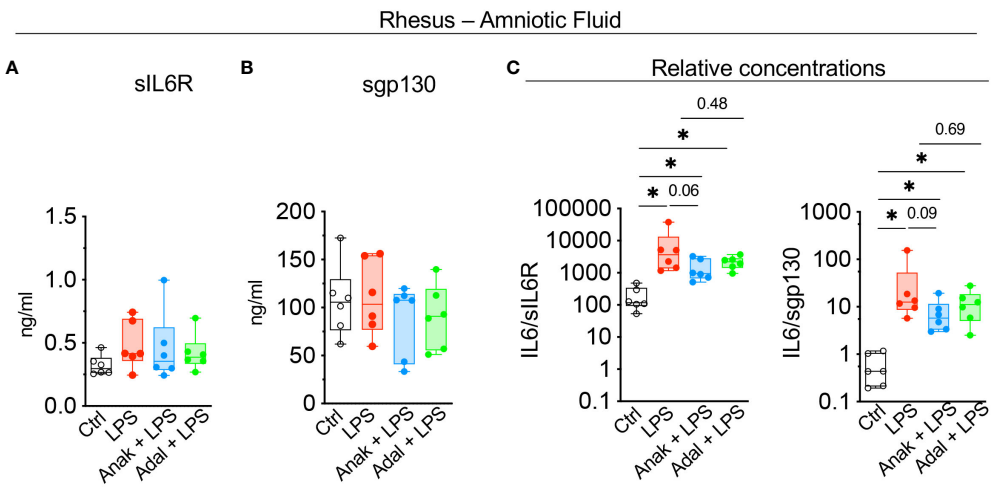


FIGURE 6
LPS-exposure significantly increased IL6/sIL6R and IL6/sgp130 ratios in the amniotic fluid. Cytokine concentrations were measured in amniotic fluid by ELISA. **(A)** Soluble IL6R (sIL6R) and **(B)** soluble gp130 (sgp130) protein levels did not change after LPS exposure. **(C)** Graph shows a significant increase in IL6/sIL6R and IL6/sgp130 ratio after LPS-exposure. Data are mean ± SEM, *p < 0.05 (Mann–Whitney U-test) (Ctrl n=6; LPS n=6; Anak + LPS n=6; Adal + LPS n=6).

10.3389/fimmu.2024.1416162

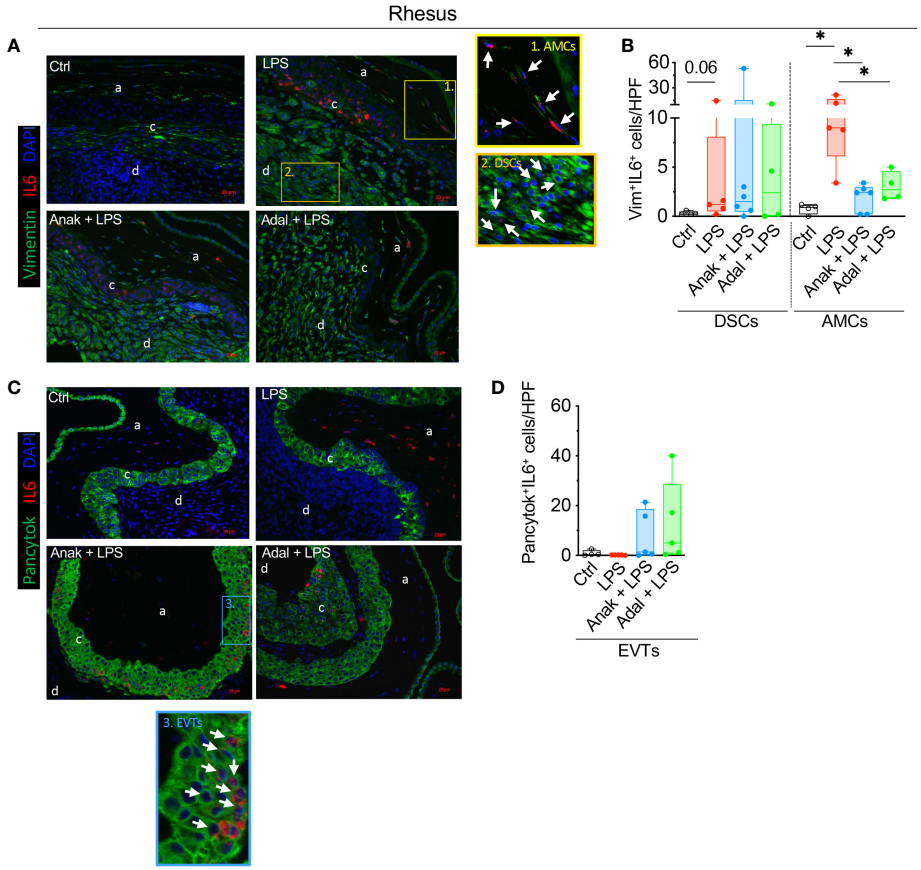


FIGURE 7
LPS exposure increased significantly the number of rhesus *IL6*$^+$ stroma cells in the decidua parietalis and *IL6*$^+$ amnion mesenchymal cells. Fixed fetal membranes (chorion-amnion-decidua parietalis) paraffin embedded sections were stained by immuno-colocalization. **(A)** Representative images showing *IL6* mRNA identified by RNAscope *in situ* hybridization and Vim colocalization by immunofluorescence. *IL6* is shown in red and Vim in green. White arrows in the magnified yellow inset 1 indicate Vim$^+$*IL6*$^+$ Amnion mesenchymal cells (AMCs), while white arrows in the magnified orange inset 2 indicate Vim$^+$IL6$^+$ Decidua stroma cells (DSCs) in LPS-exposed animals. **(B)** Quantification of Vim$^+$ DCSs and Vim$^+$ AMCs expressing *IL6* in amnion, chorion, and decidua. **(C)** Representative images showing *IL6* mRNA identified by RNAscope *in situ* hybridization and Pancytok colocalization by immunofluorescence. *IL6* is shown in red and Pancytok in green. White arrows in the magnified blue inset #3 indicate Pancytok$^+$*IL6*$^+$ EVTs in CAMD section of Anak+LPS animal. subjects. DAPI indicates nuclear staining (blue) in all images. **(D)** Quantification of Pancytok$^+$ extra villous trophoblast (EVT) cells expressing *IL6* in amnion, chorion, and decidua. For quantification, an average of 5 randomly selected HPF fields were plotted as the representative value for the animal. Counts were performed in a blinded manner. HPF, high-power field. Data are mean ± SEM, *p < 0.05 (Mann–Whitney U-test) (Ctrl n=4; LPS n=5; Anak+LPS n=5; Adal+LPS n=5). HPF, high-power field; a, amnion; c, chorion, and d, decidua.

detectable but much less prominent compared to AMC and DSC. Amnion expression of IL6 has been reported before but most of the data are from *in vitro* culture studies (38). Interestingly, neutrophils and macrophage expression of IL6 was not detected in both human and NHP subjects during IUI (Supplementary Figures 2, 3). Our results are contradictory to a previous report of chorio-decidua macrophage expression of IL6 during IUI by Wakabayshi et al. (13). The difference is that our study determined *IL6* mRNA expression while Wakabayshi et al. detected IL6 protein by immunohistology. Neutrophil expression of IL6 is controversial, but *in vitro* IL6 expression has been detected after chromatin modification of IL6 locus during inflammation (39). These results emphasize the finding that IL6 (or other cytokine) secretion is context- and stimulus-dependent and therefore *in vivo* demonstration is important rather than assumptions based on *in vitro* studies.

Amnion cell epithelial-to-mesenchymal transition (EMT) and a "mesenchymal state" of amnion was previously shown to be associated with chorioamnionitis, predispose to rupture of membranes, and induce labor (40–43). We recently reported that a subset of AMC get activated during both Rhesus macaque and human IUI. These activated AMC have increased *NF-kB* signaling and express *CD14* and *IL6* in a TNF-dependent manner (30). Consistently, AMC expression of *IL6*, but not DSC *IL6* expression was inhibited by IL1 and TNF blockers (Figure 7). In another model of Rhesus macaque IUI induced by live *E.coli*, AMC expression of *IL6* was also reported (14). These data are consistent with TNF and NF-kB signaling dependent expression of AMC in preterm infants exposed to IUI (44). Taken together, data from multiple different Rhesus macaque models and human subjects suggest that AMC secretion of IL6 is a key step in orchestrating host immune response to IUI.

IL6 signaling is relatively unique among cytokines in that a natural soluble IL6 receptor (sIL6R) exists and there is a large increase in tissue levels during inflammation (45). sIL6R is primarily generated via

10.3389/fimmu.2024.1416162

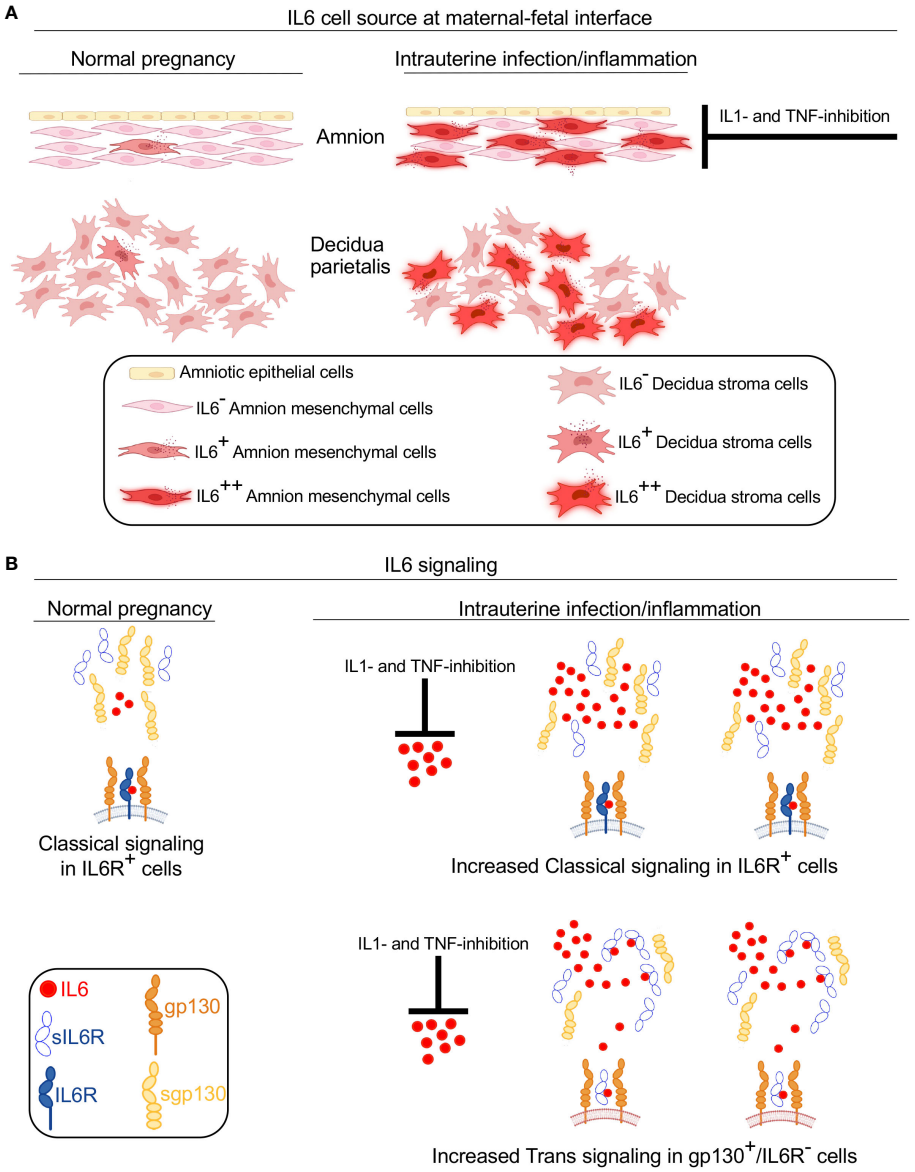


FIGURE 8
Model for IL6 signaling at the maternal-fetal interface during IUI. A schematic model based on data from Rhesus macaque and human subjects showing source and signaling of IL6 in normal pregnancy and during IUI. **(A)** Under normal conditions, a small amount of IL6 is expressed in the amnion and decidua. IUI significantly increases IL6 expression in activated amnion mesenchymal cells (AMC), decidua stroma cells, and to a small extent in chorion trophoblast cells (not shown). IL6 expression by AMC is inhibited by IL1- and TNF-inhibitors. **(B)** During normal pregnancy IL6 signaling occurs in restricted cells expressing IL6 receptor (IL6R) and gp130 and serves homeostatic function (note both IL6R and gp130 are cell membrane spanning receptors). Soluble IL6R (sIL6R) and soluble gp130 (sgp130) lacking membrane spanning domains are secreted in normal conditions. Due to low levels of IL6 in normal conditions, no significant IL6-sIL6R interactions occur. During IUI, large increase of IL6 expression occurs at the maternal-fetal interface, which is inhibited by IL1- and TNF- inhibitors. Increased IL6 concentration promotes increased number of cells signaling via classical IL6-IL6R-gp130 signaling. sIL6R secretion increases slightly during IUI and sgp130 secretion tends to decrease slightly. Increased IL6 in the inflammatory mileu increases binding to sIL6R. This complex can now bind to membrane spanning gp130 that is expressed ubiquitously. This IL6 trans-signaling increases the number and types of cells responding to IL6 and is pro-inflammatory in nature.

proteolytic cleavage enabled ectodomain sheeding of membrane bound IL6R via ADAM17 (45). Once in circulation, sIL6R binds IL6 and this complex "trans-signals" via membrane bound gp130 heterotrimerizing with IL-6/sIL6R. Since gp130 is ubiquitously expressed, this trans-signaling mechanism not only amplifies IL6 signaling, but can now signals in cells that do not express membrane bound IL6R. This trans-signaling is inhibited by soluble gp130, which is primarily generated by mRNA alternative splicing (45) (Figure 8). Recent studies have demonstrated that IL6 preferentially remains in a free form in circulation despite relatively high levels of sIL6R and sgp130 (23) but during inflammation increased IL6 and sIL6R alter the stoichiometry of IL6 complexing with IL6R and sgp130. Clinically, the ratios of IL6/sIL6R and IL6/sgp130 are used as an indicator of IL6 trans-signaling (20–22). We could not quantify IL6 trans-signaling in human subjects in our study due to difficulty in getting amniotic fluid from human subjects, although previous studies demonstrated increased sIL6R in

amniotic fluid from women diagnosed with IUI (20). In our Rhesus macaque IUI model, IL6 trans-signaling appeared to be activated as suggested by increased mRNAs for *ADAM17* and *IL6R* in fetal membranes (Figure 4), and increased amniotic fluid ratios of IL6/sIL6R and IL6/sgp130 (Figure 6). IL1, but not TNF-blockade decreased *ADAM17* expression in fetal membranes exposed to IUI (Figure 4), and tended to reduce LPS-induced IL6/sIL6R and IL6/sgp-130 ratios in the amniotic fluid (Figure 6).

Intraamniotic injection of IL1β or TNF can increase amniotic fluid and fetal IL6 levels (46, 47), suggesting that both IL1 and TNF are upstream regulators of IL6. We previously published that both IL1 and TNF inhibitors robustly decreased LPS induced amniotic fluid IL6 levels in Rhesus macaque IUI models (26, 27). Taken together, these data lend support to the notion that clinically prescribed IL1 and TNF inhibitors for inflammatory conditions (e.g. rheumatoid arthritis) could be used during pregnancy to reduce IL6 trans-signaling and IUI induced adverse consequences. Although definitive data on safety are not available, IL1 and TNF inhibitors use in pregnancy for pre-existing maternal conditions have been reported to be relatively safe (48, 49). An important consideration with any anti-cytokine therapy is to carefully balance the benefits of inhibiting mal-adaptive immune response during inflammation vs. preserving the beneficial effects of normal immunity orchestrated by the cytokines. Another strategy is to consider anti-inflammatory cytokine therapy such as IL10 for intrauterine inflammation (50, 51).

One important consequence of IUI is preterm birth. We previously reported that LPS-induced IUI does not cause preterm birth in Rhesus macaque (14). There is strong evidence in mice that both normal parturition (inflammation is involved in normal parturition) and inflammation-mediated preterm birth is decreased in the absence of IL6 signaling (11–13, 52). These studies used transgenic (IL6 knock out) or IL6R inhibitors to eliminate/knock down IL6 signaling. IL6 inhition in NHP models of preterm birth has not been reported thus far. Interestingly, intraamniotic injection of IL6 in the NHP does not cause chorioamnionitis and preterm labor (46). A possible explanation for these observations is that IL6 inhibition may be effective in conditions where IL6 trans-signaling is induced, and that injecting IL6 alone may induce classical signaling but may not be able to initiate an inflammatory cascade that results in increased IL6 trans-signaling. Although anecdotal data on IL6 inhibitors (e.g. anti-IL6R antibody Tocilizumab) in pregnant women with inflammatory disorders such as rheumatoid arthritis or severe COVID-19 during pregnancy are reported (53), systematic studies of safety and efficacy have not been reported thus far.

Although IL6 signaling is pro-inflammatory, elegant studies have also demonstrated that in gastro-intestinal mucosal surface, IL6 signaling activating gp130 mediates intestinal epithelial regeneration via YAP and NOTCH signaling (54). Whether fetal membranes repair during chorioamnionitis similar to intestinal epithelium during inflammatory bowel disease is not known.

In summary, our study established close similarities in IL6 signaling during chorioamnionitis/IUI between human subjects (both term and preterm deliveries) and a non-human primate (NHP) model, allowing understanding mechanism of IL6 regulation. During IUI, major sources of IL6 production at the maternal-fetal interface are the amnion-mesenchymal cells and decidua stroma cells and IL6 trans-signaling is induced. The amnion mesenchymal cell *IL6* expression is induced during LPS-induced IUI and inhibited by both IL1 and TNF inhibitors. The experiment adds to our understanding of IL6 biology during intrauterine infection/inflammation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Pregnant women provided a written informed consent from 2014 to 2017 under a protocol approved by the Institutional Review Boards (IRBs) of Cincinnati Children's Hospital and University of Cincinnati (#2013-2243) for use of their placenta samples. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. All animal procedures were approved by the IACUC (protocol # 22121) at the University of California Davis Institutional Animal Care and Use Committee and endorsed by the University of California, Los Angeles. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PP: Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. CR: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. PS: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. MC: Formal analysis, Investigation, Methodology, Writing – original draft. MH: Formal analysis, Methodology, Writing – original draft. LM: Writing – original draft. AJ: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. CC: Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. ED: Resources, Writing – original draft. SK: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by U01 ES029234 (CC), Burroughs Wellcome grant (CC), CCHMC Perinatal Infection and Inflammation Collaborative (CC), R21HD90856 (SK), and R01HD 98389 (SK).

Acknowledgments

We thank Sarah Davis, Jennifer Kendrick, Sarah Lockwood, Anne Gibbons, Paul-Michael Sosa, Diana Diaz, and Marie Jose-Lemoy research personnel at the CNPRC, U.C. Davis for help with the animals. Figure 8 was created with BioRender.com (https://BioRender.com).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1416162/full#supplementary-material

References

1. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet*. (2008) 371:75–84. doi: 10.1016/S0140–6736(08)60074–4

2. Been JV, Lievense S, Zimmermann LJ, Kramer BW, Wolfs TG. Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. *J Pediatr*. (2013) 162:236–42 e2. doi: 10.1016/j.jpeds.2012.07.012

3. Venkatesh KK, Leviton A, Hecht JL, Joseph RM, Douglass LM, Frazier JA, et al. Histologic chorioamnionitis and risk of neurodevelopmental impairment at age 10 years among extremely preterm infants born before 28 weeks of gestation. *Am J Obstet Gynecol*. (2020) 223(5):745.e1–745.e10. doi: 10.1016/j.ajog.2020.05.001

4. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. *Semin Fetal Neonatal Med*. (2006) 11:354–62. doi: 10.1016/j.siny.2006.03.004

5. Combs CA, Gravett M, Garite TJ, Hickok DE, Lapidus J, Porreco R, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. *Am J Obstet Gynecol*. (2014) 210:125 e1–125 e15. doi: 10.1016/j.ajog.2013.11.032

6. Chaemsaithong P, Romero R, Korzeniewski SJ, Martinez-Varea A, Dong Z, Yoon BH, et al. A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes. *J Matern Fetal Neonatal Med*. (2016) 29:349–59. doi: 10.3109/14767058.2015.1006620

7. Jacobsson B, Mattsby-Baltzer I, Hagberg H. Interleukin-6 and interleukin-8 in cervical and amniotic fluid: relationship to microbial invasion of the chorioamniotic membranes. *BJOG*. (2005) 112:719–24. doi: 10.1111/j.1471–0528.2005.00536.x

8. Romero R, Yoon B, Kenney J, Gomez R, Allison A, Sehgal P. Amniotic fluid interleukin-6 determinations are of diagnostic and prognostic value in preterm labor. *Am J Reprod Immunol*. (1993) 30:167–83. doi: 10.1111/j.1600-0897.1993.tb00618.x

9. Romero R, Miranda J, Chaiworapongsa T, Korzeniewski SJ, Chaemsaithong P, Gotsch F, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol*. (2014) 72:458–74. doi: 10.1111/aji.12296

10. Romero R, Gomez R, Ghezzi F, Yoon B, Mazor M, Edwin S, et al. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. *Am J Obstet Gynecol*. (1998) 179:186–93. doi: 10.1016/s0002-9378(98)70271-6

11. Robertson SA, Christiaens I, Dorian CL, Zaragoza DB, Care AS, Banks AM, et al. Interleukin-6 is an essential determinant of on-time parturition in the mouse. *Endocrinology*. (2010) 151:3996–4006. doi: 10.1210/en.2010–0063

12. Cappelletti M, Presicce P, Lawson MJ, Chaturvedi V, Stankiewicz TE, Vanoni S, et al. Type I interferons regulate susceptibility to inflammation-induced preterm birth. *JCI Insight*. (2017) 2:e91288. doi: 10.1172/jci.insight.91288

13. Wakabayashi A, Sawada K, Nakayama M, Toda A, Kimoto A, Mabuchi S, et al. Targeting interleukin-6 receptor inhibits preterm delivery induced by inflammation. *Mol Hum Reprod*. (2013) 19:718–26. doi: 10.1093/molehr/gat057

14. Cappelletti M, Presicce P, Feiyang M, Senthamaraikannan P, Miller LA, Pellegrini M, et al. The induction of preterm labor in rhesus macaques is determined by the strength of immune response to intrauterine infection. *PloS Biol*. (2021) 19:e3001385. doi: 10.1371/journal.pbio.3001385

15. Calabrese LH, Rose-John S. IL-6 biology: implications for clinical targeting in rheumatic disease. *Nat Rev Rheumatol*. (2014) 10:720–7. doi: 10.1038/nrrheum.2014.127

16. Vilotic A, Nacka-Aleksic M, Pirkovic A, Bojic-Trbojevic Z, Dekanski D, Jovanovic Krivokuca M. IL-6 and IL-8: an overview of their roles in healthy and pathological pregnancies. *Int J Mol Sci*. (2022) 23(23):14574. doi: 10.3390/ijms232314574

17. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta*. (2011) 1813:878–88. doi: 10.1016/j.bbamcr.2011.01.034

18. Rose-John S, Jenkins BJ, Garbers C, Moll JM, Scheller J. Targeting IL-6 trans-signalling: past, present and future prospects. *Nat Rev Immunol*. (2023) 23(10):666–81. doi: 10.1038/s41577–023–00856-y

19. Jostock T, Mullberg J, Ozbek S, Atreya R, Blinn G, Voltz N, et al. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. *Eur J Biochem*. (2001) 268:160–7. doi: 10.1046/j.1432–1327.2001.01867.x

20. Lee SY, Buhimschi IA, Dulay AT, Ali UA, Zhao G, Abdel-Razeq SS, et al. IL-6 trans-signaling system in intra-amniotic inflammation, preterm birth, and preterm premature rupture of the membranes. *J Immunol*. (2011) 186:3226–36. doi: 10.4049/jimmunol.1003587

21. Rabe B, Chalaris A, May U, Waetzig GH, Seegert D, Williams AS, et al. Transgenic blockade of interleukin 6 transsignaling abrogates inflammation. *Blood*. (2008) 111:1021–8. doi: 10.1182/blood-2007–07-102137

22. von Bismarck P, Claass A, Schickor C, Krause MF, Rose-John S. Altered pulmonary interleukin-6 signaling in preterm infants developing bronchopulmonary dysplasia. *Exp Lung Res*. (2008) 34:694–706. doi: 10.1080/01902140802389693

23. Baran P, Hansen S, Waetzig GH, Akbarzadeh M, Lamertz L, Huber HJ, et al. The balance of interleukin (IL)-6, IL-6.soluble IL-6 receptor (sIL-6R), and IL-6.sIL-6R.sgp130 complexes allows simultaneous classic and trans-signaling. *J Biol Chem*. (2018) 293:6762–75. doi: 10.1074/jbc.RA117.001163

24. Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol*. (2010) 10:89–102. doi: 10.1038/nri2691

25. Ridker PM, Rane M. Interleukin-6 signaling and anti-interleukin-6 therapeutics in cardiovascular disease. *Circ Res*. (2021) 128:1728–46. doi: 10.1161/CIRCRESAHA.121.319077

26. Presicce P, Park CW, Senthamaraikannan P, Bhattacharyya S, Jackson C, Kong F, et al. IL-1 signaling mediates intrauterine inflammation and chorio-decidua neutrophil recruitment and activation. *JCI Insight*. (2018) 3(6):e98306. doi: 10.1172/jci.insight.98306

27. Presicce P, Cappelletti M, Senthamaraikannan P, Ma F, Morselli M, Jackson CM, et al. TNF-signaling modulates neutrophil-mediated immunity at the feto-maternal interface during LPS-induced intrauterine inflammation. *Front Immunol*. (2020) 11:558. doi: 10.3389/fimmu.2020.00558

28. Bukowski R, Sadovsky Y, Goodarzi H, Zhang H, Biggio JR, Varner M, et al. Onset of human preterm and term birth is related to unique inflammatory transcriptome profiles at the maternal fetal interface. *PeerJ*. (2017) 5:e3685. doi: 10.7717/peerj.3685

29. Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C, et al. Amniotic infection syndrome: nosology and reproducibility of placental reaction patterns. *Pediatr Dev Pathol*. (2003) 6:435–48. doi: 10.1007/s10024–003-7070-y

30. Presicce P, Cappelletti M, Morselli M, Ma F, Senthamaraikannan P, Protti G, et al. Amnion responses to intrauterine inflammation and effects of inhibition of TNF signaling in preterm Rhesus macaque. *iScience*. (2023) 26:108118. doi: 10.1016/j.isci.2023.108118

31. Jackson CM, Demmert M, Mukherjee S, Isaacs T, Thompson R, Chastain C, et al. A potent myeloid response is rapidly activated in the lungs of premature Rhesus macaques exposed to intra-uterine inflammation. *Mucosal Immunol*. (2022) 15(4):730–44. doi: 10.1038/s41385–022–00495-x

32. Presicce P, Senthamaraikannan P, Alvarez M, Rueda CM, Cappelletti M, Miller LA, et al. Neutrophil recruitment and activation in decidua with intra-amniotic IL-1beta in the preterm rhesus macaque. *Biol Reprod*. (2015) 92:56. doi: 10.1095/biolreprod.114.124420

33. Heatley MK, Maxwell P, Toner PG. The immunophenotype of human decidua and extra-uterine decidual reactions. *Histopathology*. (1996) 29:437–42. doi: 10.1046/j.1365–2559.1996.d01–516.x

34. Daya D, Sabet L. The use of cytokeratin as a sensitive and reliable marker for trophoblastic tissue. *Am J Clin Pathol*. (1991) 95:137–41. doi: 10.1093/ajcp/95.2.137

35. Prairie E, Cote F, Tsakpinoglou M, Mina M, Quiniou C, Leimert K, et al. The determinant role of IL-6 in the establishment of inflammation leading to spontaneous preterm birth. *Cytokine Growth Factor Rev*. (2021) 59:118–30. doi: 10.1016/j.cytogfr.2020.12.004

36. Romero R, Pacora P, Kusanovic JP, Jung E, Panaitescu B, Maymon E, et al. Clinical chorioamnionitis at term X: microbiology, clinical signs, placental pathology, and neonatal bacteremia - implications for clinical care. *J Perinat Med*. (2021) 49:275–98. doi: 10.1515/jpm-2020–0297

37. Lockwood CJ, Murk WK, Kayisli UA, Buchwalder LF, Huang SJ, Arcuri F, et al. Regulation of interleukin-6 expression in human decidual cells and its potential role in chorioamnionitis. *Am J Pathol*. (2010) 177:1755–64. doi: 10.2353/ajpath.2010.090781

38. Keelan JA, Sato T, Mitchell MD. Interleukin (IL)-6 and IL-8 production by human amnion: regulation by cytokines, growth factors, glucocorticoids, phorbol esters, and bacterial lipopolysaccharide. *Biol Reprod*. (1997) 57:1438–44. doi: 10.1095/biolreprod57.6.1438

39. Zimmermann M, Aguilera FB, Castellucci M, Rossato M, Costa S, Lunardi C, et al. Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun*. (2015) 6:6061. doi: 10.1038/ncomms7061

40. Richardson LS, Taylor RN, Menon R. Reversible EMT and MET mediate amnion remodeling during pregnancy and labor. *Sci Signal*. (2020) 13(618):eaay1486. doi: 10.1126/scisignal.aay1486

41. Janzen C, Sen S, Lei MY, Gagliardi de Assumpcao M, Challis J, Chaudhuri G. The role of epithelial to mesenchymal transition in human amniotic membrane rupture. *J Clin Endocrinol Metab*. (2017) 102:1261–9. doi: 10.1210/jc.2016–3150

42. Richardson L, Menon R. Proliferative, migratory, and transition properties reveal metastate of human amnion cells. *Am J Pathol*. (2018) 188:2004–15. doi: 10.1016/j.ajpath.2018.05.019

43. Weed S, Armistead B, Coleman M, Liggit HD, Johnson B, Tsai J, et al. MicroRNA signature of epithelial-mesenchymal transition in group B streptococcal infection of the placental chorioamniotic membranes. *J Infect Dis*. (2020) 222:1713–22. doi: 10.1093/infdis/jiaa280

44. Toda A, Sawada K, Fujikawa T, Wakabayashi A, Nakamura K, Sawada I, et al. Targeting Inhibitor of kappaB Kinase beta Prevents Inflammation-Induced Preterm Delivery by Inhibiting IL-6 Production from Amniotic Cells. *Am J Pathol*. (2016) 186:616–29. doi: 10.1016/j.ajpath.2015.11.004

45. Jones SA, Scheller J, Rose-John S. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *J Clin Invest*. (2011) 121:3375–83. doi: 10.1172/JCI57158

46. Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ. Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. *Am J Obstet Gynecol*. (2006) 195:1578–89. doi: 10.1016/j.ajog.2006.06.072

47. Kallapur SG, Presicce P, Senthamaraikannan P, Alvarez M, Tarantal AF, Miller LM, et al. Intra-amniotic IL-1beta induces fetal inflammation in rhesus monkeys and alters the regulatory T cell/IL-17 balance. *J Immunol*. (2013) 191:1102–9. doi: 10.4049/jimmunol.1300270

48. Brien ME, Gaudreault V, Hughes K, Hayes DJL, Heazell AEP, Girard S. A systematic review of the safety of blocking the IL-1 system in human pregnancy. *J Clin Med*. (2021) 11(1):225. doi: 10.3390/jcm11010225

49. De Felice KM, Kane S. Safety of anti-TNF agents in pregnancy. *J Allergy Clin Immunol*. (2021) 148:661–7. doi: 10.1016/j.jaci.2021.07.005

50. Thaxton JE, Sharma S. Interleukin-10: a multi-faceted agent of pregnancy. *Am J Reprod Immunol*. (2010) 63:482–91. doi: 10.1111/j.1600–0897.2010.00810.x

51. Kammala AK, Mosebarger A, Radnaa E, Rowlinson E, Vora N, Fortunato SJ, et al. Extracellular Vesicles-mediated recombinant IL-10 protects against ascending infection-associated preterm birth by reducing fetal inflammatory response. *Front Immunol*. (2023) 14:1196453. doi: 10.3389/fimmu.2023.1196453

52. Farias-Jofre M, Romero R, Galaz J, Xu Y, Miller D, Garcia-Flores V, et al. Blockade of IL-6R prevents preterm birth and adverse neonatal outcomes. *EBioMedicine*. (2023) 98:104865. doi: 10.1016/j.ebiom.2023.104865

53. Jorgensen SCJ, Lapinsky SE. Tocilizumab for coronavirus disease 2019 in pregnancy and lactation: a narrative review. *Clin Microbiol Infect*. (2022) 28:51–7. doi: 10.1016/j.cmi.2021.08.016

54. Taniguchi K, Wu LW, Grivennikov SI, de Jong PR, Lian I, Yu FX, et al. A gp130-Src-YAP module links inflammation to epithelial regeneration. *Nature*. (2015) 519:57–62. doi: 10.1038/nature14228

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 18 June 2024
DOI 10.3389/fimmu.2024.1415794

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Kurt Schesser,
University of Miami, United States
Jonatan Leffler,
University of Western Australia, Australia

*CORRESPONDENCE
Xuan Shao
shaoxuan@ioz.ac.cn
Yan-Ling Wang
wangyl@ioz.ac.cn
Amanda N. Sferruzzi-Perri
ans48@cam.ac.uk

†These authors have contributed equally to this work and share the first authorship

RECEIVED 11 April 2024
ACCEPTED 03 June 2024
PUBLISHED 18 June 2024

CITATION
Fan M, Wu H, Sferruzzi-Perri AN, Wang Y-L and Shao X (2024) Endocytosis at the maternal-fetal interface: balancing nutrient transport and pathogen defense. *Front. Immunol.* 15:1415794. doi: 10.3389/fimmu.2024.1415794

COPYRIGHT
© 2024 Fan, Wu, Sferruzzi-Perri, Wang and Shao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Endocytosis at the maternal-fetal interface: balancing nutrient transport and pathogen defense

Mingming Fan[1,2,3†], Hongyu Wu[1,2,3†], Amanda N. Sferruzzi-Perri[4*], Yan-Ling Wang[1,2,3,5,6*] and Xuan Shao[1,2,3,5,6*]

[1]State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [2]Key Laboratory of Organ Regeneration and Reconstruction, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [3]University of Chinese Academy of Sciences, Beijing, China, [4]Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom, [5]Beijing Institute for Stem Cell and Regenerative Medicine, Beijing, China, [6]Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing, China

Endocytosis represents a category of regulated active transport mechanisms. These encompass clathrin-dependent and -independent mechanisms, as well as fluid phase micropinocytosis and macropinocytosis, each demonstrating varying degrees of specificity and capacity. Collectively, these mechanisms facilitate the internalization of cargo into cellular vesicles. Pregnancy is one such physiological state during which endocytosis may play critical roles. A successful pregnancy necessitates ongoing communication between maternal and fetal cells at the maternal-fetal interface to ensure immunologic tolerance for the semi-allogenic fetus whilst providing adequate protection against infection from pathogens, such as viruses and bacteria. It also requires transport of nutrients across the maternal-fetal interface, but restriction of potentially harmful chemicals and drugs to allow fetal development. In this context, trogocytosis, a specific form of endocytosis, plays a crucial role in immunological tolerance and infection prevention. Endocytosis is also thought to play a significant role in nutrient and toxin handling at the maternal-fetal interface, though its mechanisms remain less understood. A comprehensive understanding of endocytosis and its mechanisms not only enhances our knowledge of maternal-fetal interactions but is also essential for identifying the pathogenesis of pregnancy pathologies and providing new avenues for therapeutic intervention.

KEYWORDS

endocytosis, placenta, decidua, macropinocytosis, nutrient, pathogen

1 Introduction

A successful pregnancy necessitates a continuous dialogue between the mother and developing conceptus. This communication is facilitated by intricate interactions between maternal and fetal cells at specific sites in the uterus, collectively referred to as the "maternal-fetal interface." The placenta plays an instrumental role in fetal growth and health by enabling nutrient uptake from the mother's blood, facilitating waste removal from the fetus's blood, ensuring immunological tolerance for the semi-allogenic fetus, and acting as a protective barrier against toxins and pathogens (1–3). The distribution of nutrients between the mother and fetus during pregnancy is predominantly influenced by these maternal-fetal interactions, which are largely regulated by the placenta. Nutrient transport across the maternal-fetal interface occurs through various pathways: passive transport, facilitated diffusion, active transport, and endocytosis (4). While the mechanisms of placental transfer via passive diffusion or membrane transporters have been extensively studied, the roles of alternative mechanisms remain less understood (4). This review aims to provide a comprehensive understanding of endocytosis at the maternal-fetal interface, with a particular focus on the placenta, to elucidate the role of endocytosis in balancing nutrient transfer and pathogen defense.

2 Functional units at the maternal-fetal interfaces in human pregnancy

The well-organized differentiation of trophoblasts and the complex interactions among trophoblasts and a multitude of maternal cells at the maternal-fetal interface results in the formation of several functional units. The first unit involves the uterine decidua, where the interactions among decidual stromal cells, maternal immune cells, and trophoblast cells of fetal origin ensure immune tolerance to the semi-allogenic fetus, while protect against placental/fetal infections (2, 3, 5). The second functional unit is the placental villi, which are fundamental for maternal-fetal material exchange. The multinucleated syncytiotrophoblast (STB), which lines the outer surface of the placental villi, is formed through the fusion of mononucleated cytotrophoblast (CTB) cells. The STB serves as the primary site for maternal-fetal nutrient transport and waste removal, while also acts as a barrier to prevent pathogen infection (2, 3, 6). The third functional unit is the transformed uterine spiral arteries, which enable the perfusion of maternal blood into the maternal-fetal interface. Interactions between extravillous trophoblasts (EVTs; interstitial and endovascular), maternal immune cells, and vascular smooth muscle cells are needed for the transformation/remodeling of the uterine spiral arteries into high-capacity, low-resistance arteries (2, 3, 7) (Figure 1).

2.1 Maternal decidua—functional unit of immune adaptation at the maternal-fetal interface

One main placental function balancing sustained immunologic tolerance to the semi-allogenic fetus while providing adequate protective immunity against infection, which have been interpreted as the "paradox of pregnancy" (8). During early pregnancy, maternal immune cells populate the uterine mucosa, and primarily comprise decidual natural killer (dNK) cells (approximately 50%–70%), followed by macrophages (around 20%), and T cells (between 10%–20%), with dendritic cells (DC), mast cells, and B cells having a minor presence (5, 8). Notably, dNK cells, characterized by their low cytotoxicity and robust cytokine-producing capacity, perform multiple functions related to immune tolerance, embryo development, and protection against

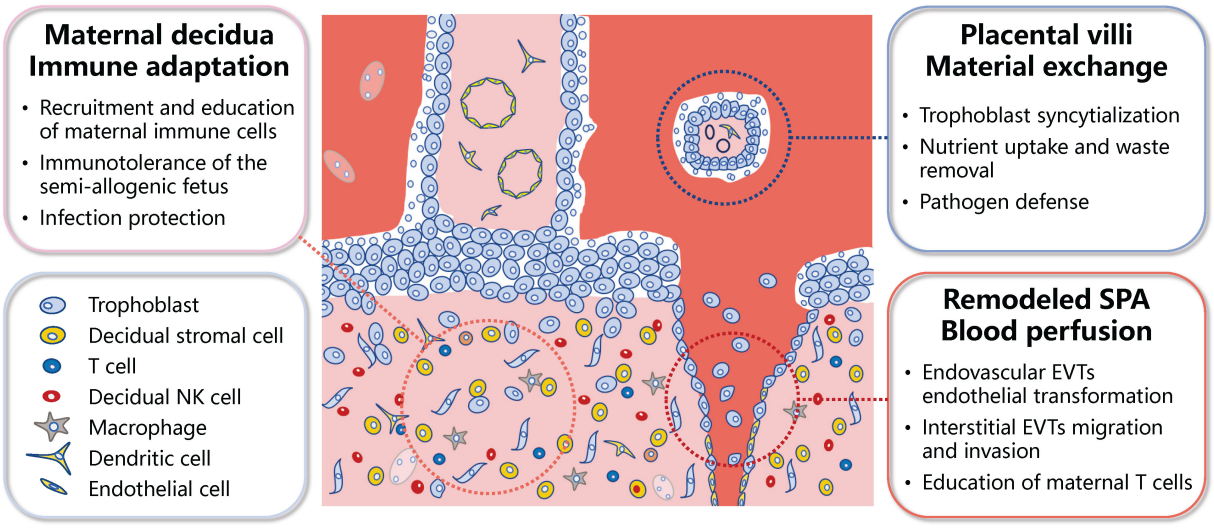


FIGURE 1
Schematic representation of the primary functional units of the human placenta. The meticulous differentiation of placental trophoblasts, coupled with the dynamic interplay between these trophoblasts and a myriad of maternal cells at the maternal-fetal interface, culminates in several functional units within the placenta. These primarily encompass the immune adaptation unit at the maternal-fetal interface, the maternal-fetal material exchange unit, and unit of the blood perfusion into the maternal-fetal interface. SPA, spiral artery; EVTs, extravillous trophoblasts; NK, natural killer.

infection. EVTs express human leukocyte antigen-C (HLA-C), HLA-G, and HLA-E (9–11). These are recognized by inhibitory receptors on dNK cells, thereby reducing cytotoxicity and enhancing the secretion capability of dNK cells, contributing to the regulation of trophoblast invasion and differentiation, remodeling of uterine spiral arteries, and lymphatic mimicry (12–14). The interaction of immune checkpoint inhibitor programmed death-1 (PD-1) and its ligand programmed death ligand-1 (PD-L1) also plays a significant role in establishing immune tolerance at the maternal-fetal interface (2). PD-L1 is predominantly expressed by EVTs and villous STB (15) and its recognition through interacting with PD-1 on decidual immune cells, including macrophages and $CD8^+$ T cells, limits an immune response from being activated. It could also contribute to the formation of M2 polarized macrophages and inhibit the cytotoxicity of $CD8^+$ T cells (16). Decidual macrophages function to clear apoptotic trophoblasts through their phagocytic activity (8). They also produce various factors, including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP9), which promote angiogenesis and tissue remodeling (8). Decidual macrophages also regulate both adaptive T cell responses and innate NK cell response (17).

2.2 Placental villi—functional unit of maternal-fetal material exchange

The syncytium, the outermost layer of the placental villi, is a continuous surface that is estimated to extend 12 to 14 m^2 at term. It is directly exposed to maternal blood and thus ideally poised to act as the primary site for regulating maternal-fetal gas, nutrient, and waste exchange, whilst serving as the frontline for pathogen defense (2, 3, 6). The STB possesses a range of transporters for glucose, amino acids, and fatty acids, indicating their preferential uptake of these low molecular-weight nutrients (6). Most amino acids are transported from the maternal circulation into the fetal circulation via active transport systems across the placenta. Of particular interest are the Na^+- dependent and -independent amino acid transporters, which have been identified in both the apical (maternal) and basal (fetal) facing membranes of the STB of the human placenta (18–20). Total transport rate of a specific amino acid is influenced by its concentration in the maternal versus fetal circulation, and placental surface area, abundance, affinity, and activity of amino acid transporters (21–23). In addition to transporters, recent research has revealed that macropinocytosis, a fluid-phase endocytosis characterized by the formation of large vesicles at the sites of membrane ruffling, functions in the STB to modulate amino acid uptake (6). Although the STB also functions as a barrier to pathogens, some pathogens can pass through STB and infect the fetus. These include Zika virus (ZIKV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (24–29).

2.3 Remodeled uterine spiral arteries—functional unit of blood perfusion into the maternal-fetal interface

In placental mammals, the process of invasion by EVTs into the decidua and the subsequent remodeling of uterine spiral arteries is most prominently observed in humans (30). This phenomenon is postulated to ensure an ample supply of nutrients and oxygen for the intrauterine development of the large human brain (31). The enEVTs play a pivotal role in this process as they invade the spiral arteries, eliminate the smooth muscle cells, and replace the endothelial cells (32–35). Additionally, iEVTs are instrumental as they infiltrate the decidual stroma and contribute to the depletion of vascular smooth muscle cells (36, 37). Following remodeling, the spiral arteries display lymphatic markers such as PROX1 (prospero homeobox gene 1), LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1), and VEGFR3 (vascular endothelial growth factor C (VEGF-C)/VEGF receptor 3) (12). This is partly dependent on dNK cells, which secrete VEGFC and is needed for the activation of VEGFR3 and remodeling of spiral arteries (12). Recent studies have also highlighted the immune regulatory properties of enEVTs. These cells produce transforming growth factor-β1 (TGF-β1) and induce the differentiation of maternal Treg which perform a central role in shielding the fetus from maternal immune attack (7). Transcriptomic analysis also suggests enEVTs have a gene expression profile particularly mirroring that of M2 macrophages and produce various cytokines and chemokines (31).

3 Receptor-mediated endocytosis at the maternal-fetal interface

Receptor-mediated endocytosis (RME) is the most comprehensively characterized pathway for macromolecules and macromolecular complexes (ligands) to enter cells. Upon binding to their respective transmembrane receptors, ligands are internalized into endocytic vesicles that fuse to form early endosomes (38, 39). The sorting process of these internalized ligand-receptor complexes depends on their type; metabolic receptors are recycled back to the plasma membrane, whereas signaling receptors and their associated ligands (e.g. receptor tyrosine kinases or receptors coupled with tyrosine kinase) are delivered to the internal vesicles of multivesicular late endosomes, which are degraded after interaction with lysosomes. During this process, endosomes move from the periphery of the cell to the juxtanuclear region, where they undergo multiple fusion, invagination, tabulation, and membrane fission events (38, 39). Clathrin-mediated endocytosis (CME) is the most extensively studied RME, although clathrin independent endocytosis (CIE) also plays a significant role. This section will provide a comprehensive review of the advancements in research concerning these two endocytosis pathways at the maternal-fetal interface.

3.1 Clathrin-mediated endocytosis

3.1.1 Brief introduction

CME is a receptor-mediated endocytosis during which cargo is packaged into vesicles with the assistance of a clathrin coat. The complexity of the clathrin-mediated endocytic pathway is illustrated by the increasing number of alternative cofactors, adaptors, and tethering proteins that are involved in the

formation of clathrin-coated pits and vesicles (40). The process involves several stages, including initiation, stabilization, maturation, fission, and uncoating (41). Initially, cargo is selected and bound by coat-associated clathrin-adaptor proteins, such as the AP2 complex and scaffold proteins. Clathrin triskelia are recruited directly from the cytosol to the adaptor region of the plasma membrane. Subsequently, the membrane bends, transforming the flat plasma membrane into a "clathrin-coated pit" (CCP), where clathrin polymerizes in hexagons and pentagons around a developing vesicle bud, thereby stabilizing membrane curvature. Following this, GTPase dynamin is recruited to the neck of the CCP by BAR domain-containing proteins and catalyzes the scission of the vesicle from the plasma membrane, releasing the CCP as a clathrin-coated vesicle (42). When clathrin-coated vesicle scission occurs, a gap in the clathrin cage forms, which facilitates the uncoating apparatus, including auxilin and HSC70, to initiate the process of removing the clathrin coat. Ultimately, the disassembly of the clathrin coat releases the nascent cargo-filled vesicle, allowing it to be trafficked further within the cell or transported out of the cell via transcytosis (42, 43). The clathrin machinery is returned back into the cytoplasm to be enlisted and reused for another cycle of clathrin-coated vesicle formation. Depending on cargo versatility, CME has various functions, including nutrient transport into cells, sampling of the cell's environment for growth and guidance cues, and the control of signaling pathway activation (42). Notably, pathogens, including certain viruses like SARS-CoV-2 and ZIKV can also enter cells facilitated by this process (42).

3.1.2 CME functions as a mechanism for nutrition and drug delivery at the maternal-fetal interface

Transmission electron microscopy has revealed that CME occurs within the placental STB, with coated vesicles observed at the microvillous membrane of the STB (44). Transferrin-bound iron and albumin, which are well-established cargo in the placental STB, are transported via CME (45–47). The transferrin-bound iron is internalized by transferrin receptor 1 (TfR1) at the microvillous membrane of the STB (48). Within endosomes, iron then separates from transferrin, undergoes reduction, and then is released into the cytoplasm (49). Albumin, a serum protein, binds a variety of cargos in the blood, including vitamin D and fatty acids (50). Albumin complexes can be transported to the placenta by megalin and cubilin, which are expressed in placental STB (45) and increase during gestation (51). These data suggest that megalin and cubilin may play a significant role in maternal-fetal transfer.

Megalin is a 600-kDa multi-ligand receptor of the low-density lipoprotein receptor-related protein family, characterized by a large amino-terminal extracellular domain, a single transmembrane domain and a short carboxy-terminal cytoplasmic tail believed to have signaling functions (52). Cubilin is a 460-kDa peripheral membrane glycoprotein, with an initial amino-terminal of 110 amino acids, eight epidermal growth factor (EGF)-like repeats and 27 CUB (complement subcomponents C1r/C1s, EGF-related sea urchin protein and bone morphogenic protein-1) domains (53). Cubilin lacks the transmembrane intracellular domain and always works in conjunction with megalin to perform its endocytic function (54). These two multi-ligand receptors have been extensively studied within the kidneys, where they are prominently expressed in the apical region of epithelial cells in the proximal tubule and co-localized with clathrin coated pits and endosomes (55). As previously reported, their potential ligands encompass vitamin B12 and D, folic acid, retinoic acid, albumin, transferrin, apolipoproteins, cholesterol, insulin, EGF, calcium, and aminoglycosides (56). It is postulated that megalin and cubilin may similarly participate in the transport of these cargo by the placenta. However, the molecular mechanisms governing megalin and/or cubilin-mediated transplacental transport of most substrates remain uncharacterized at the maternal-fetal interface.

Furthermore, megalin plays a role in the placental transport of aminoglycoside antibiotics, including gentamicin, used for treating of intra-amniotic infections (57). Consequently, megalin holds potential as a ligand-modified drug nanocarriers designed to deliver therapeutic molecules to the placenta. Of note, PEGylated lipids modified with gentamicin have been shown to undergo clathrin-mediated uptake that involve megalin (58). Furthermore, megalin is needed for the uptake of a liposome encapsulated fluorescence probe in BeWo cells (a choriocarcinoma cell line) (57).

3.1.3 CME can facilitate the entry of viruses at the maternal-fetal interface

In addition to its role in nutrient transport and drug delivery, CME can also serve as a mechanism for the entry of specific viruses into the placenta. SARS-CoV-2, a novel coronavirus responsible for the severe disease known as Coronavirus Disease 2019 (COVID-19), has spread to over 200 countries globally. A multitude of studies have identified the presence of SARS-CoV-2 virus in placental tissue from infected pregnant women, suggesting that villous STB may be the primary target of infection (24–27). The entry factors for SARS-CoV-2 infection, angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) (59), are also expressed by the human placenta (60, 61). Nevertheless, infection can still occur if TMPRSS2 is inadequate, during which the virus–ACE2 complex is internalized via CME (62).

ZIKV has the potential to vertically transmit from an infected mother to her fetus, thereby significantly impacting fetal health. This can lead to conditions such as microcephaly, congenital malformations, and fetal demise (28, 29). Studies conducted using the choriocarcinoma cell lines JEG-3 have shown that ZIKV crosses the placental barrier via both the paracellular pathway and the endocytic and transcytotic pathways (63). In the paracellular pathway, ZIKV infection can disrupt tight junctions, thereby allowing the virus to traverse the compromised barrier. As shown by the use of inhibitors, the endocytic and transcytotic pathways mediating viral entry are facilitated by CME, caveolae-dependent endocytosis and macropinocytosis (63). However, there is a need to understand which specific cell surface receptor(s) are involved in this process. Efforts employing single-cell RNA-sequencing and immunohistochemistry have identified AXL as a potential ZIKV entry receptor in neural stem cells (64). Despite this, knockout of AXL did not protect human neural progenitor cells and cerebral

organoids from ZIKV infection (65). However, recent research using gene knockout and overexpression strategies in human trophoblast stem cells and organoids has shown that AXL and TIM-1, another putative ZIKV entry receptor contribute to the high sensitivity of the placenta to ZIKV infection (66). Therefore, AXL and TIM-1 likely work together to enable ZIKV infection at the maternal-fetal interface, but further research is required.

3.1.4 CME can serve as a bridge for intercellular communication at the maternal-fetal interface

CME is crucial for the communication between the mother and fetus. As previously mentioned, the largest population of leukocytes at the maternal-fetal interface are dNK cells, which exhibit low cytotoxicity. dNK cells interact with HLA-G$^+$ EVTs as the EVTs invade the maternal decidua. Prior studies have shown that upon interaction with EVTs, dNKs acquire HLA-G via trogocytosis, a specific form of endocytosis (67). Mechanistically, filamentous projections from HLA-G-enriched EVTs establish synapses with dNK cells, facilitating direct HLA-G transfer (67). The HLA-G then interacts with dNK cell expressed KIR2DL4, forming a complex that undergoes GTP-dependent internalization via dynamin-mediated CME (68). Data suggests that intracellular HLA-G–KIR2DL4 complexes can lead to sustained intracellular signaling following the abrogation of the immune synapse and uptake of HLA-G. Cytokine activation promotes HLA-G degradation and coincides with increased cytotoxicity by dNK cells. This uptake of EVT HLA-G by trogocytosis confers an immuno-suppressive characteristic to dNK cells, reducing their cytotoxicity. Notably, the disruption of this process by viral products leads to activation of cytotoxicity of dNK cells and the control over virus infection at the maternal-fetal interface (67). Thus, this process provides both maternal-fetal immune tolerance and anti-viral immune function by dNK cells (67).

Maternal Treg cells are pivotal in the immune tolerance of the semi-allograft fetus. These CD4$^+$CD25$^+$Foxp3$^+$ Tregs constitutively and significantly express the immune checkpoint receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4) (69). The protective effect of Tregs on the fetus involve CTLA-4 and the formation of immune synapses on these Treg cells. These interactions facilitate the depletion of CD80/CD86 costimulatory molecules on antigen presenting cells (APCs) through CTLA-4-dependent trogocytosis (69). Furthermore, the downregulation of CD80 by Treg-expressed membrane CTLA-4 disrupts cis-CD80/PD-L1 heterodimers on DCs. This disruption results in the release of soluble PD-L1, which inhibits the activation of PD-1-expressing effector T cells (69). Consequently, this ensures immunotolerance towards the semi-allograft fetus.

Other studies have revealed that the placenta is able to internalize macrophage-derived exosomes via CME, which subsequently modulates the production of cytokines by the placenta, including interleukin (IL)-6, IL-8, IL-10, and IL-12 (70). This mechanism by which immune cells can signal to the placental unit may aid responses to maternal inflammation and infection and thereby, serve to limit harm to the fetus. Indeed, researchers have highlighted that potential role of macrophage-derived exosomes as therapeutics for clinical translation (71). However, further work is needed to study their benefit at the maternal-fetal interface and in improving pregnancy outcomes.

3.2 Clathrin independent endocytosis

3.2.1 Brief introduction

While the CIE plays a crucial role in the cellular communication between cells with their surrounding environment, its understanding lags significantly behind that of the CME. The known functions of CIE encompass extracellular lipid/protein/virus uptake and removal of activated receptors from the plasma membrane to regulate cell polarization, spreading, and migration (72). Importantly, CIE regulates the cellular entry of over twenty viruses (including HIV, Herpes, Ebola, SV40 and Dengue viruses), various toxins, bacteria and prions (such as cholera and Shiga toxins), Streptolysin O and VacA (73). Furthermore, CIE has been identified in numerous *in vitro* cell lines and *in vivo* in mouse, worm, fly, plant, and yeast species (74). However, the mechanisms of CIE remain poorly characterized, particularly at the maternal-fetal interface.

CIE encompasses various types, including caveolin-mediated endocytosis, clathrin-independent carrier (CLIC)/glycosylphosphatidylinositol-anchored protein enriched early endocytic compartment (GEEC) endocytosis (both clathrin and dynamin independent), fast endophilin-mediated endocytosis (FEME, a clathrin-independent but dynamin-dependent pathway for rapid ligand-driven endocytosis of specific membrane proteins), and phagocytosis (75). Although caveolae can bud from the plasma membrane, few cargos rely on them for uptake. Due to the absence of specific endocytosis inhibitors, it is currently not possible to fully distinguish between the FEME or CLIC/GEEC pathways and the caveolae pathway.

3.2.2 Caveolin-mediated endocytosis at the maternal-fetal interface

Caveolae-mediated endocytosis is characterized by their flask-shaped invagination of the plasma membrane. These structures are distinguished by their high concentrations of membrane cholesterol, sphingolipids, and integral membrane caveolin proteins. These components contribute to the curvature of the plasma membrane (76). The ATPase Eps15 homology (EH) domain-containing protein 2 (EHD2), a membrane remodeling protein, has been suggested to form a ring-like oligomer that encircles the caveolar neck. This protein plays a crucial role in stabilizing caveolae at the plasma membrane and regulating endocytosis (77). Following membrane invagination, caveolin-rich vesicles are extracted from the membrane via dynamin action (76). A wealth of literature has established a significant correlation between caveolae and endocytosis. This includes studies in vascular endothelia, suggesting that caveolae facilitate transcellular transport from the vessel lumen into tissues (78). Furthermore, beyond their role in endothelial cells, caveolae also play a comparable function in numerous endocytic processes, such

as the uptake of toxins, viruses, entire bacteria, lipids, and various nanoparticles (75).

Caveolin-1 is expressed in the CTB of term placentas and its expression has also been observed in endothelial cells, pericytes and stromal cells of fetal capillaries but not in STB (79). Thus, caveolin-1-mediated endocytosis may be involved in nutrient uptake by the fetus. In a recent study, intracellular accumulation of caveolin-1 was observed with colocalization of Met in trophoblast isolated from early-onset preeclamptic placentas (80). Additionally, prolonged hypoxic stress was found to enhance endocytosis and inhibit ubiquitin-mediated Met degradation, resulting in impaired regulation of trophoblast invasion by hepatocyte growth factor (HGF)/Met signaling (80).

Riboflavin (vitamin B2) is involved in essential cellular redox reactions. In addition to CME, riboflavin may be taken up into the placenta via other endocytosis pathways. Studies in BeWo cells have demonstrated that riboflavin is co-localized with both clathrin- and Rab5-positive endosomes (81). Furthermore, the uptake of riboflavin required GTPase dynamin activity, suggesting an endocytic uptake mechanism (81). Colocalization of fluorescent ligand endocytosis assays with rhodamine-riboflavin and immuno-stained caveolin-1 in the BeWo cell line has suggested that the uptake of riboflavin involves multiple distinct endocytosis pathways (81). However, these results require confirmation in primary trophoblast or placental villous tissue. Riboflavin transport via specific carrier-mediated mechanisms has also been identified, with the human riboflavin transporter (hRFT1) being observed to be expressed by the human placenta (82). hRFT1 is typically found at the basal membrane of polarized epithelia (83, 84) and therefore, in the placenta, may facilitate riboflavin transport across the basal membrane from the STB to fetal blood. However, this has yet to be studied.

Endocytosis inhibitors, including those that affect caveolae, can block ZIKV transcytosis, suggesting that caveolae may also contribute to ZIKV transcytosis (63). Moreover, the most studied viruses that use caveolar/raft-dependent pathways belong to the polyomavirus family, which includes SV40, mouse polyomavirus (mPy), and two human pathogens, namely BK and JC viruses (85). Each of these viruses use different gangliosides as receptors (86–88). For instance, SV40 requires ganglioside GM1 for its binding, internalization and infection (89), whereas recent studies on mPy have shown that the receptor ganglioside GD1α is not required for binding and endocytosis and instead guides the virus from late endosomes to early endosomes (90). Although previous studies based on some observations have suggested that the uptake of SV40 into different host cells was associated with caveolae, subsequent *in vitro* cell line experiments and studies using mouse primary cells showed that most surface-bound SV40 and mPy in various cell types were not associated with plasma membrane caveolae (90–100). However, the fraction of virus that uses caveolar entry might differ between cell types and the virus; this is supported by the observation that when caveolin-1 was expressed in caveolin-deficient Jurkat cells, infection rates by mPy increased significantly (99). These results suggest that a small percentage of SV40, mPy and possibly other polyomaviruses enter the cytoplasm via the caveolin pathway (101).

While caveolin-mediated endocytosis is crucial for nutrient transport and pathogen entry at the maternal-fetal interface, the significance of caveolae-facilitated endocytosis remains largely unexplored. Further research is required to fully elucidate this matter.

3.2.3 Other endocytosis pathways at the maternal-fetal interface

In addition to the previously mentioned caveolin-mediated endocytosis, numerous other CIE pathways exist. The nutritional demand for folate (vitamin B9) escalates to meet the developmental needs of the fetus during pregnancy. Folate uptake primarily occurs through glycosylphosphatidylinositol (GPI)-modified receptors and folate receptors, which are predominantly expressed in the microvillous membrane of the STB, and involve the CLIC/GEEC pathway (102, 103). Following uptake of folate by the STB, it is transported to the fetus via the STB basal membrane, a process potentially facilitated by the multi-drug resistance protein 1 (MRP1) (102). Endocytosis may also be involved in this process, as the endocytosis inhibitor monensin inhibited uptake of folate by the BeWo cell line (104). The CLIC/GEEC pathway also aids in the endocytosis of GPI-anchored proteins within uncoated tubulovesicular membrane structures, thereby contributing to fluid uptake (76). However, direct evidence for the uptake of cargo by STB through CLIC/GEEC pathway remains to be further investigated.

Transfer of maternal IgG into the fetal circulation confers passive immunity to the fetus. Prior research has established that in human intestinal epithelial cells, IgG is internalized, at least partially, through hFcRn-mediated endocytosis; mechanisms that are both clathrin- and caveolin-independent (105). Studies have shown that hFcRn plays a significant role in the transport of IgG via ex vivo perfused placenta (106). Moreover, it has been verified that IgG exhibits a high affinity for hFcRn within the acidic pH environment of endosomes, while IgG fails to bind to the cell surface under neutral pH conditions (106). Additionally, research in the BeWo cell line suggests that IgG is internalized via fluid-phase endocytosis, with the tight binding of IgG to hFcRn (107). Notably, hFcRn has been identified in the fetal capillary endothelium and STB of human placenta (108). The Fc gamma receptor IIb (FCGR2B) is also expressed by the villous fetal endothelium, however the mechanism underlying the uptake of IgG by the STB and subsequent transport of IgG across the fetal capillary endothelium remains incompletely understood (106, 108). Furthermore, the hFcRn receptor may play a role in the transplacental transport of human cytomegalovirus, which can lead to severe disabilities (4).

Flotillins, a category of integral membrane proteins linked with lipid microdomains, have been implicated in endocytosis processes independent of both clathrin and caveolae. Studies have indicated that flotillins, FLOT1 and FLOT2 are highly expressed in term villous CTBs and endothelial cells, while their expression in the STB is comparatively less (4, 109). This implies that flotillin-dependent endocytosis may not be the primary pathway in the STB, but could play functional roles in the CTB and endothelium. Additionally, flotillins are essential for the endocytosis of molecules including

membrane-bound receptors like CD59, which is implicated in the control of complement activation (4, 109). Further investigations in the human placenta are therefore strongly recommended.

The FEME pathway, a recently identified critical route for the rapid endocytosis of specific transmembrane receptors, is characterized by its independence from clathrin and dependence on dynamin. This pathway plays a pivotal role in a renal cell line (BSC1; a cell line isolated from the kidney of an African green monkey) migration and growth factor signaling (110). Despite these findings, the significance of the FEME pathway at the maternal-fetal interface remains ambiguous and warrants further exploration.

Studies of RME at the maternal-fetal interface are both extensive and challenging. However, as summarized above, current evidence indicates that RME may be critical for facilitating the transfer of various nutrients from the maternal blood to the fetal circulation, whilst also by providing a route for drug and pathogen entry into trophoblast cells.

4 Fluid-phase endocytosis

4.1 Brief introduction of macropinocytosis

Although the mechanisms described in the preceding section can be described as RME, which is primarily responsible for internalizing solid substances, pinocytosis describes a form of fluid-phase endocytosis. Fluid-phase uptake in cells occurs through two distinct processes, namely micropinocytosis and macropinocytosis. Micropinocytosis is a non-specific process that results in small vesicles of less than 0.1 µm in diameter and involves both clathrin-coated and non-coated vesicles (111, 112). Meanwhile, macropinocytosis, a specialized process exclusive to certain cell types, involves the formation of large vesicles measuring between 0.2–5 µm in diameter that form at sites of membrane ruffling (111, 113). Macropinocytic vacuoles (macropinosomes) are formed when membrane ruffles fold back onto the plasma membrane to form fluid-filled cavities that close by membrane fusion. Macropinosome formation is not guided by a particle or a cytoplasmic coat, and this gives rise to their irregular size and shape. Their formation is associated with a transient 5- to 10-fold increase in cellular fluid uptake (101, 114). The cellular components required for macropinocytosis are intricate. While it is independent of clathrin, macropinocytosis necessitates the presence of actin, Rac family small GTPase 1 (RAC1), and Phosphoinositide 3-kinase (PI3K) (111). The verification of fluid-phase uptake via macropinocytosis presents challenges due to the non-specific nature of its cargo and the absence of signature marker proteins. However, the uptake of large molecules currently serves as the most reliable indicator of macropinocytosis. Tracer molecules exceeding 70 kDa are predominantly internalized by macropinocytosis, given that the small size of micropinosomes or endosomes restricts both the magnitude and quantity of high molecular weight molecules (111, 115). After formation, macropinosomes move deeper into the cytoplasm, where they can undergo acidification, as well as homo- and heterotypic fusion events. Depending on the cell type, they either recycle back to the cell surface or feed into the endosome network and mature before fusing with lysosomes (101, 114).

Macropinocytosis is not a universal cellular process. In immature DCs, macrophages, and podocytes, it is constitutively active (116, 117), whereas in other cells, macropinocytosis needs to be activated by extracellular stimuli, such as EGF (118). Macropinocytosis facilitates the efficient internalization of extracellular fluids, with the larger size of the vesicles also aiding in the uptake of large molecules (111). The role of macropinocytosis also varies between cell types. In immature DCs, it is employed to survey the surrounding environment for foreign pathogens (117). In *Dictyostelium discoideum* (amoeba) and Ras-transformed human pancreatic cancer cells, macropinocytosis serves as a primary route for uptake of nutrients, including albumin (119, 120). In podocytes, macropinocytosis is proposed to be essential for maintaining the function of glomerular basement membrane and filtration of the kidney by removing proteins that cross the glomerular basement membrane (111).

4.2 Macropinocytosis serves as a strategy of nutrient uptake in trophoblasts

Amino acids serve as a crucial input for mechanistic target of rapamycin complex 1 (mTORC1) activation in STB, and in turn, mTORC1 signaling is indispensable for amino acid uptake (121). Studies have demonstrated the importance of placental mTOR activation to facilitate plasma membrane expression of specific system A (SNAT2, SLC38A2) and system L (LAT1, SLC7A5) transporter isoforms (122, 123). Furthermore, recent research indicates that macropinocytosis may also occur in placental STB (6). During brief periods of nutrient deprivation, the inactivation of mTORC1 triggers the formation of autophagosomes to engulf intracellular components, including proteins, thereby recycling amino acids for adaptive protein synthesis and ensuring cell survival (124); while during prolonged nutrient deprivation, the persistent suppression of mTORC1 activation of Ras signaling allows the STB to utilize pinocytotic extracellular proteins as an amino acid source, addressing the increased biomass demand for sustained cell viability (124). In primary human trophoblasts and in the BeWo cell line, differentiation towards a syncytium triggers macropinocytosis, which is significantly enhanced during amino acid shortage, as induced by inhibiting mTOR signaling (6). Furthermore, inhibiting mTOR in pregnant mice significantly stimulates macropinocytosis in the STB of the placenta, whilst blocking macropinocytosis worsens the fetal growth restriction caused by mTOR-inhibition (6). Amino acids prompt the recruitment of mTORC1 to lysosomal membranes. Subsequent activation of mTORC1 by growth factor signaling enables lysosomal mTORC1 to monitor the recovery of amino acids from proteins that have been delivered via endocytosis or autophagy (124). Within the distinctive microenvironment of the placental STB, where nutrient supply is entirely reliant on maternal blood perfusion into the intervillous space, sensing of nutrient status in placental syncytium through mTOR activity may hence serve to balance transporter-mediated amino acid uptake and extracellular

protein macropinocytosis (6). This mechanism offers a flexible strategy, enabling the placenta to adapt efficiently to nutrient fluctuations whilst consistently providing biosynthetic substrates for the growing fetus.

A significant unresolved issue is the fate of scavenged large molecules in STB. It is plausible that these molecules may be transported to lysosomes for degradation, subsequently serving as an energy source or recycled building blocks for cellular components, as evidenced in podocytes and tumor cells (111, 119). A deficiency in amino acids (Arg, Lys, and Glu) augments macropinocytosis in syncytialized trophoblasts, and inhibiting macropinocytosis with 5-(N-ethyl-N-isopropyl) amiloride (EIPA) has been shown to diminish fetal and placental weight, reinforcing the idea that large macropinocytosed molecules in the placental STB could potentially serve as a nutrient source for the developing conceptus (6).

4.3 Macropinocytosis serves as a pathway for the entry of pathogens into trophoblasts

Although typically associated with growth factor–induced fluid uptake, a variety of particles, including apoptotic bodies, necrotic cells, bacteria, viruses, and protozoans can induce dramatic, cell-wide plasma membrane ruffling. This process facilitates their macropinocytic internalization alongside fluid uptake (101). Viruses exploit macropinocytosis as a mechanism for cell entry, this includes ZIKV, SARS-CoV-2, mature vaccinia virus virions, species B human adenovirus serotype 3, echovirus 1, group B Coxsackieviruses, herpes simplex virus 1, Kaposi's sarcoma-associated herpesvirus, and human immunodeficiency virus-1 (HIV-1) (2, 27, 101). The evolution of viruses to utilize macropinocytosis for internalization and entry may be attributed to several factors; however, the primary reason is likely particle size, especially in the case of vaccinia and herpes simplex virus 1. These viruses are presumably too large to be taken-up through most other forms of endocytosis. For other viruses, macropinocytic entry could potentially serve as a strategy to expand their host range or tissue specificity (101).

Despite the formidable nature of placental defenses, which can resist most microorganisms, select pathogens such as those from the TORCH group (Toxoplasma, Others, Rubella, Cytomegalovirus, and Herpes simplex virus) can circumvent and/or weaken these barriers for vertical transmission (125–127). The recent explosive outbreak of ZIKV, a new addition to the TORCH pathogens, has been identified as a significant threat to pregnancy, causing severe maternal and fetal outcomes including miscarriage and microcephaly. These outcomes have not previously been associated with other infectious diseases related to flaviviruses (2, 128, 129). The placenta and fetus have been found to be more susceptible to ZIKV infection during early gestation, as no apparent fetal demise or disease has been observed when ZIKV exposure occurs in the later stages of pregnancy (130, 131). This suggests that the maturation of the placental barrier over time may independently restrict fetal infection and mitigate ZIKV-related diseases (130, 131). Using gene manipulating mouse models, it was discovered that type I interferon (IFN-I) plays a crucial role in limiting ZIKV replication and transmission systemically (2). Similarly, human trophoblasts from full-term placentas have been shown to release IFNλ1 to protect the placenta from ZIKV infection through both autocrine and paracrine mechanisms (132). Despite this, several potential cell surface receptors including the TAM (TYRO3, AXL, and MER) receptor family, as well as protein S (PROS1) and growth arrest-specific gene 6 (GAS6) have been proposed to facilitate ZIKV entry into trophoblasts (133). In addition, transcytosis of ZIKV by the trophoblast cell line JEG-3 was significantly reduced by inhibitors of macropinocytosis and caveolae- and clathrin-mediated endocytosis (63). These data suggest that placental transmission of the virus may occur through multiple endocytosis pathways.

Several studies have confirmed the transplacental transmission of SARS-CoV-2 during pregnancy through the use of immunohistochemistry and *in situ* hybridization techniques to detect viral antigens or viral nucleic acid within fetal cells of the placenta (24, 134–140). However, this form of virus transmission is rare (141, 142). Despite this, patients afflicted with SARS-CoV-2 infection display cellular and molecular markers indicative of placenta insufficiency, coupled with a significant antiviral and pro-inflammatory response at the maternal-fetal interface (27). Histological observations including maternal vascular mal-perfusion, fetal vascular mal-perfusion, chronic histiocytic intervillositis, or increased intervillous fibrin have been identified in a limited number of placentas from infected women (27). As previously stated, SARS-CoV-2 primarily enters the human placenta via ACE2 and TMPRSS2, or it is internalized via CME as a virus-ACE2 complex (59–62). Although macropinocytosis is not the primary route of entry for SARS-CoV-2 (143, 144), the virus can activate the signaling pathways that trigger macropinocytosis. This allows the virus to enter the cell by promoting actin-mediated membrane ruffling and lamellipodia formation at sites of membrane perturbation. Consequently, this leads to the closure and formation of large, irregular vesicles (145–147).

The best-documented example of virus entry via macropinocytosis so far is the vaccinia virus (148). Vaccinia fetalis, a rare but frequently fatal complication of primary vaccinia virus (smallpox) vaccination during pregnancy, involves the vertical transfer of the vaccinia virus from mother to fetus (149). Using liver cells as a model, researchers have discovered that vaccinia-induced macropinocytosis exhibits several distinct characteristics. Notably, the association of mature virions with cells triggers the formation of large transient plasma membrane blebs, rather than classical lamellipodial ruffles (101). Furthermore, the membrane of vaccinia is enriched in phosphatidylserine, a phospholipid essential for the macropinocytic clearance of apoptotic debris (150, 151). This suggests that vaccinia virions stimulate a macropinocytic response in host cells by simulating apoptotic bodies (148). However, further research is required to determine whether this virus is able to enter trophoblast and the human placenta through macropinocytosis.

Listeria monocytogenes, a Gram-positive bacterium, is the pathogen responsible for listeriosis, a foodborne disease.

Symptoms of listeriosis can range from gastroenteritis and meningitis to encephalitis, maternal-fetal infections, and septicemia (152). The diverse clinical manifestations of *Listeria monocytogenes* infection underscore its ability to penetrate tight barriers, including the placental barrier in humans (152). Once ingested, the bacterium can traverse the fetoplacental barrier in pregnant women, leading to outcomes such as early pregnancy loss, stillbirth, or fetal infection (153, 154). Both traditional phagocytes, like macrophages, as well as non-phagocytic cells, including epithelial cells, endothelial cells and hepatocytes, internalize *Listeria monocytogenes* (155, 156). The entry of *Listeria monocytogenes* into non-phagocytic cells is facilitated by the bacterial surface-associated internalins A and B (InlA and InlB). InlA and InlB employ the adherens junction protein E-cadherin and the Met tyrosine kinase receptor as host ligands, and exploit the endocytic recycling machinery of these receptors, instigating cytoskeletal remodeling and facilitating bacterial internalization (157, 158). *Listeria monocytogenes* can colonize the placenta through ActA-dependent cell-to-cell spread, transitioning from infected macrophages to the STB layer, or via direct invasion of the trophoblast facilitated by InlA and InlB (159). Furthermore, InlP, a novel virulence factor for listeriosis, interacts with the host protein afadin (160), specifically enhancing *Listeria monocytogenes* transcytosis (161). The colonization of the placenta by *Listeria monocytogenes* occurs through hematogenous spread. Despite the STB being extensively exposed to maternal blood, it maintains relative resistance to *Listeria monocytogenes* infection (161). The ability of EVTs to restrict *Listeria monocytogenes* may be associated with the capacity of dNK cells to directly transfer the antimicrobial peptide granulysin to trophoblasts via nanotubes. This nanotube transfer specifically targets intracellular *Listeria monocytogenes* without damaging the trophoblast, thereby preserving the integrity of the maternal-fetal barrier (162). Other work has reported that *Listeria monocytogenes* can be taken up by human hematopoietic stem cells undergoing myeloid/monocytic differentiation through macropinocytosis (163). However, whether *Listeria monocytogenes* can be internalized by STB through macropinocytosis requires further investigation.

In addition to viruses and bacteria, protozoans can also infiltrate cells via macropinocytosis. The most well-documented example of this is *Toxoplasma gondii*, the causative agent of toxoplasmosis. This obligate intracellular protozoan parasite can invade and proliferate within any nucleated cell of a broad spectrum of homeothermic hosts (164). Predominantly, transmission occurs to the fetus in women who contract their primary infection during pregnancy. Although the majority of infants appear to be healthy at birth, significant long-term consequences can become apparent months or years later (165). The standard internalization of *Toxoplasma gondii*, often referred to as active penetration, involves several stages; initial recognition of the host cell surface, followed by sequential secretion of proteins from parasite micronemes and then rhoptries that assemble a macromolecular complex forming a specialized and transient moving junction (164, 166). The parasite is subsequently internalized through an endocytic process, leading to the formation of a parasitophorous vacuole that does not fuse with lysosomes, where the parasites persist and multiply (167, 168). Treatments that interfere with macropinocytosis, such as incubation with amiloride or IPA-3 (inhibitor targeting PAK1 activation-3), have been shown to increase parasite attachment to the host cell surface, but significantly inhibit parasite internalization when tested on various cell types (164). Immunofluorescence microscopy has revealed that markers of macropinocytosis, such as the Rab5 effector rabankyrin 5 and Pak1, are associated with parasite-containing cytoplasmic vacuoles, suggesting that macropinocytosis plays a role in the entry of *Toxoplasma gondii* into host cells (164). Beyond *Toxoplasma gondii*, other like *Leishmania Mexicana* (169), *Leishmania donovani* (170), and *Trypanosoma cruzi* (171, 172), along with certain bacteria, including *Pseudomonas aeruginosa* (173), may also enter host cells via phagocytosis. Therefore, macropinocytosis might be more frequent than currently appreciated (174). However, further research is required to determine whether parasites can gain entry into trophoblast through macropinocytosis.

5 *In vivo* evidence underscores the pivotal role of endocytosis at the maternal-fetal interface

The current understanding of the role of endocytosis in pregnancy maintenance and pathogen defense is largely derived from cell culture models. However, *in vivo* studies are necessary to extrapolate these findings into a physiological context. Previous research has demonstrated that RGS (G protein signaling)-PX1, also known as sorting nexin 13, is a member of the regulators of RGS and sorting nexin (SNX) protein families that can inhibit Gs-mediated signaling through its RGS domain, thereby regulating endocytosis and EGFR degradation (175, 176). In mice, genetic deficiency of Snx13 has been shown to result in severe fetal growth retardation, neural tube closure defects, blood vessel formation defects and placental labyrinth layer formation defects (177). Whole body Snx13-null embryos also showed embryonic lethality around mid-gestation (177). The visceral yolk sac endoderm cells of Snx13-null conceptuses also showed significant changes in the organization of endocytic compartments, including abnormal localization of several endocytic markers such as megalin, ARH (autosomal recessive hypercholesterolemia), LAMP2 (lysosome-associated membrane glycoprotein 2) and LC3 (microtubule-associated protein light chain 3) (177). These results suggest that Snx13-null embryos have impaired nutrient uptake and transport, which may contribute to the developmental defects and lethality observed (177).

Although direct *in vivo* evidence of the role of endocytosis in pregnancy outcomes is scarce, there is a wealth of indirect evidence. The cystic fibrosis transmembrane conductance regulator (CFTR) is an example of such a gene. It encodes a cAMP-regulated chloride channel and its mutation is the primary cause of cystic fibrosis (178). CFTR is expressed ubiquitously throughout the female reproductive tract including the cervix, ovary, oviduct and uterus (179, 180). *Cftr*$^{-/-}$ mice have been shown to develop chronic inflammation and gastrointestinal disease, slower growth rates and delayed puberty onset compared with wild type mice (181). Two independently derived mouse models of cystic fibrosis were used to study female infertility associated with the disease. Both

models showed reduced fertility as indicated by decreased litter number and litter size. Other reproductive phenotypes in the cystic fibrosis female mice, such as small ovarian and uterine size, irregular estrous cycles and reduced oocyte ovulation rates, were also found to contribute to the reduced fertility (182). Evidence additionally indicates that CFTR plays an important role in proximal tubular endocytosis. This has been demonstrated both in the cystic fibrosis mouse model and patients with cystic fibrosis (183, 184). For instance, mice deficient in CFTR show impaired uptake of ^{125}I-β2-microglobulin, reduced cubilin receptor expression in their kidneys and increased excretion of cubilin and its low molecular weight ligand into urine. These data highlight the importance of CFTR in RME within proximal tubular cells for renal handling of low molecular weight proteins (183, 184). Whether or not CFTR deficiency affects pregnancy outcomes through impaired placental endocytosis remains to be determined.

6 Conclusion

The ongoing dialogue and interaction between maternal and fetal cells are essential for the immunotolerance of the semi-allogeneic fetus, protection from pathogen infection, and nutrient transfer. These processes are mediated by various mechanisms including passive transport, facilitated diffusion, active transport, and multiple endocytosis pathways such as CME, CIE, and pinocytosis. Additionally, chemicals, drugs, and pathogens can also cross this barrier via endocytosis. Thus, endocytosis plays a critical role in successful pregnancies (Figure 2). Despite these findings, there remain substantial gaps in our understanding of the endocytic mechanisms that occur at the maternal-fetal interface. While research on endocytosis has advanced rapidly in other tissues, progress in human placenta has been notably slower. In addition, most of the studies have relied on the use of trophoblast cell lines rather than primary trophoblast, trophoblast organoids, or human placenta specimens. Moreover, some specific endocytosis pathways may be more common than currently thought (e.g. macropinocytosis), which has been hampered by the lack of detailed investigation into their specific pathway components and deficiency of selective inhibitors. Therefore, additional studies of endocytosis at the maternal-fetal interface are required to ensure normal fetal development through nutrient transfer and immune tolerance and to prevent pathogen infections in pregnant women and avoid adverse pregnancy outcomes.

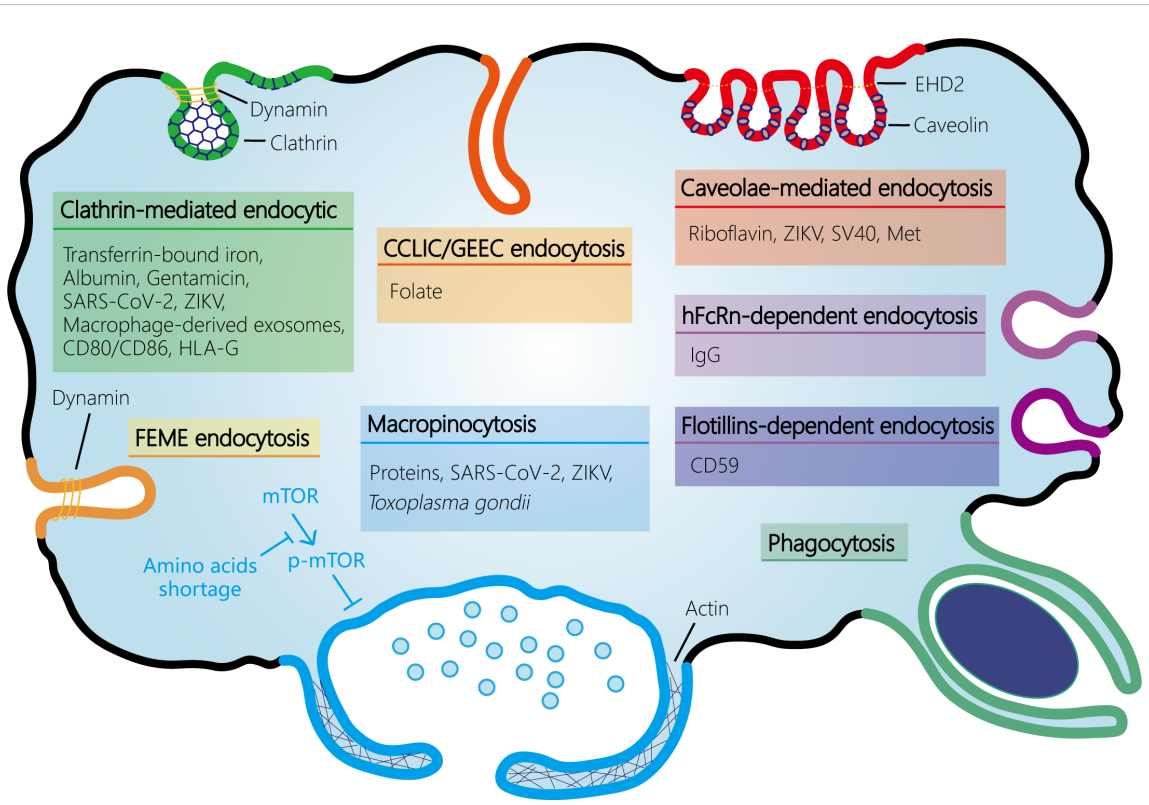


FIGURE 2
Schematic representation of the endocytosis pathways at the maternal-fetal interface. At the maternal-fetal interface, multiple endocytosis pathways exist, including receptor-mediated endocytosis (RME) and fluid-phase endocytosis. RME is subdivided into clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). CIE encompasses several types such as caveolin-mediated endocytosis, clathrin-independent carrier (CLIC)/glycosylphosphatidylinositol-anchored protein enriched early endocytic compartment (GEEC) endocytosis (which is independent of both clathrin and dynamin), fast endophilin-mediated endocytosis (FEME, a pathway for rapid ligand-driven endocytosis of specific membrane proteins that is independent of clathrin but dependent on dynamin), hFcRn- or flotillins-dependent endocytosis, and phagocytosis. Fluid-phase uptake in cells occurs through two separate processes: micropinocytosis and macropinocytosis, which are classified based on the size of the vesicles. Amino acids shortage activates macropinocytosis by inhibiting mTOR phosphorylation and activation in syncytiotrophoblast. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ZIKV, Zika virus; EHD2, Eps15 homology (EH) domain-containing protein 2.

Author contributions

MF: Writing – original draft, Visualization. HW: Writing – original draft, Visualization. AS: Writing – review & editing. YW: Writing – review & editing, Funding acquisition. XS: Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the grants from National Key Research and Development Program of China (2022YFC2702400, 2022YFC2702500, 2022YFC2704700), and National Natural Science Foundation in China (82192872, 32171115).

References

1. Ji L, Brkic J, Liu M, Fu G, Peng C, Wang YL. Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia. *Mol Aspects Med*. (2013) 34:981–1023. doi: 10.1016/j.mam.2012.12.008

2. Shao X, Yu W, Yang Y, Wang F, Yu X, Wu H, et al. The mystery of the life tree: the placentasdagger. *Biol Reprod*. (2022) 107:301–16. doi: 10.1093/biolre/ioac095

3. Yu X, Wu H, Yang Y, Wang F, Wang Y-L, Shao X. Placental development and pregnancy-associated diseases. *Maternal-Fetal Med*. (2022) 4:36–51. doi: 10.1097/FM9.0000000000000134

4. Cooke LDF, Tumbarello DA, Harvey NC, Sethi JK, Lewis RM, Cleal JK. Endocytosis in the placenta: An undervalued mediator of placental transfer. *Placenta*. (2021) 113:67–73. doi: 10.1016/j.placenta.2021.04.014

5. Wang F, Jia W, Fan M, Shao X, Li Z, Liu Y, et al. Single-cell immune landscape of human recurrent miscarriage. *Genomics Proteomics Bioinf*. (2021) 19:208–22. doi: 10.1016/j.gpb.2020.11.002

6. Shao X, Cao G, Chen D, Liu J, Yu B, Liu M, et al. Placental trophoblast syncytialization potentiates macropinocytosis via mTOR signaling to adapt to reduced amino acid supply. *Proc Natl Acad Sci U S A*. (2021) 118:e2017092118. doi: 10.1073/pnas.2017092118

7. Ma Y, Yang Q, Fan M, Zhang L, Gu Y, Jia W, et al. Placental endovascular extravillous trophoblasts (enEVTs) educate maternal T-cell differentiation along the maternal-placental circulation. *Cell Prolif*. (2020) 53:e12802. doi: 10.1111/cpr.12802

8. Ander SE, Diamond MS, Coyne CB. Immune responses at the maternal-fetal interface. *Sci Immunol*. (2019) 4:eaat6114. doi: 10.1126/sciimmunol.aat6114

9. Hiby SE, Walker JJ, O'Shaughnessy KM, Redman CW, Carrington M, Trowsdale J, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med*. (2004) 200:957–65. doi: 10.1084/jem.20041214

10. Moffett A, Chazara O, Colucci F. Maternal allo-recognition of the fetus. *Fertil Steril*. (2017) 107:1269–72. doi: 10.1016/j.fertnstert.2017.05.001

11. Apps R, Murphy SP, Fernando R, Gardner L, Ahad T, Moffett A. Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology*. (2009) 127:26–39. doi: 10.1111/j.1365-2567.2008.03019.x

12. Pawlak JB, Balint L, Lim L, Ma W, Davis RB, Benyo Z, et al. Lymphatic mimicry in maternal endothelial cells promotes placental spiral artery remodeling. *J Clin Invest*. (2019) 129:4912–21. doi: 10.1172/JCI120446

13. Bai Y, Liang J, Liu W, Wang F, Li C. Possible roles of HLA-G regulating immune cells in pregnancy and endometrial diseases via KIR2DL4. *J Reprod Immunol*. (2020) 142:103176. doi: 10.1016/j.jri.2020.103176

14. Moffett A, Colucci F. Co-evolution of NK receptors and HLA ligands in humans is driven by reproduction. *Immunol Rev*. (2015) 267:283–97. doi: 10.1111/imr.12323

15. Lu B, Teng X, Fu G, Bao L, Tang J, Shi H, et al. Analysis of PD-L1 expression in trophoblastic tissues and tumors. *Hum Pathol*. (2019) 84:202–12. doi: 10.1016/j.humpath.2018.10.001

16. Wang SC, Li YH, Piao HL, Hong XW, Zhang D, Xu YY, et al. PD-1 and Tim-3 pathways are associated with regulatory CD8+ T-cell function in decidua and maintenance of normal pregnancy. *Cell Death Dis*. (2015) 6:e1738. doi: 10.1038/cddis.2015.112

17. Houser BL. Decidual macrophages and their roles at the maternal-fetal interface. *Yale J Biol Med*. (2012) 85:105–18.

18. Lin G, Wang X, Wu G, Feng C, Zhou H, Li D, et al. Improving amino acid nutrition to prevent intrauterine growth restriction in mammals. *Amino Acids*. (2014) 46:1605–23. doi: 10.1007/s00726-014-1725-z

19. Regnault TR, Friedman JE, Wilkening RB, Anthony RV, Hay WW Jr. Fetoplacental transport and utilization of amino acids in IUGR–a review. *Placenta*. (2005) 26 Suppl A:S52–62. doi: 10.1016/j.placenta.2005.01.003

20. Avagliano L, Garo C, Marconi AM. Placental amino acids transport in intrauterine growth restriction. *J Pregnancy*. (2012) 2012:972562. doi: 10.1155/2012/972562

21. Christensen HN. Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol Rev*. (1990) 70:43–77. doi: 10.1152/physrev.1990.70.1.43

22. Teasdale F, Jean-Jacques G. Morphometric evaluation of the microvillous surface enlargement factor in the human placenta from mid-gestation to term. *Placenta*. (1985) 6:375–81. doi: 10.1016/S0143-4004(85)80014-X

23. Regnault TR, de Vrijer B, Battaglia FC. Transport and metabolism of amino acids in placenta. *Endocrine*. (2002) 19:23–41. doi: 10.1385/ENDO:19:1

24. Hosier H, Farhadian SF, Morotti RA, Deshmukh U, Lu-Culligan A, Campbell KH, et al. SARS-CoV-2 infection of the placenta. *J Clin Invest*. (2020) 130:4947–53. doi: 10.1172/JCI139569

25. Algarroba GN, Rekawek P, Vahanian SA, Khullar P, Palaia T, Peltier MR, et al. Visualization of severe acute respiratory syndrome coronavirus 2 invading the human placenta using electron microscopy. *Am J Obstet Gynecol*. (2020) 223:275–8. doi: 10.1016/j.ajog.2020.05.023

26. Morotti D, Cadamuro M, Rigoli E, Sonzogni A, Gianatti A, Parolin C, et al. Molecular pathology analysis of SARS-CoV-2 in syncytiotrophoblast and hofbauer cells in placenta from a pregnant woman and fetus with COVID-19. *Pathogens*. (2021) 10:479. doi: 10.3390/pathogens10040479

27. Chen J, Du L, Wang F, Shao X, Wang X, Yu W, et al. Cellular and molecular atlas of the placenta from a COVID-19 pregnant woman infected at midgestation highlights the defective impacts on fetal health. *Cell Prolif*. (2022) 55:e13204. doi: 10.1111/cpr.13204

28. Hoen B, Schaub B, Funk AL, Ardillon V, Boullard M, Cabie A, et al. Pregnancy outcomes after ZIKV infection in French territories in the Americas. *N Engl J Med*. (2018) 378:985–94. doi: 10.1056/NEJMoa1709481

29. Brasil P, Pereira JP Jr., Moreira ME, Ribeiro Nogueira RM, Damasceno L, Wakimoto M, et al. Zika virus infection in pregnant women in Rio de Janeiro. *N Engl J Med*. (2016) 375:2321–34. doi: 10.1056/NEJMoa1602412

30. Cindrova-Davies T, Sferruzzi-Perri AN. Human placental development and function. *Semin Cell Dev Biol*. (2022) 131:66–77. doi: 10.1016/j.semcdb.2022.03.039

31. Ma Y, Yu X, Ye S, Li W, Yang Q, Li YX, et al. Immune-regulatory properties of endovascular extravillous trophoblast cells in human placenta. *Placenta*. (2024) 145:107–16. doi: 10.1016/j.placenta.2023.12.009

32. De Wolf F, De Wolf-Peeters C, Brosens I, Robertson WB. The human placental bed: electron microscopic study of trophoblastic invasion of spiral arteries. *Am J Obstet Gynecol*. (1980) 137:58–70. doi: 10.1016/0002-9378(80)90387-7

33. Pijnenborg R, Dixon G, Robertson WB, Brosens I. Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta*. (1980) 1:3–19. doi: 10.1016/S0143-4004(80)80012-9

34. Ramsey EM, Houston ML, Harris JW. Interactions of the trophoblast and maternal tissues in three closely related primate species. *Am J Obstet Gynecol*. (1976) 124:647–52. doi: 10.1016/0002-9378(76)90068-5

35. Damsky CH, Fisher SJ. Trophoblast pseudo-vasculogenesis: faking it with endothelial adhesion receptors. *Curr Opin Cell Biol*. (1998) 10:660–6. doi: 10.1016/S0955-0674(98)80043-4

36. Ma Y, Yu X, Zhang L, Liu J, Shao X, Li YX, et al. Uterine decidual niche modulates the progressive dedifferentiation of spiral artery vascular smooth muscle cells during human pregnancy dagger. *Biol Reprod*. (2021) 104:624–37. doi: 10.1093/biolre/ioaa208

37. Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta*. (2006) 27:939–58. doi: 10.1016/j.placenta.2005.12.006

38. Eshbach ML, Weisz OA. Receptor-mediated endocytosis in the proximal tubule. *Annu Rev Physiol*. (2017) 79:425–48. doi: 10.1146/annurev-physiol-022516-034234

39. Kornilova ES. Receptor-mediated endocytosis and cytoskeleton. *Biochem (Mosc)*. (2014) 79:865–78. doi: 10.1134/S0006297914090041

40. Marsh M, Helenius A. Virus entry: open sesame. *Cell*. (2006) 124:729–40. doi: 10.1016/j.cell.2006.02.007

41. Robinson MS. Forty years of clathrin-coated vesicles. *Traffic*. (2015) 16:1210–38. doi: 10.1111/tra.12335

42. McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol*. (2011) 12:517–33. doi: 10.1038/nrm3151

43. Lemmon SK. Clathrin uncoating: Auxilin comes to life. *Curr Biol*. (2001) 11:R49–52. doi: 10.1016/S0960-9822(01)00010-0

44. Ockleford CD, Whyte A. Differentiated regions of human placental cell surface associated with exchange of materials between maternal and fetal blood: coated vesicles. *J Cell Sci*. (1977) 25:293–312. doi: 10.1242/jcs.25.1.293

45. Lambot N, Lybaert P, Boom A, Delogne-Desnoeck J, Vanbellinghen AM, Graff G, et al. Evidence for a clathrin-mediated recycling of albumin in human term placenta. *Biol Reprod*. (2006) 75:90–7. doi: 10.1095/biolreprod.105.050021

46. Pearse BM. Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. *Proc Natl Acad Sci U S A*. (1982) 79:451–5. doi: 10.1073/pnas.79.2.451

47. Booth AG, Wilson MJ. Human placental coated vesicles contain receptor-bound transferrin. *Biochem J*. (1981) 196:355–62. doi: 10.1042/bj1960355

48. Bastin J, Drakesmith H, Rees M, Sargent I, Townsend A. Localization of proteins of iron metabolism in the human placenta and liver. *Br J Hematol*. (2006) 134:532–43. doi: 10.1111/j.1365-2141.2006.06216.x

49. Akour AA, Kennedy MJ, Gerk P. Receptor-mediated endocytosis across human placenta: emphasis on megalin. *Mol Pharm*. (2013) 10:1269–78. doi: 10.1021/mp300609c

50. Bouillon R, Schuit F, Antonio L, Rastinejad F. Vitamin D binding protein: A historic overview. *Front Endocrinol (Lausanne)*. (2019) 10:910. doi: 10.3389/fendo.2019.00910

51. Burke KA, Jauniaux E, Burton GJ, Cindrova-Davies T. Expression and immunolocalization of the endocytic receptors megalin and cubilin in the human yolk sac and placenta across gestation. *Placenta*. (2013) 34:1105–9. doi: 10.1016/j.placenta.2013.08.003

52. Saito A, PietroMonaco S, Loo AK, Farquhar MG. Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family. *Proc Natl Acad Sci U S A*. (1994) 91:9725–9. doi: 10.1073/pnas.91.21.9725

53. Moestrup SK, Kozyraki R, Kristiansen M, Kaysen JH, Rasmussen HH, Brault D, et al. The intrinsic factor-vitamin B12 receptor and target of teratogenic antibodies is a megalin-binding peripheral membrane protein with homology to developmental proteins. *J Biol Chem*. (1998) 273:5235–42. doi: 10.1074/jbc.273.9.5235

54. Christensen EI, Birn H. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol*. (2001) 280:F562–73. doi: 10.1152/ajprenal.2001.280.4.F562

55. Verroust PJ, Christensen EI. Megalin and cubilin–the story of two multipurpose receptors unfolds. *Nephrol Dial Transpl*. (2002) 17:1867–71. doi: 10.1093/ndt/17.11.1867

56. Christensen EI, Birn H. Megalin and cubilin: multifunctional endocytic receptors. *Nat Rev Mol Cell Biol*. (2002) 3:256–66. doi: 10.1038/nrm778

57. Akour AA, Kennedy MJ, Gerk PM. The role of megalin in the transport of gentamicin across BeWo cells, an *in vitro* model of the human placenta. *AAPS J*. (2015) 17:1193–9. doi: 10.1208/s12248-015-9778-9

58. Alfaifi AA, Heyder RS, Bielski ER, Almuqbil RM, Kavdia M, Gerk PM, et al. Megalin-targeting liposomes for placental drug delivery. *J Control Release*. (2020) 324:366–78. doi: 10.1016/j.jconrel.2020.05.033

59. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. (2020) 181:271–80.e8. doi: 10.1016/j.cell.2020.02.052

60. Ouyang Y, Bagalkot T, Fitzgerald W, Sadovsky E, Chu T, Martinez-Marchal A, et al. Term human placental trophoblasts express SARS-CoV-2 entry factors ACE2, TMPRSS2, and furin. *mSphere*. (2021) 6:e00250-21. doi: 10.1128/mSphere.00250-21

61. Li M, Chen L, Zhang J, Xiong C, Li X. The SARS-CoV-2 receptor ACE2 expression of maternal-fetal interface and fetal organs by single-cell transcriptome study. *PLoS One*. (2020) 15:e0230295. doi: 10.1371/journal.pone.0230295

62. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. *Nat Rev Mol Cell Biol*. (2022) 23:3–20. doi: 10.1038/s41580-021-00418-x

63. Chiu CF, Chu LW, Liao IC, Simanjuntak Y, Lin YL, Juan CC, et al. The mechanism of the Zika virus crossing the placental barrier and the blood-brain barrier. *Front Microbiol*. (2020) 11:214. doi: 10.3389/fmicb.2020.00214

64. Nowakowski TJ, Pollen AA, Di Lullo E, Sandoval-Espinosa C, Bershteyn M, Kriegstein AR. Expression analysis highlights AXL as a candidate Zika virus entry receptor in neural stem cells. *Cell Stem Cell*. (2016) 18:591–6. doi: 10.1016/j.stem.2016.03.012

65. Wells MF, Salick MR, Wiskow O, Ho DJ, Worringer KA, Ihry RJ, et al. Genetic ablation of AXL does not protect human neural progenitor cells and cerebral organoids from Zika virus infection. *Cell Stem Cell*. (2016) 19:703–8. doi: 10.1016/j.stem.2016.11.011

66. Wu H, Huang XY, Sun MX, Wang Y, Zhou HY, Tian Y, et al. Zika virus targets human trophoblast stem cells and prevents syncytialization in placental trophoblast organoids. *Nat Commun*. (2023) 14:5541. doi: 10.1038/s41467-023-41158-0

67. Tilburgs T, Evans JH, Crespo AC, Strominger JL. The HLA-G cycle provides for both NK tolerance and immunity at the maternal-fetal interface. *Proc Natl Acad Sci U S A*. (2015) 112:13312–7. doi: 10.1073/pnas.1517724112

68. Rajagopalan S, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I, et al. Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol*. (2006) 4:e9. doi: 10.1371/journal.pbio.0040009

69. Tekguc M, Wing JB, Osaki M, Long J, Sakaguchi S. Treg-expressed CTLA-4 depletes CD80/CD86 by trogocytosis, releasing free PD-L1 on antigen-presenting cells. *Proc Natl Acad Sci U S A*. (2021) 118:e2023739118. doi: 10.1073/pnas.2023739118

70. Holder B, Jones T, Sancho Shimizu V, Rice TF, Donaldson B, Bouqueau M, et al. Macrophage exosomes induce placental inflammatory cytokines: A novel mode of maternal-placental messaging. *Traffic*. (2016) 17:168–78. doi: 10.1111/tra.12352

71. Song Y, Hu J, Ma C, Liu H, Li Z, Yang Y. Macrophage-derived exosomes as advanced therapeutics for inflammation: current progress and future perspectives. *Int J Nanomed*. (2024) 19:1597–627. doi: 10.2147/IJN.S449388

72. Sandvig K, Pust S, Skotland T, van Deurs B. Clathrin-independent endocytosis: mechanisms and function. *Curr Opin Cell Biol*. (2011) 23:413–20. doi: 10.1016/j.ceb.2011.03.007

73. Yamauchi Y, Helenius A. Virus entry at a glance. *J Cell Sci*. (2013) 126:1289–95. doi: 10.1242/jcs.119685

74. Doherty GJ, McMahon HT. Mechanisms of endocytosis. *Annu Rev Biochem*. (2009) 78:857–902. doi: 10.1146/annurev.biochem.78.081307.110540

75. Rennick JJ, Johnston APR, Parton RG. Key principles and methods for studying the endocytosis of biological and nanoparticle therapeutics. *Nat Nanotechnol*. (2021) 16:266–76. doi: 10.1038/s41565-021-00858-8

76. Mayor S, Parton RG, Donaldson JG. Clathrin-independent pathways of endocytosis. *Cold Spring Harb Perspect Biol*. (2014) 6:a016758. doi: 10.1101/cshperspect.a016758

77. Matthaeus C, Sochacki KA, Dickey AM, Puchkov D, Haucke V, Lehmann M, et al. The molecular organization of differentially curved caveolae indicates bendable structural units at the plasma membrane. *Nat Commun*. (2022) 13:7234. doi: 10.1038/s41467-022-34958-3

78. McIntosh DP, Schnitzer JE. Caveolae require intact VAMP for targeted transport in vascular endothelium. *Am J Physiol*. (1999) 277:H2222–32. doi: 10.1152/ajpheart.1999.277.6.H2222

79. Linton EA, Rodriguez-Linares B, Rashid-Doubell F, Ferguson DJ, Redman CW. Caveolae and caveolin-1 in human term villous trophoblast. *Placenta*. (2003) 24:745–57. doi: 10.1016/S0143-4004(03)00106-1

80. Li G, Wang Y, Cao G, Ma Y, Li YX, Zhao Y, et al. Hypoxic stress disrupts HGF/Met signaling in human trophoblasts: implications for the pathogenesis of preeclampsia. *J BioMed Sci*. (2022) 29:8. doi: 10.1186/s12929-022-00791-5

81. D'Souza VM, Foraker AB, Free RB, Ray A, Shapiro PS, Swaan PW. cAMP-Coupled riboflavin trafficking in placental trophoblasts: a dynamic and ordered process. *Biochemistry*. (2006) 45:6095–104. doi: 10.1021/bi060138f

82. Yao Y, Yonezawa A, Yoshimatsu H, Masuda S, Katsura T, Inui K. Identification and comparative functional characterization of a new human riboflavin transporter hRFT3 expressed in the brain. *J Nutr*. (2010) 140:1220–6. doi: 10.3945/jn.110.122911

83. Yonezawa A, Masuda S, Katsura T, Inui K. Identification and functional characterization of a novel human and rat riboflavin transporter, RFT1. *Am J Physiol Cell Physiol*. (2008) 295:C632–41. doi: 10.1152/ajpcell.00019.2008

84. Subramanian VS, Subramanya SB, Rapp L, Marchant JS, Ma TY, Said HM. Differential expression of human riboflavin transporters -1, -2, and -3 in polarized epithelia: a key role for hRFT-2 in intestinal riboflavin uptake. *Biochim Biophys Acta*. (2011) 1808:3016–21. doi: 10.1016/j.bbamem.2011.08.004

85. Eash S, Manley K, Gasparovic M, Querbes W, Atwood WJ. The human polyomaviruses. *Cell Mol Life Sci*. (2006) 63:865–76. doi: 10.1007/s00018-005-5454-z

86. Tsai B, Gilbert JM, Stehle T, Lencer W, Benjamin TL, Rapoport TA. Gangliosides are receptors for murine polyoma virus and SV40. *EMBO J*. (2003) 22:4346–55. doi: 10.1093/emboj/cdg439

87. Smith AE, Lilie H, Helenius A. Ganglioside-dependent cell attachment and endocytosis of murine polyomavirus-like particles. *FEBS Lett*. (2003) 555:199–203. doi: 10.1016/S0014-5793(03)01220-1

88. Low JA, Magnuson B, Tsai B, Imperiale MJ. Identification of gangliosides GD1b and GT1b as receptors for BK virus. *J Virol*. (2006) 80:1361–6. doi: 10.1128/JVI.80.3.1361-1366.2006

89. Ewers H, Romer W, Smith AE, Bacia K, Dmitrieff S, Chai W, et al. GM1 structure determines SV40-induced membrane invagination and infection. *Nat Cell Biol*. (2010) 12:11–8; sup pp 1–2. doi: 10.1038/ncb1999

90. Qian M, Cai D, Verhey KJ, Tsai B. A lipid receptor sorts polyomavirus from the endolysosome to the endoplasmic reticulum to cause infection. *PLoS Pathog*. (2009) 5: e1000465. doi: 10.1371/journal.ppat.1000465

91. Tagawa A, Mezzacasa A, Hayer A, Longatti A, Pelkmans L, Helenius A. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. *J Cell Biol*. (2005) 170:769–79. doi: 10.1083/jcb.200506103

92. Roy S, Luetterforst R, Harding A, Apolloni A, Etheridge M, Stang E, et al. Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. *Nat Cell Biol*. (1999) 1:98–105. doi: 10.1038/10067

93. Pelkmans L, Puntener D, Helenius A. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science*. (2002) 296:535–9. doi: 10.1126/science.1069784

94. Pelkmans L, Helenius A. Insider information: what viruses tell us about endocytosis. *Curr Opin Cell Biol*. (2003) 15:414–22. doi: 10.1016/S0955-0674(03)00081-4

95. Pelkmans L, Fava E, Grabner H, Hannus M, Habermann B, Krausz E, et al. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature*. (2005) 436:78–86. doi: 10.1038/nature03571

96. Parton RG, Lindsay M. Exploitation of major histocompatibility complex class I molecules and caveolae by simian virus 40. *Immunol Rev*. (1999) 168:23–31. doi: 10.1111/j.1600-065X.1999.tb01280.x

97. Parton RG. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J Histochem Cytochem*. (1994) 42:155–66. doi: 10.1177/42.2.8288861

98. Norkin LC. Caveolae in the uptake and targeting of infectious agents and secreted toxins. *Adv Drug Delivery Rev*. (2001) 49:301–15. doi: 10.1016/S0169-409X(01)00143-0

99. Liebl D, Difato F, Hornikova L, Mannova P, Stokrova J, Forstova J. Mouse polyomavirus enters early endosomes, requires their acidic pH for productive infection, and meets transferrin cargo in Rab11-positive endosomes. *J Virol*. (2006) 80:4610–22. doi: 10.1128/JVI.80.9.4610-4622.2006

100. Damm EM, Pelkmans L, Kartenbeck J, Mezzacasa A, Kurzchalia T, Helenius A. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell Biol*. (2005) 168:477–88. doi: 10.1083/jcb.200407113

101. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. *Annu Rev Biochem*. (2010) 79:803–33. doi: 10.1146/annurev-biochem-060208-104626

102. Solanky N, Requena Jimenez A, D'Souza SW, Sibley CP, Glazier JD. Expression of folate transporters in human placenta and implications for homocysteine metabolism. *Placenta*. (2010) 31:134–43. doi: 10.1016/j.placenta.2009.11.017

103. Sabharanjak S, Sharma P, Parton RG, Mayor S. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell*. (2002) 2:411–23. doi: 10.1016/S1534-5807(02)00145-4

104. Keating E, Lemos C, Azevedo I, Martel F. Comparison of folic acid uptake characteristics by human placental choriocarcinoma cells at acidic and physiological pH. *Can J Physiol Pharmacol*. (2006) 84:247–55. doi: 10.1139/y05-129

105. Sato K, Nagai J, Mitsui N, Ryoko Y, Takano M. Effects of endocytosis inhibitors on internalization of human IgG by Caco-2 human intestinal epithelial cells. *Life Sci*. (2009) 85:800–7. doi: 10.1016/j.lfs.2009.10.012

106. Firan M, Bawdon R, Radu C, Ober RJ, Eaken D, Antohe F, et al. The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of gamma-globulin in humans. *Int Immunol*. (2001) 13:993–1002. doi: 10.1093/intimm/13.8.993

107. Ellinger I, Schwab M, Stefanescu A, Hunziker W, Fuchs R. IgG transport across trophoblast-derived BeWo cells: a model system to study IgG transport in the placenta. *Eur J Immunol*. (1999) 29:733–44. doi: 10.1002/(ISSN)1521-4141

108. Latvala S, Jacobsen B, Otteneder MB, Herrmann A, Kronenberg S. Distribution of FcRn across species and tissues. *J Histochem Cytochem*. (2017) 65:321–33. doi: 10.1369/0022155417705095

109. Walton JR, Frey HA, Vandre DD, Kwiek JJ, Ishikawa T, Takizawa T, et al. Expression of flotillins in the human placenta: potential implications for placental transcytosis. *Histochem Cell Biol*. (2013) 139:487–500. doi: 10.1007/s00418-012-1040-2

110. Boucrot E, Ferreira AP, Almeida-Souza L, Debard S, Vallis Y, Howard G, et al. Endophilin marks and controls a clathrin-independent endocytic pathway. *Nature*. (2015) 517:460–5. doi: 10.1038/nature14067

111. Chung JJ, Huber TB, Godel M, Jarad G, Hartleben B, Kwoh C, et al. Albumin-associated free fatty acids induce macropinocytosis in podocytes. *J Clin Invest*. (2015) 125:2307–16. doi: 10.1172/JCI79641

112. Damke H, Baba T, van der Bliek AM, Schmid SL. Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J Cell Biol*. (1995) 131:69–80. doi: 10.1083/jcb.131.1.69

113. Kerr MC, Teasdale RD. Defining macropinocytosis. *Traffic*. (2009) 10:364–71. doi: 10.1111/j.1600-0854.2009.00878.x

114. Hewlett LJ, Prescott AR, Watts C. The coated pit and macropinocytic pathways serve distinct endosome populations. *J Cell Biol*. (1994) 124:689–703. doi: 10.1083/jcb.124.5.689

115. Li L, Wan T, Wan M, Liu B, Cheng R, Zhang R. The effect of the size of fluorescent dextran on its endocytic pathway. *Cell Biol Int*. (2015) 39:531–9. doi: 10.1002/cbin.10424

116. Norbury CC, Hewlett LJ, Prescott AR, Shastri N, Watts C. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity*. (1995) 3:783–91. doi: 10.1016/1074-7613(95)90067-5

117. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med*. (1995) 182:389–400. doi: 10.1084/jem.182.2.389

118. Haigler HT, McKanna JA, Cohen S. Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J Cell Biol*. (1979) 83:82–90. doi: 10.1083/jcb.83.1.82

119. Commisso C, Davidson SM, Soydaner-Azeloglu RG, Parker SJ, Kamphorst JJ, Hackett S, et al. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. *Nature*. (2013) 497:633–7. doi: 10.1038/nature12138

120. Hacker U, Albrecht R, Maniak M. Fluid-phase uptake by macropinocytosis in Dictyostelium. *J Cell Sci*. (1997) 110:105–12. doi: 10.1242/jcs.110.2.105

121. Milanesi R, Coccetti P, Tripodi F. The regulatory role of key metabolites in the control of cell signaling. *Biomolecules*. (2020) 10:862. doi: 10.3390/biom10060862

122. Rosario FJ, Powell TL, Jansson T. mTOR folate sensing links folate availability to trophoblast cell function. *J Physiol*. (2017) 595:4189–206. doi: 10.1113/JP272424

123. Rosario FJ, Powell TL, Jansson T. Mechanistic target of rapamycin (mTOR) regulates trophoblast folate uptake by modulating the cell surface expression of FR-alpha and the RFC. *Sci Rep*. (2016) 6:31705. doi: 10.1038/srep31705

124. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*. (2010) 141:290–303. doi: 10.1016/j.cell.2010.02.024

125. Megli CJ, Coyne CB. Infections at the maternal-fetal interface: an overview of pathogenesis and defense. *Nat Rev Microbiol*. (2022) 20:67–82. doi: 10.1038/s41579-021-00610-y

126. Costa ML, de Moraes Nobrega G, Antolini-Tavares A. Key infections in the placenta. *Obstet Gynecol Clin North Am*. (2020) 47:133–46. doi: 10.1016/j.ogc.2019.10.003

127. Chenge S, Ngure H, Kanoi BN, Sferruzzi-Perri AN, Kobia FM. Infectious and environmental placental insults: from underlying biological pathways to diagnostics and treatments. *Pathog Dis*. (2023) 81:ftad024. doi: 10.1093/femspd/ftad024

128. Baud D, Gubler DJ, Schaub B, Lanteri MC, Musso D. An update on Zika virus infection. *Lancet*. (2017) 390:2099–109. doi: 10.1016/S0140-6736(17)31450-2

129. Coyne CB, Lazear HM. Zika virus - reigniting the TORCH. *Nat Rev Microbiol*. (2016) 14:707–15. doi: 10.1038/nrmicro.2016.125

130. Chen J, Liang Y, Yi P, Xu L, Hawkins HK, Rossi SL, et al. Outcomes of congenital Zika disease depend on timing of infection and maternal-fetal interferon action. *Cell Rep*. (2017) 21:1588–99. doi: 10.1016/j.celrep.2017.10.059

131. Jagger BW, Miner JJ, Cao B, Arora N, Smith AM, Kovacs A, et al. Gestational stage and IFN-lambda signaling regulate ZIKV infection in utero. *Cell Host Microbe*. (2017) 22:366–76.e3. doi: 10.1016/j.chom.2017.08.012

132. Bayer A, Lennemann NJ, Ouyang Y, Bramley JC, Morosky S, Marques ET Jr., et al. Type III interferons produced by human placental trophoblasts confer protection against Zika virus infection. *Cell Host Microbe*. (2016) 19:705–12. doi: 10.1016/j.chom.2016.03.008

133. Hastings AK, Yockey LJ, Jagger BW, Hwang J, Uraki R, Gaitsch HF, et al. TAM receptors are not required for Zika virus infection in mice. *Cell Rep*. (2017) 19:558–68. doi: 10.1016/j.celrep.2017.03.058

134. Kotlyar AM, Grechukhina O, Chen A, Popkhadze S, Grimshaw A, Tal O, et al. Vertical transmission of coronavirus disease 2019: a systematic review and meta-analysis. *Am J Obstet Gynecol*. (2021) 224:35–53 e3. doi: 10.1016/j.ajog.2020.07.049

135. Fenizia C, Biasin M, Cetin I, Vergani P, Mileto D, Spinillo A, et al. Analysis of SARS-CoV-2 vertical transmission during pregnancy. *Nat Commun*. (2020) 11:5128. doi: 10.1038/s41467-020-18933-4

136. Vivanti AJ, Vauloup-Fellous C, Prevot S, Zupan V, Suffee C, Do Cao J, et al. Transplacental transmission of SARS-CoV-2 infection. *Nat Commun*. (2020) 11:3572. doi: 10.1038/s41467-020-17436-6

137. Sukhikh G, Petrova U, Prikhodko A, Starodubtseva N, Chingin K, Chen H, et al. Vertical transmission of SARS-CoV-2 in second trimester associated with severe neonatal pathology. *Viruses*. (2021) 13:447. doi: 10.3390/v13030447

138. Shende P, Gaikwad P, Gandhewar M, Ukey P, Bhide A, Patel V, et al. Persistence of SARS-CoV-2 in the first trimester placenta leading to transplacental transmission and fetal demise from an asymptomatic mother. *Hum Reprod*. (2021) 36:899–906. doi: 10.1093/humrep/deaa367

139. Valk JE, Chong AM, Uhlemann AC, Debelenko L. Detection of SARS-CoV-2 in placental but not fetal tissues in the second trimester. *J Perinatol*. (2021) 41:1184–6. doi: 10.1038/s41372-020-00877-8

140. Valdespino-Vazquez MY, Helguera-Repetto CA, Leon-Juarez M, Villavicencio-Carrisoza O, Flores-Pliego A, Moreno-Verduzco ER, et al. Fetal and placental infection with SARS-CoV-2 in early pregnancy. *J Med Virol*. (2021) 93:4480–7. doi: 10.1002/jmv.26965

141. Raschetti R, Vivanti AJ, Vauloup-Fellous C, Loi B, Benachi A, De Luca D. Synthesis and systematic review of reported neonatal SARS-CoV-2 infections. *Nat Commun*. (2020) 11:5164. doi: 10.1038/s41467-020-18982-9

142. Schwartz DA, Baldewijns M, Benachi A, Bugatti M, Collins RRJ, De Luca D, et al. Chronic histiocytic intervillositis with trophoblast necrosis is a risk factor associated with placental infection from coronavirus disease 2019 (COVID-19) and intrauterine maternal-fetal severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) transmission in live-born and stillborn infants. *Arch Pathol Lab Med*. (2021) 145:517–28. doi: 10.5858/arpa.2020-0771-SA

143. Ripa I, Andreu S, Lopez-Guerrero JA, Bello-Morales R. Membrane rafts: portals for viral entry. *Front Microbiol*. (2021) 12:631274. doi: 10.3389/fmicb.2021.631274

144. Desplanques AS, Nauwynck HJ, Vercauteren D, Geens T, Favoreel HW. Plasma membrane cholesterol is required for efficient pseudorabies virus entry. *Virology*. (2008) 376:339–45. doi: 10.1016/j.virol.2008.03.039

145. Alkafaas SS, Abdallah AM, Ghosh S, Loutfy SA, Elkafas SS, Abdel Fattah NF, et al. Insight into the role of clathrin-mediated endocytosis inhibitors in SARS-CoV-2 infection. *Rev Med Virol*. (2023) 33:e2403. doi: 10.1002/rmv.2403

146. Van Hamme E, Dewerchin HL, Cornelissen E, Verhasselt B, Nauwynck HJ. Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin. *J Gen Virol*. (2008) 89:2147–56. doi: 10.1099/vir.0.2008/001602-0

147. Glebov OO. Understanding SARS-CoV-2 endocytosis for COVID-19 drug repurposing. *FEBS J*. (2020) 287:3664–71. doi: 10.1111/febs.15369

148. Mercer J, Helenius A. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science*. (2008) 320:531–5. doi: 10.1126/science.1155164

149. Benning N, Hassett DE. Vaccinia virus infection during murine pregnancy: a new pathogenesis model for vaccinia fetalis. *J Virol*. (2004) 78:3133–9. doi: 10.1128/JVI.78.6.3133-3139.2004

150. Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. *Curr Biol*. (2001) 11:R795–805. doi: 10.1016/S0960-9822(01)00474-2

151. Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, et al. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J Cell Biol*. (2001) 155:649–59. doi: 10.1083/jcb.200108080

152. Charlier C, Perrodeau E, Leclercq A, Cazenave B, Pilmis B, Henry B, et al. Clinical features and prognostic factors of listeriosis: the MONALISA national prospective cohort study. *Lancet Infect Dis*. (2017) 17:510–9. doi: 10.1016/S1473-3099(16)30521-7

153. Acres AE, Listeriosis: prevention and control. *CMAJ*. (1988) 138:439.

154. Hamon M, Bierne H, Cossart P. Listeria monocytogenes: a multifaceted model. *Nat Rev Microbiol*. (2006) 4:423–34. doi: 10.1038/nrmicro1413

155. MacDonald TT, Carter PB. Cell-mediated immunity to intestinal infection. *Infect Immun*. (1980) 28:516–23. doi: 10.1128/iai.28.2.516-523.1980

156. Racz P, Tenner K, Mero E. Experimental Listeria enteritis. I. An electron microscopic study of the epithelial phase in experimental listeria infection. *Lab Invest*. (1972) 26:694–700.

157. Shen Y, Naujokas M, Park M, Ireton K. InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell*. (2000) 103:501–10. doi: 10.1016/S0092-8674(00)00141-0

158. Braun L, Cossart P. Interactions between Listeria monocytogenes and host mammalian cells. *Microbes Infect*. (2000) 2:803–11. doi: 10.1016/S1286-4579(00)90365-4

159. Le Monnier A, Autret N, Join-Lambert OF, Jaubert F, Charbit A, Berche P, et al. ActA is required for crossing of the fetoplacental barrier by Listeria monocytogenes. *Infect Immun*. (2007) 75:950–7. doi: 10.1128/IAI.01570-06

160. Faralla C, Rizzuto GA, Lowe DE, Kim B, Cooke C, Shiow LR, et al. InlP, a new virulence factor with strong placental tropism. *Infect Immun*. (2016) 84:3584–96. doi: 10.1128/IAI.00625-16

161. Faralla C, Bastounis EE, Ortega FE, Light SH, Rizzuto G, Gao L, et al. Listeria monocytogenes InlP interacts with afadin and facilitates basement membrane crossing. *PLoS Pathog*. (2018) 14:e1007094. doi: 10.1371/journal.ppat.1007094

162. Crespo AC, Mulik S, Dotiwala F, Ansara JA, Sen Santara S, Ingersoll K, et al. Decidual NK cells transfer granulysin to selectively kill bacteria in trophoblasts. *Cell*. (2020) 182:1125–39.e18. doi: 10.1016/j.cell.2020.07.019

163. Kolb-Maurer A, Wilhelm M, Weissinger F, Brocker EB, Goebel W. Interaction of human hematopoietic stem cells with bacterial pathogens. *Blood*. (2002) 100:3703–9. doi: 10.1182/blood-2002-03-0898

164. Portes J, Barrias E, Travassos R, Attias M, de Souza W. Toxoplasma gondii mechanisms of entry into host cells. *Front Cell Infect Microbiol*. (2020) 10:294. doi: 10.3389/fcimb.2020.00294

165. Montoya JG, Remington JS. Management of Toxoplasma gondii infection during pregnancy. *Clin Infect Dis*. (2008) 47:554–66. doi: 10.1086/590149

166. Morisaki JH, Heuser JE, Sibley LD. Invasion of Toxoplasma gondii occurs by active penetration of the host cell. *J Cell Sci*. (1995) 108:2457–64. doi: 10.1242/jcs.108.6.2457

167. Guerin A, Corrales RM, Parker ML, Lamarque MH, Jacot D, El Hajj H, et al. Efficient invasion by Toxoplasma depends on the subversion of host protein networks. *Nat Microbiol*. (2017) 2:1358–66. doi: 10.1038/s41564-017-0018-1

168. Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. *Cell Mol Life Sci*. (2014) 71:1245–63. doi: 10.1007/s00018-013-1491-1

169. Oghumu S, Satoskar AR. PI3K-gamma inhibitors in the therapeutic intervention of diseases caused by obligate intracellular pathogens. *Commun Integr Biol*. (2013) 6:e23360. doi: 10.4161/cib.23360

170. Mukherjee M, Basu Ball W, Das PK. Leishmania donovani activates SREBP2 to modulate macrophage membrane cholesterol and mitochondrial oxidants for establishment of infection. *Int J Biochem Cell Biol*. (2014) 55:196–208. doi: 10.1016/j.biocel.2014.08.019

171. Vieira M, Dutra JM, Carvalho TM, Cunha-e-Silva NL, Souto-Padron T, Souza W. Cellular signaling during the macrophage invasion by Trypanosoma cruzi. *Histochem Cell Biol*. (2002) 118:491–500. doi: 10.1007/s00418-002-0477-0

172. Woolsey AM, Sunwoo L, Petersen CA, Brachmann SM, Cantley LC, Burleigh BA. Novel PI 3-kinase-dependent mechanisms of trypanosome invasion and vacuole maturation. *J Cell Sci*. (2003) 116:3611–22. doi: 10.1242/jcs.00666

173. Lovewell RR, Patankar YR, Berwin B. Mechanisms of phagocytosis and host clearance of Pseudomonas aeruginosa. *Am J Physiol Lung Cell Mol Physiol*. (2014) 306:L591–603. doi: 10.1152/ajplung.00335.2013

174. de Carvalho TM, Barrias ES, de Souza W. Macropinocytosis: a pathway to protozoan infection. *Front Physiol*. (2015) 6:106. doi: 10.3389/fphys.2015.00106

175. Carlton J, Bujny M, Peter BJ, Oorschot VM, Rutherford A, Mellor H, et al. Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high- curvature membranes and 3-phosphoinositides. *Curr Biol*. (2004) 14:1791–800. doi: 10.1016/j.cub.2004.09.077

176. Kurten RC, Cadena DL, Gill GN. Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science*. (1996) 272:1008–10. doi: 10.1126/science.272.5264.1008

177. Zheng B, Tang T, Tang N, Kudlicka K, Ohtsubo K, Ma P, et al. Essential role of RGS-PX1/sorting nexin 13 in mouse development and regulation of endocytosis dynamics. *Proc Natl Acad Sci U.S.A*. (2006) 103:16776–81. doi: 10.1073/pnas.0607974103

178. Davis PB, Drumm M, Konstan MW. Cystic fibrosis. *Am J Respir Crit Care Med*. (1996) 154:1229–56. doi: 10.1164/ajrccm.154.5.8912731

179. Trezise AE, Linder CC, Grieger D, Thompson EW, Meunier H, Griswold MD, et al. CFTR expression is regulated during both the cycle of the seminiferous epithelium and the oestrous cycle of rodents. *Nat Genet*. (1993) 3:157–64. doi: 10.1038/ng0293-157

180. Tizzano EF, Silver MM, Chitayat D, Benichou JC, Buchwald M. Differential cellular expression of cystic fibrosis transmembrane regulator in human reproductive tissues. Clues for the infertility in patients with cystic fibrosis. *Am J Pathol*. (1994) 144:906–14.

181. Jin R, Hodges CA, Drumm ML, Palmert MR. The cystic fibrosis transmembrane conductance regulator (Cftr) modulates the timing of puberty in mice. *J Med Genet*. (2006) 43:e29. doi: 10.1136/jmg.2005.032839

182. Hodges CA, Palmert MR, Drumm ML. Infertility in females with cystic fibrosis is multifactorial: evidence from mouse models. *Endocrinology*. (2008) 149:2790–7. doi: 10.1210/en.2007-1581

183. Jouret F, Devuyst O. CFTR and defective endocytosis: new insights in the renal phenotype of cystic fibrosis. *Pflugers Arch*. (2009) 457:1227–36. doi: 10.1007/s00424-008-0594-2

184. Jouret F, Bernard A, Hermans C, Dom G, Terryn S, Leal T, et al. Cystic fibrosis is associated with a defect in apical receptor-mediated endocytosis in mouse and human kidney. *J Am Soc Nephrol*. (2007) 18:707–18. doi: 10.1681/ASN.2006030269

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 20 June 2024
DOI 10.3389/fimmu.2024.1411315

Check for updates

OPEN ACCESS

EDITED BY
Nandor Gabor Than,
Hungarian Academy of Sciences (MTA), Hungary

REVIEWED BY
Yohei Sato,
Jikei University School of Medicine, Japan
Aliz Barakonyi,
University of Pécs, Hungary

*CORRESPONDENCE
Christian Marcel Karsten
Christian.karsten@uksh.de

RECEIVED 02 April 2024
ACCEPTED 27 May 2024
PUBLISHED 20 June 2024

CITATION
Froehlich F, Landerholm K, Neeb J, Meß A-K, Seiler DL, Tilburgs T and Karsten CM (2024) Emerging role of C5aR2: novel insights into the regulation of uterine immune cells during pregnancy.
Front. Immunol. 15:1411315.
doi: 10.3389/fimmu.2024.1411315

COPYRIGHT
© 2024 Froehlich, Landerholm, Neeb, Meß, Seiler, Tilburgs and Karsten. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Emerging role of C5aR2: novel insights into the regulation of uterine immune cells during pregnancy

Fenna Froehlich[1], Konstanze Landerholm[1], Johanna Neeb[1], Ann-Kathrin Meß[1], Daniel Leonard Seiler[1], Tamara Tilburgs[2,3] and Christian Marcel Karsten[1*]

[1]Institute for Systemic Inflammation Research (ISEF), University of Lübeck, Luebeck, Germany, [2]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States, [3]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, United States

Pregnancy is a fascinating immunological phenomenon because it allows allogeneic fetal and placental tissues to survive inside the mother. As a component of innate immunity with high inflammatory potential, the complement system must be tightly regulated during pregnancy. Dysregulation of the complement system plays a role in pregnancy complications including pre-eclampsia and intrauterine growth restriction. Complement components are also used as biomarkers for pregnancy complications. However, the mechanisms of detrimental role of complement in pregnancy is poorly understood. C5a is the most potent anaphylatoxin and generates multiple immune reactions via two transmembrane receptors, C5aR1 and C5aR2. C5aR1 is pro-inflammatory, but the role of C5aR2 remains largely elusive. Interestingly, murine NK cells have been shown to express C5aR2 without the usual co-expression of C5aR1. Furthermore, C5aR2 appears to regulate IFN-γ production by NK cells *in vitro*. As IFN-γ produced by uterine NK cells is one of the major factors for the successful development of a vital pregnancy, we investigated the role anaphylatoxin C5a and its receptors in the establishment of pregnancy and the regulation of uterine NK cells by examinations of murine *C5ar2*$^{-/-}$ pregnancies and human placental samples. *C5ar2*$^{-/-}$ mice have significantly reduced numbers of implantation sites and a maternal C5aR2 deficiency results in increased IL-12, IL-18 and IFN-γ mRNA expression as well as reduced uNK cell infiltration at the maternal-fetal interface. Human decidual leukocytes have similar C5a receptor expression patterns showing clinical relevance. In conclusion, this study identifies C5aR2 as a key contributor to dNK infiltration and pregnancy success.

KEYWORDS

anaphylatoxins, C5aR2, pregnancy, uterine NK cells, uterine DCs, INF-γ

Abbreviations: IS, implantation sites; pNK cells, peripheral NK cells; uNK cells, uterine NK cells; uDCs, uterine DCs

1 Introduction

Pregnancy presents a complex immunological challenge because of the need to protect the immunologically foreign fetus from the maternal immune system without increasing her susceptibility to pathogens (1). As a result, the maternal immune system undergoes innumerable adaptions that allow implantation and placentation (2, 3). An ancient and highly conserved component of innate immunity is the complement system, which provides a first line of defense against pathogens (4). Complement activation leads to a self-amplifying cascade that ultimately results in, among other things (opsonization and formation of the membrane attack complex, MAC), the release of highly pro-inflammatory anaphylatoxins, of which C5a is the most potent (5, 6). Due to its high inflammatory potential, pregnancy requires tight regulation of the complement system, as excessive activation has been implicated in the pathogenesis of pregnancy complications including early pregnancy loss, fetal growth restriction, hypertensive disorders of pregnancy, and preterm birth (7). However, uncomplicated pregnancy is also characterized by systemic activation of the complement system (8), suggesting a physiological role during pregnancy. Previous studies have shown that complement activation and function are involved in implantation, placental development, normal fetal development, and labor (9). To date, two receptors for C5a, C5aR1 (CD88) and C5aR2 (C5L2), have been identified (10, 11). While it is known that C5aR1 is a G-protein-coupled seven-transmembrane receptor that triggers pro-inflammatory processes such as chemotaxis, cytokine release and oxidative burst of immune cells upon C5a binding (12), the role of C5aR2 remains enigmatic (13, 14). C5aR2 lacks G-protein coupling and has therefore often been described as a decoy receptor that modulates C5aR1 signaling (15). Interestingly, however, studies using C5aR1 and C5aR2 reporter mice have shown that C5aR2, but not C5aR1, is expressed on murine NK cells, suggesting a specific function of C5aR2 on NK cells that is independent of C5aR1 (16, 17). Furthermore, a large body of recent research findings contradicts the decoy receptor hypothesis (18–20), which is supported by our findings that C5aR2 has been shown to be able to regulate the production of interferon-γ (IFN-γ) by peripheral natural killer (pNK) cells (17).

A distinct subset of NK cells are the uterine NK (uNK) cells, which constitute the largest leukocyte population in the murine and human decidua during early pregnancy. uNK cells play a unique role in placental development, placental immunity and pregnancy success (21, 22). UNK cells differ from blood pNK cells in size, function, and surface molecules (23). Murine uNK cells can be distinguished by their unique reactivity to Dolichus biflorus agglutinin (DBA), which is not found in other cell types or species (24). In contrast, human uNK cells – also called decidual NK (dNK) cells – are characterized by their $CD56^{bright}$ $CD16^{-}$ phenotype (25). Murine and human uNK cells have lower cytotoxicity than pNK cells (26–29) and are high producers of cytokines, chemokines, and angiogenic factors that mediate key physiological processes during placentation (30).

Although uNK cells are in direct contact with semi-allograft fetal trophoblast cells, they do not exert any cytolytic functions against these cells due to the expression of non-classical MHC-I molecules (31, 32). Nevertheless, uNK cells have important functions in protecting the fetus from viral or bacterial infections of the placenta via Killer Ig-Like Receptors (KIRs) or through the production of the antimicrobial peptide granulysin (22, 33). In addition to their protective role, uNK cells are critically involved in the establishment of normal implantation sites and in adequate remodeling of the decidual spiral arteries in mice, which is required for adequate blood flow within the placenta. This process is mainly mediated by uNK cell-derived IFN-γ, indicating the importance of uNK cell functions in the development of a vital pregnancy (34, 35). In addition to IFN-γ, human and mouse uNK cells produce a variety of other cytokines, growth, and angiogenic factors that support the developmental processes within the placenta, suggesting that sufficient uNK cell activation may be necessary to reduce the risk of pregnancy complications such as pre-eclampsia (30). Within the decidual environment, uNK cells interact with uterine dendritic cells (uDCs), which are capable of producing NK cell-activating factors such as IL-12, IL-15, and IL-18 (36). This crosstalk and direct cell-cell contact is required for proper uNK cell function, as inhibition of uDC recruitment to the pregnant uterus results in impaired uNK cell development, a reduction in their IFN-γ production, and structural abnormalities of the spiral arteries (37).

Based on previous findings (16, 17), we hypothesize that C5aR2 is a novel regulator of uNK cell function and plays an important role during pregnancy.

2 Materials and methods

2.1 Breeding analysis

Data from our internal breeding facility were analyzed for breeding efficiency of syngeneic matings of C57BL/6j wild type (WT x WT), C5aR2-deficient (*C5ar2*$^{-/-}$ x *C5ar2*$^{-/-}$), and C5aR1-deficient (*C5ar1*$^{-/-}$ x *C5ar1*$^{-/-}$) mice based on their litter size. Knock-out strains were based on the C57BL/6j background. Mouse breeding was performed and documented by trained personnel. Litters within one year were included in the analysis. Mice used for internal breeding were maintained in a pathogen-free environment with a 12-h:12-h light-dark cycle. The number of live-born pups and pups found dead shortly after parturition per litter was used to compare their breeding efficiency. Furthermore, the total number of healthy implantation sites (IS) in the harvested uteri of WT and *C5ar2*$^{-/-}$ x WT matings used for further experiments was analyzed.

2.2 Animals, mating procedure and organ harvest

C57BL/6j wild-type (WT), C5aR2 knock-out (*C5ar2*$^{-/-}$), and tdTomato floxed C5aR2-knock-in (C5aR2 reporter) mice on C57BL/6j background were bred in the breeding facility of the University of Lübeck. All animals were used at 12–20 weeks of age, and the handling was performed according to the appropriate

institutional and national guidelines. Mice were maintained in a pathogen-free environment with a 12-h:12-h light-dark cycle. To make females susceptible to mating taking advantage of the Whitten effect, some stray containing the male's urine was placed in their cage three days before mating to induce estrus. Female mice of both genotypes were mated with WT males overnight, checked for a vaginal plug the next morning defined as gestation day (gd) 1 and terminated at gd 8 to gd 10 by cervical dislocation. The IS were harvested and placed in 6-well plates containing RPMI medium after removal of surrounding fat and vessels. Their number was documented, and photographs were taken. Isolated IS were stored on ice until further processing. The procedure was approved by the local authorities of the Animal Care and Use Committee (Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein, Kiel, Germany). All experiments were performed by certified personnel. The mice were kindly provided by Jörg Köhl, Institute for Systemic Inflammation Research (ISEF), Luebeck.

2.3 Immunofluorescence staining and confocal microscopy

IS of gd 8 were dissected, frozen in liquid nitrogen and stored at -80°C until further use. Serial cryosections of 6 µm were made using the Leica Kryostat CM3050 S and fixed in acetone (JT Baker). They were stored at -20°C until further use. Before staining, sections were thawed, encircled using a DAKO liquid fat pen and washed three times in PBS. They were blocked for 15 minutes with F_c-block (anti-CD16/CD32; dilution 1:100) for 15 minutes in a StainTray (eBioscience) and then washed again three times in PBS. The staining mastermix containing DAPI (Life Technologies; dilution 1:1000), PE-CF594-conjugated anti-CD11c antibody (BD Biosciences; dilution 1:100) or PE-conjugated anti-Thy1.2 (BD Pharmingen) and fluorescein-conjugated DBA (Vector laboratories; dilution 1:500) was applied and incubated for 45 minutes in the dark. The washing procedure was repeated, and coverslips (Glaswarenfabrik Karl Hecht GmbH und Co. KG) were placed on the sections using Fluoroshield mounting medium (Sigma-Aldrich).

Slides stained against CD11c, and DBA were viewed with the Keyence BZ 9000 und photographed with the BZ Viewer software. Image merges and overlays were created, and scales inserted using the BZ analyzer software or the FIJI software. Only every tenth section was used for analysis to avoid double counting of cells. At least three different sections per specimen and six specimens per genotype were analyzed for frequency, infiltration area and depth of DBA^+ uNK cells using the FIJI software. The diameters and surface areas of DBA^+ uNK cells were measured using the same software. A minimum of 50 centrally sectioned uNK cells per section, three sections per specimen, and six specimens per genotype were analyzed.

Sufficient image quality of the staining of Thy1.2 and DBA could only be achieved using the Confocal Laser-Scanning-Mikroscope FluoView V1000 by Olympus. Images were analyzed using the FIJI software. At least three different sections per specimen and five specimens per genotype were analyzed for frequency of $Thy1.2^+$ and DBA^+ uNK cells.

2.4 Flow cytometry

IS of WT and *C5ar2*$^{-/-}$ x WT matings were freshly dissected at gd 8, cleared from surrounding tissue, and transferred to 5 mL of RPMI medium. DNase and Liberase TL (both Hoffmann – La Roche AG) were added at concentrations of 0.5 mg/ml and 0.1 mg/ml, respectively. The tissue was minced and enzymatically digested for 45 minutes at 37°C on a waving shaker. The tissue was then forced through a 100-µm cell strainer, washed with PBS, and centrifuged. After a 15-minute blocking step with F_c-block (anti-mouse CD16/CD32 antibody) and incubation with the fixable viability dye eFluor780 (Invitrogen; dilution 1:1000), cells from IS of WT and *C5ar2*$^{-/-}$ x WT matings were stained with the antibodies for the NK cell (Table 1) and the DC panel (Table 2), respectively, for 15 minutes at 4°C in the dark. Antibodies were diluted to the appropriate concentrations in PBS containing 1% BSA. After a washing step, cells were fixed, permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and washed with PermWash buffer (BD Biosciences) according to the manufacturer's instructions. Cells used for the NK cell panel were then incubated with fluorescein-conjugated DBA (Vector Laboratories) for 45 minutes and washed before analysis. Positive staining was identified by *Fluorescence-minus-one* (FMO) controls for each target. Data were acquired using the BD LSR II and analyzed using FlowJo software version 10.7.1.

2.5 Real-time-PCR

To isolate RNA from IS, the Qiagen RNeasy Mini Kit was used according to the manufacturer's instructions. Tissue was

TABLE 1 Anti-murine antibodies used for the analysis of murine NK cells.

Antigen	Target	Isotype	Manufacturer	Fluorochrome	Dilution
CD3	T cells	Rat IgG_{2b}, κ	BioLegend	BV510	1:400
CD16/32	Fc-block	Rat IgG2a, λ	Invitrogen	–	1:300
CD45	30-F11	Rat IgG_{2b}, κ	eBioscience	AF700	1:800
CD122	TM-β1	Rat IgG2a, κ	BD Biosciences	BV421	1:400
DBA	NK cells	–	Vector Laboratories	Fluorescein	1:500

TABLE 2 Anti-murine antibodies used for the analysis of murine dendritic cells.

Antigen	Target	Isotype	Manufacturer	Fluorochrome	Dilution
CD3e	T cells (dump channel)	Armenian Hamster IgG_1, κ	BioLegend	FITC	1:400
CD16/32	Fc-block	Rat IgG2a, λ	Invitrogen	–	1:300
CD19	B cells (dump channel)	Rat Lewis IgG_{2a}, κ	BD Pharmingen	FITC	1:400
CD49b	NK cells (dump channel)	Rat Lewis IgM, κ	BD Pharmingen	FITC	1:400
F4/80	Macrophages (dump channel)	Rat IgG2a, κ	Invitrogen	FITC	1:300
Ly6G	Neutrophils	Rat Lewis IgG_{2a}, κ	BD Pharmingen	FITC	1:400
MHC-II	Dendritic cells	Armenian Hamster IgG_1, λ2	BioLegend	PE-Cy7	1:500
CD11c	Dendritic cells	Rat IgG2a, κ	BD Biosciences	APC	1:400

homogenized in RLT buffer for approximately 30 seconds using the ULTRA-TURRAX T8 homogenizer prior to performing the protocol provided. For transcription of RNA into cDNA, the PrimeScript RT Reagent Kit with gDNA Eraser was used according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (qPCR) was performed targeting mRNAs transcribed from *interleukin 15* (*Il15*), *interleukin 18* (*Il18*), *interferon gamma* (*Ifng*), and *transforming growth factor beta 1* (*Tgfb1*). *β-Actin (Actb)* was used as a housekeeping gene to normalize the expression of the target genes. The following primers used for qPCR were purchase from Eurofins Genomics Germany GmbH (Ebersberg, Germany): IFN-γ forward 5'-CCA TCC TTT TGC CAG TTC CTC-3', IFN-γ reverse 5'-ATG AAC GCT ACA CAC TGC ATC-3', IL-15 forward 5'-ACA TCC ATC TCG TGC TAC TTG T-3', IL-15 reverse 5'-GCC TCT GTT TTA GGG AGA CCT-3', TGF-β1 forward 5'-AGC TGG TGA AAC GGA AGC G-3', and TGF-β1 reverse 5'-GCG AGC CTT AGT TTG GAC AGG-3'. The primers IL-18 forward 5'-GTA TTA CTG CGG TTG TAC AGT G-3' and IL-18 reverse 5'-GCG AGC CTT AGT TTG GAC AGG-3' were obtained from MWG-Biotech AG (Ebersberg, Germany). The primers β-Actin forward 5'-AAA TAG CAG CCT GGA TAG CAA C-3' and β-Actin reverse 5'-GCA CCA CAC CTT CTA CAA TGA G-3' were used for the housekeeping gene (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The Bio-Rad iQ™ SYBR® Green Supermix was used for dye-based real-time quantification according to the manufacturer's instructions. For the quantification of mRNA levels of *placental growth factor* (*Plgf*), a TaqMan probe assay (assay ID qMmuCIP0033405, purchased from Bio-Rad) was used with *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) serving as a housekeeping gene (assay ID: Mm99999915_g1, purchased from Applied Biosystems). For the assay, the Taqman Gene Expression Mastermix (Applied Biosystems) was used according to the manufacturer's instructions. Samples were pipetted in triplicates. Afterwards, thermocycling protocols were performed using the 7900 HT Fast Real Time PCR System.

To calculate the induced fold changes, the ΔΔCt method by Livak and Schmittgen was applied to the samples (38). The values of the WT x WT matings served as reference controls for the calculation of fold changes within the samples of *C5ar2*$^{-/-}$ x WT matings. Statistical analysis of the data was performed by comparing the ΔCt values of the respective targets.

2.6 Analysis of inner lumen:outer diameter ratio

IS of gd 10 were dissected, frozen in liquid nitrogen and stored at -80°C until further use. Serial cryosections of 6 µm were generated using the Leica Kryostat CM3050 S and fixed in acetone (JT Baker). They were stored at -20°C until further use. Prior to staining, the sections were thawed, encircled using a DAKO liquid fat pen and washed three times in PBS. They were stained with Epredia™ Shandon™ Kwik-Diff™ Stain Kit according to the manufacturer's instructions. Microscopic images were taken and the inner lumen:outer diameter ratio was calculated as described (39).

2.7 Preparation of human blood and placentas and flow cytometry analysis

In cooperation with the Department of Gynecology and Obstetrics of the University Hospital Schleswig-Holstein, Campus Lübeck, woman giving birth were recruited to provide their placenta and a blood sample after birth for the examination of the leukocytes contained therein. Samples from 8 term pregnancies were included in the study. The mothers were between 23 and 42 years old. In 7 pregnancies, the mode of delivery was caesarean section due to maternal risk factors such as previous caesarean section or maternal cardiopulmonary disease. One birth was by vacuum extraction. The gestational age at birth was between 34 + 1 and 40 + 2 weeks. 3 women were diagnosed with gestational diabetes. One mother had pre-existing arterial hypertension. None of the women were diagnosed with pre-eclampsia, HELLP syndrome or other pregnancy-related hypertensive disorders. The placenta was stored refrigerated until processing. For isolation of the *decidua basalis*, the placenta was positioned with the maternal side up and

individual pieces were cut from the tissue. The placental villi were carefully removed until only the thin mucosal layer remained. This was then transferred into a 50-mL tube filled with PBS. For isolation of the *decidua parietalis*, the placenta was positioned with the fetal side facing up and the amnion was carefully detached. Then, a piece of the remaining membranes (chorion and *decidua parietalis*) was cut off from the rest of the placenta, and the *decidua parietalis* was carefully detached using sterile forceps. The decidual tissue was washed several times with PBS until no more blood was macroscopically detectable in the liquid phase. The tissue was then minced using dissecting scissors and washed with PBS as described before. Centrifugation was performed and the supernatant was discarded. RPMI medium containing DNase I (Hoffmann – La Roche, 0.5 mg/ml) and collagenase IV (Sigma-Aldrich, 1 mg/ml) was added. The tissue was incubated with the added enzymes in a shaking water bath at a temperature of 37°C for 90 minutes and then filtered using a cell dissociation sieve ("cell dissociation sieve - tissue grinder kit"). The solubilized cells were then filtered sequentially through cell sieves with a respective pore size of first 100 µm and then 40 µm. Samples were then centrifuged and resuspended in 20 ml RPMI and then layered over 10 ml of Histopaque-1077 (Sigma-Aldrich) in a new tube. This was followed by density gradient centrifugation. Mononuclear cells were isolated from the cell layer in the middle and washed twice with PBS. After leukocyte isolation was completed, cells were stained for flow cytometric analysis. Because CD16 was also stained as a marker, blockade of Fc receptors (CD32/CD16) was omitted. Cells were incubated with 1:1000 diluted eFluor780 fixable viability dye. After a brief centrifugation, cells were incubated with the antibodies for extracellular staining for 15 minutes (Table 3). After another washing step, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for intracellular staining and then washed with PermWash buffer (BD Biosciences) according to the manufacturer's instructions. This was followed by a 45-minute incubation with antibodies used for intracellular staining (Anti-C5aR1 and C5aR2, Table 3). After another wash step, samples were resuspended in 350 µL PBS/1%BSA and transferred to FACS tubes. Mononuclear cells from peripheral blood samples of the same donors were isolated by density gradient centrifugation and then washed and stained according to the same protocol. Samples were analyzed using the BD LSRII and gated using FlowJo software version 10.7.1. Gating was verified using fluorescence-minus-one (FMO) controls and isotype controls for C5aR1 and C5aR2.

2.8 Statistical analysis

Data was analyzed using GraphPad Prism version 8.0.1. Breeding data is shown as box-whisker-plots indicating mean, quartiles, and range of the data sets. RT-PCR data is expressed as mean ± SEM. Data on murine uterine NK cells and dendritic cells as well as human decidual cells are given as mean ± SEM. We tested the data sets for normal distribution using the Shapiro-Wilk test and used the Mann-Whitney U-test or the unpaired T-test depending on the result. $P<0.05$ was considered statistically significant.

2.9 Study approval

Animal experiments were approved by the local authorities of the Animal Care and Committee (Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein, Kiel, Germany). The use of human material was approved by the ethics committee of the University of Luebeck (Ethikkomission der Universität zu Lübeck).

3 Results

3.1 Maternal C5aR2-deficiency impairs breeding efficiency in mice

To investigate whether loss of the C5ar2 gene affects pregnancy outcome in mice, breeding data from our animal facility were analyzed for the number of live-born pups per litter in homozygous matings of C57BL/6j wild-type (WT) versus C57BL/6j *C5ar2*$^{-/-}$ mice. Over a period of one year, 98 litters of WT and 60 litters of *C5ar2*$^{-/-}$ pregnancies were documented. Interestingly, the litter size of WT matings averaged 5.3 (± 2.9) live-born pups, while a litter from *C5ar2*$^{-/-}$ matings averaged only 3.4 (± 2.6) live-born pups (Figure 1A). Notably, no abnormalities in reproductive efficiency were observed in *C5ar1*$^{-/-}$ mice compared to WT matings (Supplementary Figure S1A). In addition to litter size, the survival of individual offspring from each litter was also analyzed. We found that 20.5% of all pups from homozygous *C5ar2*$^{-/-}$ matings were found dead shortly after parturition (53 of 259 pups), compared to 10.3% of all pups in the WT group (61 of

TABLE 3 Anti-human antibodies used for the analysis of human decidual and peripheral leukocytes.

Antigen	Target	Isotype	Manufacturer	Fluorochrome	Dilution
CD3	T cells	Mouse IgG_1, κ	BioLegend	PerCP	1:400
CD16	NK cells	Mouse IgG_1, κ	BioLegend	PE-Cy7	1:200
CD45	Leukocytes	Mouse IgG_{2a}, κ	Invitrogen	Superbright 780	1:300
CD56	NK cells	Mouse IgG_1, κ	BioLegend	BV711	1:100
CD88 (C5aR1)	C5aR1+ cells	Mouse IgG_{2a}, κ	BioLegend	APC	1:300
C5L2 (C5aR2)	C5aR2+ cells	Mouse IgG_{2a}, κ	BioLegend	PE	1:400

10.3389/fimmu.2024.1411315

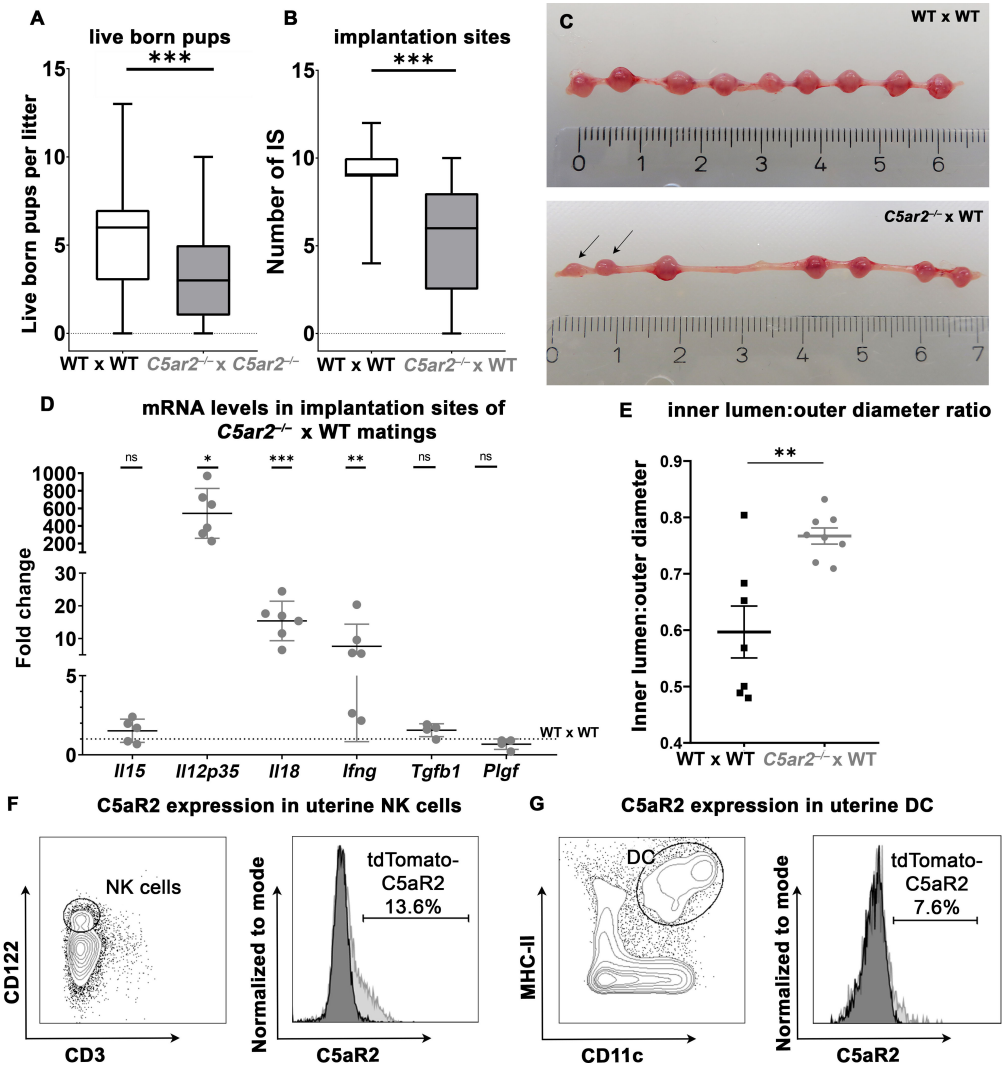


FIGURE 1
Breeding efficiency of WT x WT, *C5ar2*$^{-/-}$ x *C5ar2*$^{-/-}$, and *C5ar2*$^{-/-}$ x WT matings. **(A)** Depicted is the number of live born pups per litter in WT x WT matings (n = 98) compared to *C5ar2*$^{-/-}$ x *C5ar2*$^{-/-}$ matings (n = 60). **(B)** Depicted is the number of healthy implantation sites (IS) at gd 8 to gd 10 in *C5ar2*$^{-/-}$ females mated with WT males (*C5ar2*$^{-/-}$ x WT, n = 27) compared with WT females mated with WT males (WT x WT, n = 17). Boxplots depict median and interquartile range. **(C)** Representative pictures of a pregnant uterus of a WT x WT (top panel) and *C5ar2*$^{-/-}$ x WT (bottom panel) mating at gd 8. Incipient resorptions are marked with arrows. **(D)** mRNA levels of *Il15*, *Il12p35*, *Il18*, *Ifng*, *Tgfb1*, and *Plgf* in IS of *C5ar2*$^{-/-}$ x WT matings (n ≥ 4 normalized to mRNA levels in IS of WT x WT matings (n ≥ 5). Statistical analysis was performed by comparison of the delta ct values of each target. **(E)** Depicts the inner lumen to outer diameter ratio in IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 10. **(F)** Representative FACS plots of CD3^{-} CD122^{+} uNK cells C5aR2-tdTomato signal of uNK cells from C5aR2-reporter x WT matings (light grey histogram) compared to WT x WT matings (dark grey histogram) at gd 8. **(G)** Representative plots of CD11c^{+} MHC-II^{+} uDCs and C5aR2-tdTomato signal in uDC cells from C5aR2-reporter x WT matings (light grey histogram) compared to WT x WT matings (dark grey histogram) at gd8. Lines represent mean and SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns nonsignificant.

590) and 6.8% in the *C5ar1*$^{-/-}$ group (13 of 191; Supplementary Figure S1B).

As we saw striking effects of C5aR2 deficiency on breeding efficiency in the data of our breeding facility, we wondered whether these effects were mechanistically related to C5aR2 deficiency in maternal tissue. To exclude potential effects caused by homozygous C5aR2 deficiency in the offspring, heterozygous matings with *C5ar2*$^{-/-}$ females and WT males were performed. A significant reduction in the number of healthy IS was observed in pregnant uteri of *C5ar2*$^{-/-}$ females mated with WT males (5.53 ± 3.17) compared to WT x WT matings (9.07 ± 1.64) (Figures 1B, C), demonstrating the detrimental effect of a maternal C5aR2 loss on pregnancy success.

3.2 Maternal C5aR2-deficiency alters mRNA levels of cytokines involved in NK cell and DC function in IS

To determine whether maternal C5aR2 deficiency affects cytokine expression in the IS, mRNA levels of pro- and anti-inflammatory cytokines and growth factors important for

10.3389/fimmu.2024.1411315

placentation were determined in the IS of WT x WT or *C5ar2*$^{-/-}$ x WT matings at gd 8 Transcripts of the pro-inflammatory cytokines *Il18*, *Ifng*, and *Il12p35* were increased statistically significant by 15-, 5-, and 543-fold, respectively, in the IS of *C5ar2*$^{-/-}$ x WT matings. The expression level of placental growth factor (*Plgf*) in the IS of *C5ar2*$^{-/-}$ x WT matings was nonsignificantly reduced by approximately 33%, compared to the IS of WT x WT matings. No significant difference in expression was observed for anti-inflammatory cytokine *Tgfb1* and the NK cell activating cytokine *Il15* (Figure 1D, Supplementary Figure S2). Thus, a maternal C5aR2 deficiency significantly increases pro-inflammatory factors ate the maternal-fetal interface.

3.3 Maternal C5aR2 deficiency causes hyperdilation of decidual arteries in IS

Successful pregnancy requires remodeling of the spiral arteries to enable adequate blood supply to the developing fetus. In murine pregnancy, this process is supported by IFN-γ production by uNK cells (34). As maternal C5aR2 deficiency caused elevated mRNA levels of *Ifng* as well as alterations in uNK cell number and infiltration depth, decidual arteries in IS of *C5ar2*$^{-/-}$ x WT matings and WT x WT matings were analyzed for vessel inner lumen to outer diameter ratios (lumen:diameter ratio), an indicator of vessel dilation (23). In the IS of *C5ar2*$^{-/-}$ x WT matings, spiral arteries were significantly more dilated (mean lumen:diameter ratio of 0.8) compared with arteries in the IS of WT x WT matings (mean lumen:diameter ratio of 0.6; Figure 1E). Representative pictures of the decidual vessels are shown in Supplementary Figure S3. Thus, a maternal C5aR2 deficiency influences placentation by causing excessive dilation of decidual arteries beyond the usual remodeling process during murine pregnancy.

3.4 C5aR2 is expressed in murine uNK cells and uDCs

Since the breeding efficiency and the cytokines involved in uNK cell and uDC crosstalk were strongly affected by maternal C5aR2 deficiency, C5aR2 expression on uNK and uDC was determined by flow cytometry in matings of female floxed tdTomato C5aR2 *knock-in* reporter mice (C5aR2 reporter) with male WT mice (C5aR2 reporter x WT matings). The C5aR2 reporter mice have a floxed gene encoding the reporter protein tdTomato, which is co-expressed with C5aR2 (Karsten et al., (17)). Phenotypically, these mice do not differ from their WT counterparts, and we did not observe any abnormalities during pregnancy (data not shown). Interestingly, in C5aR2 reporter x WT matings, a subset of uNK expressed tdTomato-C5aR2, with approximately 10–15% of CD3$^-$ CD122$^+$ uNK cells staining positive for tdTomato-C5aR2 (Figure 1F). Approximately 3% of splenic NK cells from the same females showed C5aR2 expression (Supplementary Figure S4A). In lineage marker (CD3e, CD19, CD49b, Ly6G, F4/80)-negative MHCII$^+$CD11c$^+$ uDCs and splenic DCs, C5aR2 expression was found in approximately 7% of cells (Figure 1G, Supplementary Figure 4B). Thus, a subset of uNK and uDC express C5aR2 and are directly susceptible for complement signaling.

3.5 DBA$^+$ uNK cell frequency, infiltration, and size are reduced in IS of *C5ar2*$^{-/-}$mice

To gain insight into the role of C5aR2 in uNK infiltration and their activation at the maternal fetal interface, immunofluorescence staining with DBA was performed to analyze the uNK frequency and their infiltration depth (Figures 2A, B). In the IS of WT x WT matings, a mean frequency of 867.8 DBA$^+$ uNK cells per tissue section was observed at gd 8 (Figures 2A, C). In contrast, a mean frequency of 222.7 DBA$^+$ uNK cells per section was observed in the IS of *C5ar2*$^{-/-}$ x WT matings at gd 8 (Figures 2B, C). In addition, a significantly reduced infiltration area (Figure 2D) and depth (Figure 2E) of DBA$^+$ uNK was observed in the IS of *C5ar2*$^{-/-}$ x WT matings compared to WT x WT matings. In the IS of WT x WT matings, DBA$^+$ uNK cells infiltrated 40.15% of the total IS area, whereas in *C5ar2*$^{-/-}$ x WT matings, DBA+ uNK cells infiltrated only 23.36% of the IS area. Similarly, the mean infiltration depth in the IS of WT x WT matings was 41.33% of the total IS length, whereas a mean infiltration depth of 27.19% was observed in *C5ar2*$^{-/-}$ x WT matings.

Analysis of uNK cell size in the same images revealed a significant reduction in the IS of *C5ar2*$^{-/-}$ x WT matings compared to WT x WT matings (Figure 2F). Size is a measure of uNK cell maturation, with larger uNK cells being more mature (Felker and Croy (40)). Here, the DBA$^+$ uNK cells of the IS of WT x WT matings measured 262.1 μm^2, whereas in the IS of *C5ar2*$^{-/-}$ x WT matings we found significantly smaller uNK cells measuring 191.8 μm^2 (Figure 2F).

3.6 Maternal C5aR2-deficiency shifts the distribution of the uNK cell population to a predominant DBA$^-$ uNK cell phenotype

Since no lymphocyte population in any tissue outside the uterus or in virgin mice shows positive DBA reactivity, DBA is a good marker for uNK cells (41). However, there are also uNK cells that are negative for DBA. These uNK differ not only in their DBA reactivity but also in their function. While DBA$^+$ uNK cells have been shown to produce more angiogenic factors such as PlGF, DBA$^-$ uNK cells resemble pNK cells and mainly produce IFN-γ (42). To determine whether the DBA$^-$ population was also affected by maternal C5aR2 deficiency, the proportion of Thy1.2+ uNK cells (a general marker for uNK cells including DBA$^+$ and DBA$^-$ uNK cell subsets) was determined (Figures 2G-J). The number of Thy1.2$^+$ (DBA$^{+/-}$) uNK cells per 0.25mm2 was significantly lower in the IS of *C5ar2*$^{-/-}$ x WT matings compared to the IS of WT x WT matings (Figures 2G-I). In addition, the percentage of DBA$^+$ Thy1.2$^+$ uNK cells was significantly lower in the IS of *C5ar2*$^{-/-}$ x WT matings (18%) compared to WT x WT matings (42%; Figure 2J). Flow cytometric analysis confirmed the decreased proportion of DBA$^+$ uNK cells and increased proportion of DBA$^-$ uNK cells in *C5ar2*$^{-/-}$

10.3389/fimmu.2024.1411315

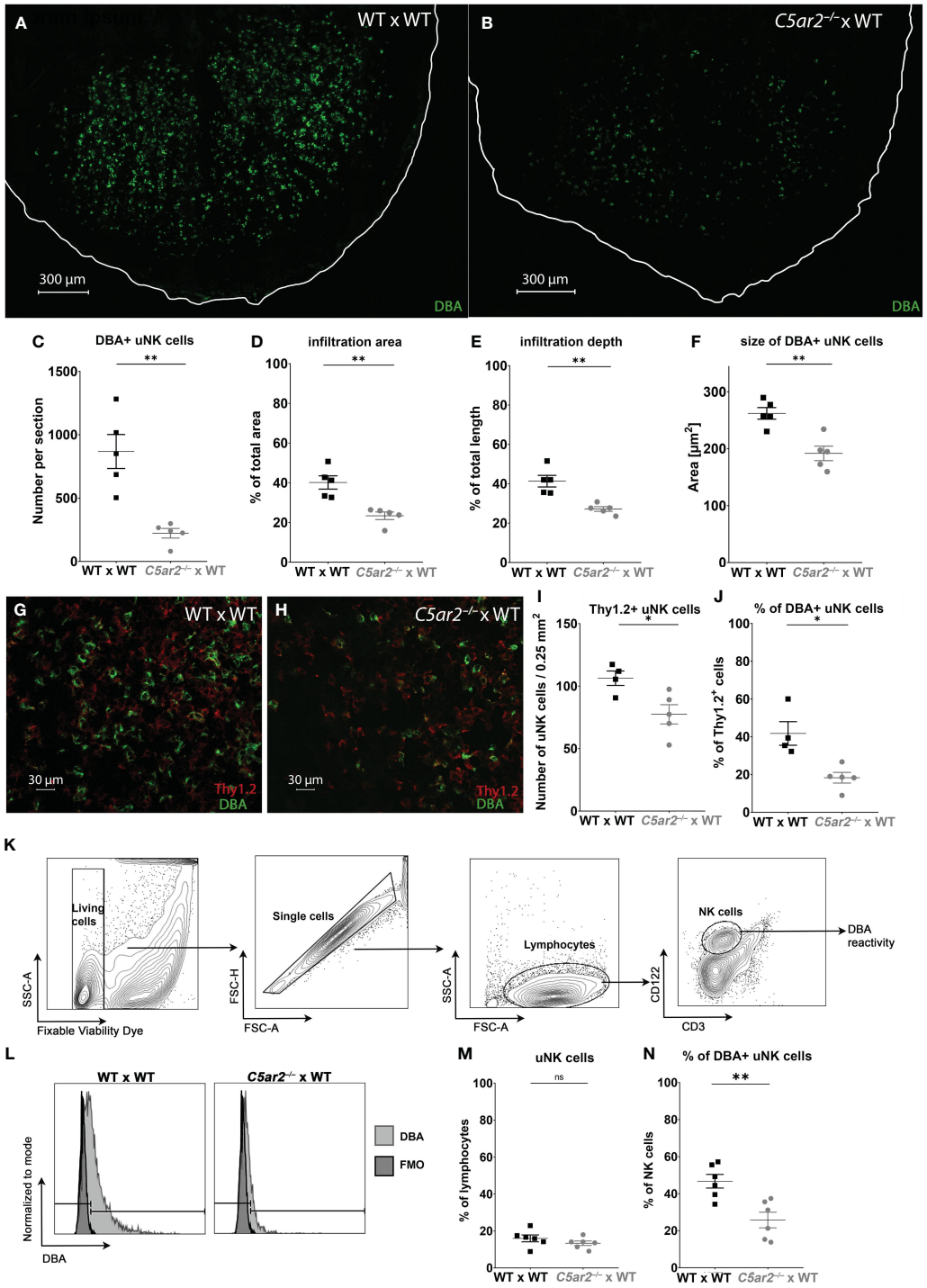


FIGURE 2
Uterine NK cell numbers and infiltration depth are reduced in the implantation sites of *C5ar2*$^{-/-}$ x WT matings. Representative picture the *decidua basalis* of a section of an IS of **(A)** WT x WT and **(B)** *C5ar2*$^{-/-}$ x WT matings stained with dolichos biflorus agglutinin (DBA) to identify DBA^{+} uNK cells at gd 8. Tissue borders are marked by the white line for orientation. Graphs depict **(C)** number, **(D)** infiltration area, **(E)** infiltration depth, **(F)** size of uNK cells in IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 8. Representative pictures of Thy1.2 (red) and DBA (green) immunofluorescence staining in the *decidua basalis* of **(G)** WT x WT and **(H)** *C5ar2*$^{-/-}$ x WT matings at gd 8. Graphs depict **(I)** the number of Thy1.2^{+} uNK cells per 0.25 mm^{2} and **(J)** the proportion of DBA^{+} uNK cells to Th1.2^{+} uNK in the *decidua basalis* of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 8. **(K)** Gating strategy used for the flow cytometric analysis of DBA reactivity of uNK cells in IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 8. **(L)** Representative FACS plots of the DBA staining in CD3^{-} CD122^{+} uNK cells compared to FMO in IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 8. Graphs depict **(M)** the share of CD3^{-} CD122^{+} uNK cells among all lymphocytes and **(N)** the share of DBA^{+} uNK cells as a percentage of all NK cells in IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings. Graphs depict mean and SEM. * $p < 0.05$; ** $p < 0.01$; ns nonsignificant.

x WT matings (Figures 2K-N). Thus, maternal C5aR2 deficiency negatively affects the number of DBA$^+$ uNK cells in the IS and shifts the uNK cell population toward a predominant DBA$^-$ uNK cell phenotype.

3.7 Frequency of CD11c$^+$ DCs and their colocalization with DBA$^+$ uNK cells are diminished in IS of *C5ar2*$^{-/-}$ x WT matings

In addition to uNK cells, uDC play a key role in balancing maternal-fetal immune tolerance and immunity (43). Further uDC also have direct functions in attracting and activating uNK cells (44). To determine if maternal C5aR2 deficiency also affects the uDC population, we analyzed CD11c$^+$ uDC frequency and co-localization of CD11c$^+$ uDCs with DBA$^+$ uNK cells in the IS of *C5ar2*$^{-/-}$ x WT matings compared to WT x WT matings at gd 8 using immunofluorescence microscopy.

In the IS of *C5ar2*$^{-/-}$ x WT matings, the frequency of CD11c$^+$ uDCs was significantly decreased compared with the frequency of CD11c$^+$ DCs in WT x WT matings (Figures 3A-C). Next, the occurrence of CD11c$^+$ uDC and DBA$^+$ uNK cell co-localizations was determined by counting the number CD11c$^+$ uDC with and without the proximity of DBA$^+$ uNK cells (Figures 3A, B). Co-localizations of CD11c$^+$ DCs with DBA$^+$ uNK cells were significantly reduced in *C5ar2*$^{-/-}$ x WT matings (19.8 per section) compared with the IS of WT x WT matings (85.7 per section) (Figure 3D). The IS of WT x WT matings showed a high density of uDC and uNK cells and many co-localizations of both cell types in proximity in the central zone of the decidua basalis (Figure 3A). A typical uNK-uDC co-localization in the IS of WT x WT matings is shown in Figure 3E.

The reduction of CD11c$^+$ uDC in the IS of *C5ar2*$^{-/-}$ x WT matings was also confirmed by flow cytometry (Figures 3F, G). In the IS of WT x WT matings, we found a mean of 9.72% CD11c$^+$, MHC-II$^+$ uDC gated within lineage-negative cells (CD3e$^-$, CD19$^-$, Ly6G$^-$, F4/80$^-$). In contrast, in IS of *C5ar2*$^{-/-}$ x WT matings a mean percentage of 6.86% uDC within the lineage-negative population was found (Figure 3F). In summary, our data show that maternal C5aR2 deficiency significantly reduced the number of CD11c$^+$ DCs and their ability to colocalize with DBA$^+$ uNK cells at the maternal-fetal interface, possibly contributing to reduced uNK cell activation by uDCs.

3.8 Human decidual NK cells, T cells and MΦ show different expression pattern of C5aR1 and C5aR2 compared to their peripheral blood counterparts

We next investigated if human decidual immune populations (dNK cells, T cells and macrophages) express C5a receptors. Human decidual leukocytes obtained from placental samples of healthy term pregnancies were analyzed for their expression of C5a receptors by flow cytometry. The gating strategy is shown in Supplementary Figure S5. Peripheral blood leukocytes were isolated from the same donors and analyzed. In both the *decidua basalis (D. basalis)*, the maternal part of the placenta at the implantation site, and the *decidua parietalis (D. parietalis)*, the maternal part of the placental membranes connected to the fetal chorion, we found a significant proportion of the dNK cells expressing C5aR2, whereas C5aR1 expression was almost completely absent (Figures 4A-C). This was in striking contrast to the expression pattern of C5aR2 and C5aR1 on peripheral blood NK cells (pNK), which were either double-negative or double-positive (Figures 4A, B).

Similar to the dNK cells, decidual T (dT) cells exclusively expressed C5aR2, whereas C5aR1 expression was virtually absent on these cells. C5a receptor expression was considerably lower in peripheral T (pT) cells compared to dT cells (Figures 4D-F). Interestingly, decidual MΦ (dMΦ) and peripheral blood monocytes (pM) both expressed C5aR1 and C5aR2 (Figures 4G, I). The frequency of C5aR1$^+$ dMΦ in *D.basalis* was significantly higher than the frequency of C5aR2$^+$ dMΦ, with an average of 53.2% cells being C5aR1$^+$ and 31.9% of cells being C5aR2$^+$ (Figure 4H). In *D. parietalis*, no significant difference was observed in the frequency of C5aR1$^+$ and C5aR2$^+$ dMΦ (Figure 4G). The pM showed high expression levels for both receptors, with mainly double-positive cells (Figure 4H). The selective expression of C5aR2 on decidual NK and T cells demonstrates the significance of the murine C5aR2 model for human pregnancy and leaves open many questions on how complement regulates decidual immune functions in healthy and complicated pregnancies.

4 Discussion

In this study, we highlight the importance of C5aR2 function in pregnancy. Pregnancy is a complex process requiring multiple adaptations of the maternal immune system to allow survival and protection of the semi-allogeneic fetus. Global maternal deficiency of the *C5ar2* gene severely impairs reproductive efficiency in mice (an effect not only observed at our institution but also reported by several collaborators working with these mice), suggesting a potential maternal dysregulation of the required adaptive mechanisms. Furthermore, we show in mice that the number of healthy IS in early gestation is significantly reduced in pregnancies with maternal C5aR2 deficiency. We identified increased mRNA levels of key pro-inflammatory cytokines involved uterine immune cell function in IS of *C5ar2*$^{-/-}$ pregnancies in combination with reduced dNK and uDC numbers, suggesting that the absence of C5aR2 signaling directly influences their function as well as infiltration into the maternal fetal interface. UNK cells are important regulators of spiral artery remodeling and trophoblast invasion. IFN-γ derived from uNK cells is the major factor inducing dilation of the spiral arteries during pregnancy (34). IFN-γ$^{-/-}$ mice show decidual abnormalities, including narrowed spiral arteries. However, in the IS of *C5ar2*$^{-/-}$ x WT matings, mRNA expression levels of *Ifng* were greatly increased, combined with a massive hyperdilation of decidual vessels, resembling the findings of Senegas et al. in pregnant mice with *T. gondii* infection, in which increased

10.3389/fimmu.2024.1411315

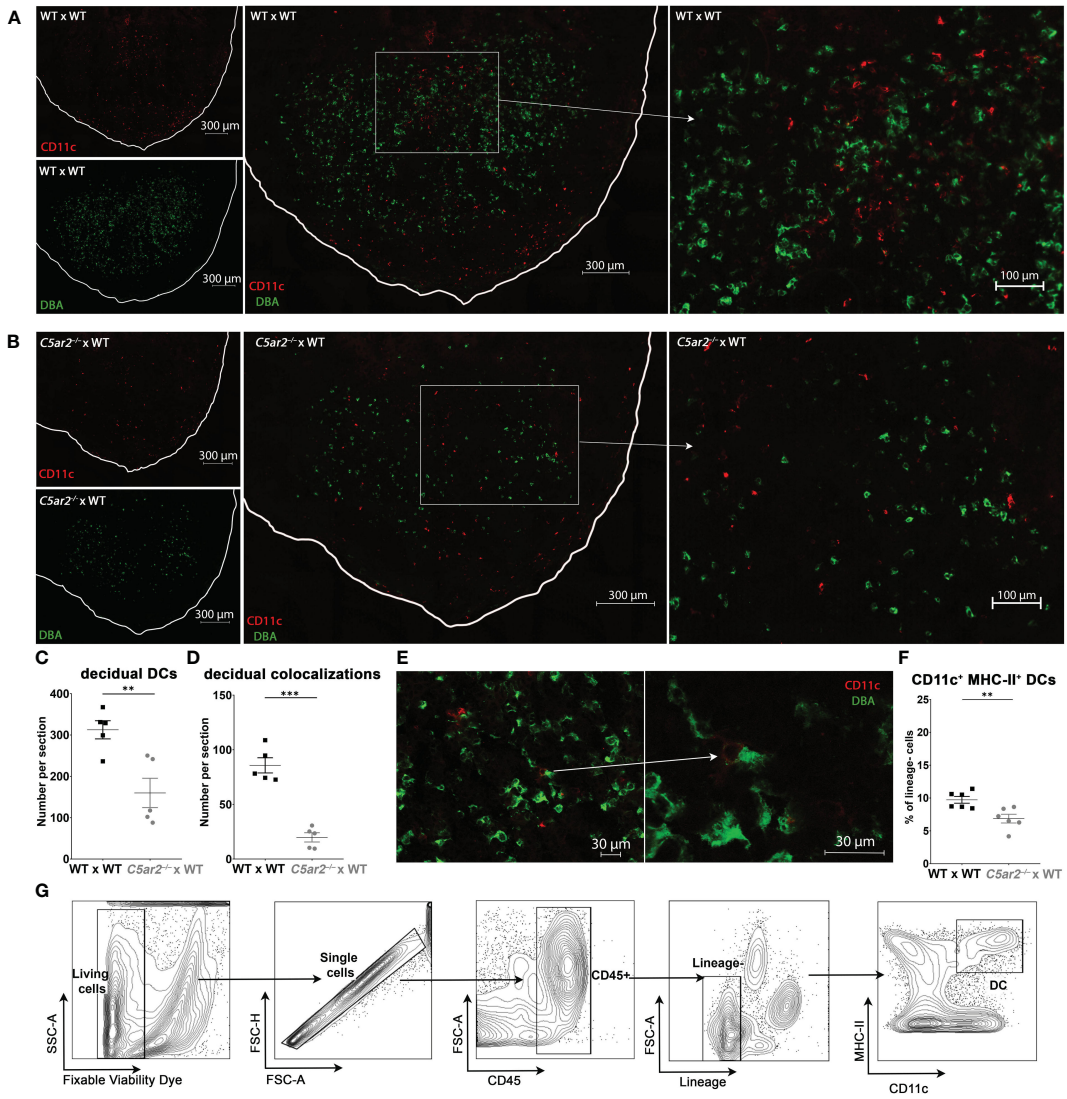


FIGURE 3
Uterine DCs and their co-localizations with DBA$^+$ uNK cells in the IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings. Representative pictures from the *decidua basalis* of IS sections from **(A)** WT x WT or **(B)** *C5ar2*$^{-/-}$ x WT matings after staining with DBA (green) and CD11c (red) to identify DBA$^+$ uNK cells and CD11c$^+$ uDC at gd 8. Tissue borders are marked by the white line for orientation. Left: CD11c$^+$ cells (red, top panel) and DBA$^+$ cells (green, bottom panel) in **(A)** WT x WT and **(B)** *C5ar2*$^{-/-}$ x WT matings. Middle panel shows the overlay of the two channels for CD11c and DBA in the overview of the decidual region in **(A)** WT x WT and **(B)** *C5ar2*$^{-/-}$ x WT matings. Enlarged details from the overview picture (marked with white rectangles) within the central zone of the *decidua basalis* of **(A)** WT x WT and **(B)** *C5ar2*$^{-/-}$ x WT matings are shown in the right panel. Graphs depict **(C)** the number of CD11c$^+$ uDCs and **(D)** the number of DBA$^+$ uNK cell-CD11c$^+$ uDC co-localizations within the decidua of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 8. **(E)** Representative co-localization of a DBA$^+$ uNK cell and a CD11c$^+$ uDC within the decidua of a WT x WT mating. **(F)** Frequency of CD11c$^+$ MHC-II$^+$ DCs among lineage-negative leukocytes in the IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings identified by flow cytometric analysis at gd 8. **(G)** Gating strategy used for the flow cytometric analysis of uDCs in the IS of WT x WT and *C5ar2*$^{-/-}$ x WT matings at gd 8. Lineage markers used for the exclusion of T cells, B cells, NK cells, Neutrophils and Macrophages were CD3e, CD19, CD49b, Ly6G, F4/80. Graphs depict mean and SEM. ** $p < 0.01$; *** $p < 0.001$.

IFN-γ levels and lower IL-15 levels caused hyperdilation of spiral arteries and fetal resorptions (39), suggesting similar mechanisms leading to pregnancy failure in these cases. C5aR2 may be directly involved in the mechanism of increasing intrauterine IFN-γ levels, as it is already known to increase IFN-γ production by pNK cells stimulated by IL-18 and IL-12 *in vitro* (17).

There are two major subsets of murine uNK cells that differ primarily in their reactivity to DBA (24), with the DBA$^+$ subset producing mainly angiogenic factors such as PlGF, while the DBA- subset secretes mainly IFN-γ (42). The reduced absolute frequency of uNK cells in the IS of *C5ar2*$^{-/-}$ pregnancies was accompanied by a phenotypic shift toward the more IFN-γ-producing DBA- subset, complementing the reduced *Plgf* and concomitantly increased *Ifng* transcript levels. In addition, the increased intrauterine *Ifng* expression of pregnant *C5ar2*$^{-/-}$ females may be related to a dysregulation of the expression of the synergistic IFN-γ inducing factors IL-18 and IL-12. Both cytokines are produced by uDCs, which have important functions in mediating immunity and tolerance during pregnancy (45). Throughout pregnancy, there is reciprocal crosstalk between uDCs and uNK cells within the

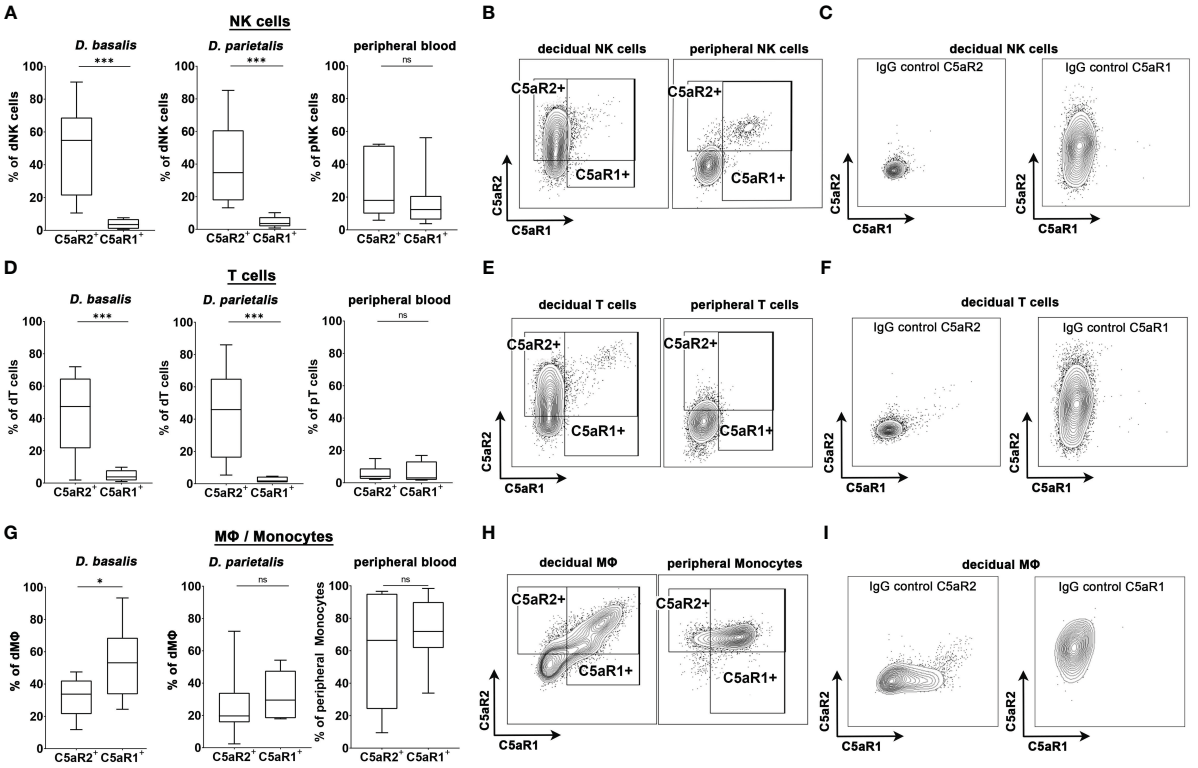


FIGURE 4
C5aR1 and C5aR2 expression patterns in human decidual cells. Graphs depict the share of C5aR1$^+$ or C5aR2$^+$ **(A)** NK cells, **(D)** T cells, and **(G)** MΦ/ monocytes in the *decidua basalis (D. basalis)*, *decidua parietalis (D. parietalis)*, and peripheral blood of healthy pregnant woman at term. Representative FACS plots and gates for C5aR1 and C5aR2 expression of **(B)** decidual and peripheral NK cells, **(E)** decidual and peripheral T cells, and **(H)** decidual MΦ and peripheral monocytes are shown. Positive staining was identified by isotype controls for C5aR1 and C5aR2, respectively. Representative plots of the staining of the isotype controls in **(C)** decidual NK cells, **(F)** decidual T cells, and **(I)** decidual MΦ. For peripheral T cells one sample was identified as an outlier by Grubbs test and excluded from the analysis. Boxplots depict median and interquartile range. ($n_{D.basalis}$=8, $n_{D.parietalis}$=8, $n_{Peripheral\ blood}$ =7). * $p < 0.05$; *** $p < 0.001$; ns nonsignificant.

decidua, either indirectly through cytokine secretion or directly through cell-to-cell contact (44). In addition to altered mRNA levels of key cytokines, we also detected decreased numbers of uNK cells, uDCs, and uNK-uDC co-localizations in the IS of *C5ar2*$^{-/-}$ x WT matings. The reduced frequency of cell co-localization in combination with the altered cytokine mRNA levels indicates a severe dysregulation of NK-DC interaction at the maternal-fetal interface in *C5ar2*$^{-/-}$ x WT matings. Previous studies have shown that the absence of DCs in the murine IS results in decreased levels of IL-15 and IL-12 *in utero*, leading to reduced abundance, size, and IFN-γ expression of uNK cells (37). However, in *C5ar2*$^{-/-}$ x WT matings, the reduced frequency of uDCs was associated with similar levels of *Il15* transcripts but a massive increase in *Il12p35*, *Il18*, and *Ifng* expression within the IS. Possible explanations might be an overcompensation of the lowered cell number, but also a direct involvement of C5aR2 in the regulation of the expression of these cytokines by uNK cells and uDCs.

Not only the frequency, but also the size, infiltration depth and phenotype of uNK cells were affected by maternal C5aR2 deficiency. Normally, uNK cells have large diameters because they contain large amounts of granules despite their low cytotoxicity (46). Decreased cell size is indicative of reduced maturation (37, 40). *In utero*, DCs are the major source for IL-15 being the key cytokine for NK cell development (47). As absence of DCs as causes decreased uNK cell number and size (37), we propose impaired uNK-uDC crosstalk as the likely major cause of the altered uNK cells abundance, phenotype, and function in the IS of *C5ar2*$^{-/-}$ x WT matings. It has been shown that depletion of PlGF also leads to morphometric anomalies of the IS as well as an increased frequency of immature uNK cells (48). Accordingly, the reduced levels of *Plgf* transcripts in the IS of *C5ar2*$^{-/-}$ x WT matings could be involved in the development of the unfavorable environmental conditions.

In murine IS, uNK cells are restricted to the mesometrial site of the uterus. From gd 5 to gd 11, uNK cells start to proliferate, gain increasing numbers of cytoplasmic granules, and form a transient lymphoid structure known as the mesometrial lymphoid aggregate of pregnancy (MLAp; Peel (49); Paffaro et al., (24)). There is evidence for the recruitment of early NK cell precursors from the blood (50, 51), but also for the invasion of mature NK cells from the periphery (52). In mice, uNK cells initially accumulate in the *D. basalis* (around gd 6.5 (53);, but subsequently settle near by the spiral arteries (between gd 8.5 and gd 13.5) and begin to cooperate with the invading trophoblast cells to ensure correct remodeling of the arteries (21, 54). However, in murine pregnancy, the arterial media is mainly modified as a result of direct infiltration of uNK cells (54). The strong reduction in the infiltration of uNK cells into the decidua of the IS of *C5ar2*$^{-/-}$ females, indicates an impaired recruitment and infiltration process.

Using the well-established C5aR2-reporter mice (Karsten et al., (17)), we detected C5aR2 expression in uterine NK cells and DCs. However, not the entirety of uNK and uDC was positive for C5aR2, but only subsets of the cell types. This may indicate that not only the direct effects of the receptor on the NK cells and DCs but also effects caused by C5aR2 deficiency on other cell types interacting and influencing NK and DC function might be responsible for the effects seen in *C5ar2*$^{-/-}$ mice. Likewise, the C5aR2^{+} cells could be of special functional importance. It is also conceivable that the cells require C5aR2 for their functionality during their development. In order to differentiate between these possible explanations, studies using mice with cell specific C5aR2 deficiency are needed to elucidate the mechanistic process causing the distinct changes in cell phenotype and function as well as the dramatically impaired reproductive efficiency in C5aR2 deficient mice.

Peripheral human NK cells intracellularly express both receptors for C5a under steady-state conditions (55). Interestingly, our findings showed that a significant proportion of dNK cells expressed C5aR2, whereas C5aR1 expression was not detected. In contrast, pNK cells were either double-negative or double-positive for C5a receptors. This marked difference in C5a receptor expression on pNK and dNK suggests different modes of receptor function in the different NK cell types. Human dNK cells are essential regulators of implantation, trophoblast invasion and spiral artery remodeling and thus contribute critically to pregnancy success (56). Alterations in dNK cell number have been found in patients with pregnancy complications such as pre-eclampsia or intrauterine growth restriction associated with impaired placentation process (57). In particular pre-eclampsia is a severe condition resulting of incomplete trophoblast invasion and spiral artery remodeling with defects in NK cell crosstalk likely to be etiologically involved (56). However, both pre-eclampsia as well as its variant HELLP Syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets) are associated with increased complement activation (58, 59). In placentas from pre-eclampsia patients, elevated C5a deposition in macrophages and increased C5aR1 expression on trophoblast cells as well as increased maternal serum C5a levels have been found (60). In HELLP syndrome, elevated C5a and C5b-9 levels are typically seen, and the condition is associated with mutations in complement genes (61). There is evidence that blocking C5 with eculizumab may be beneficial for pre-eclamptic patients (59). In one case report, treatment with eculizumab prolonged pregnancy and reduced symptoms in a HELLP patient (62). Collectively, these findings illustrate the need to control C5a-mediated inflammatory effects not during healthy pregnancy and in pregnancy complications.

C5aR2 may have a specific function in uNK cells in mice and humans, separate from C5aR1 and other cell types (18, 63). Complement components can be produced locally or intracellularly, influencing local complement activation and normal cell functions (64–67). Within cells, the function of C5a may differ from its classical chemoattractant properties. C5a produced by immune cells *in utero*, like uNK cells and uDCs, may contribute to spiral artery remodeling and trophoblast invasion, crucial for successful pregnancy. C5aR2 is mainly expressed intracellularly, including in decidual immune cells, suggesting its potential effect. Further studies are needed to understand the precise mechanisms of C5aR2 function in pregnancy.

In conclusion, our study demonstrates an essential role for C5aR2 function for a healthy pregnancy outcome. Maternal C5aR2 deficiency in mice causes severe impairments in reproductive efficiency, with reduced numbers of healthy implantation sites and altered mRNA levels of key cytokines involved in NK cell and DC function. These changes are accompanied by a dysregulation of uNK cell uDC crosstalk and a shift in uNK cell phenotype toward increased production of IFN-γ. These findings suggest that C5aR2 plays a critical role in immune system adaptations during pregnancy and may be a crucial target for improving pregnancy outcomes and reducing pregnancy complications.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethikkomission der Universität zu Lübeck. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Ministerium für Energiewende, Landwirtschaft, 110 Umwelt und ländliche Räume des Landes Schleswig-Holstein, Kiel, Germany. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FF: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. KL: Writing – original draft, Investigation. JN: Writing – original draft, Investigation. A-KM: Writing – original draft, Investigation. DS: Writing – review & editing, Writing – original draft, Validation, Supervision. TT: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology. CK: Data curation, Conceptualization, Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was funded by the Deutsche Forschungsgemeinschaft through IRTG 1911 “Immune regulation of inflammation in allergy and infection”, project B2. In addition, the publication of this article was financially supported by the Land Schleswig-Holstein through the Open Access Publikationsfonds.

Acknowledgments

We acknowledge support by the Cell Analysis Core Facility (CAnaCore) of the University of Lübeck. Furthermore, we acknowlegde the collaboration of Dr. Verena Bossung and Dr. Michael Gembicki (Department of Gynaecology, UKSH, Campus Lübeck).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1411315/full#supplementary-material

References

1. Papúchová H, Meissner TB, Li Q, Strominger JL, Tilburgs T. The dual role of HLA-C in tolerance and immunity at the maternal-fetal interface. *Front Immunol.* (2019) 10:2730. doi: 10.3389/fimmu.2019.02730

2. Mor G, Cardenas I. The immune system in pregnancy: a unique complexity. *Am J Reprod Immunol.* (2010) 63:425–33. doi: 10.1111/j.1600-0897.2010.00836.x

3. Ander SE, Diamond MS, Coyne CB. Immune responses at the maternal-fetal interface. *Sci Immunol.* (2019) 4:eaat6114. doi: 10.1126/sciimmunol.aat6114

4. Walport MJ. Complement. First of two parts. *N Engl J Med.* (2001) 344:1058–66. doi: 10.1056/NEJM200104053441406

5. Sarma JV, Ward PA. New developments in C5a receptor signaling. *Cell Health Cytoskelet.* (2012) 4:73–82. doi: 10.2147/CHC

6. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Mol Immunol.* (2011) 48:1631–42. doi: 10.1016/j.molimm.2011.04.014

7. Regal JF, Gilbert JS, Burwick RM. The complement system and adverse pregnancy outcomes. *Mol Immunol.* (2015) 67:56–70. doi: 10.1016/j.molimm.2015.02.030

8. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien JK, et al. Normal pregnancy is characterized by systemic activation of the complement system. *J Matern Fetal Neonatal Med.* (2005) 17:239–45. doi: 10.1080/14767050500072722

9. Girardi G, Lingo JJ, Fleming SD, Regal JF. Essential role of complement in pregnancy: from implantation to parturition and beyond. *Front Immunol.* (2020) 11:1681. doi: 10.3389/fimmu.2020.01681

10. Chenoweth DE, Hugli TE. Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. *Proc Natl Acad Sci U S A.* (1978) 75:3943–7. doi: 10.1073/pnas.75.8.3943

11. Ohno M, Hirata T, Enomoto M, Araki T, Ishimaru H, Takahashi TA. A putative chemoattractant receptor, C5L2, is expressed in granulocyte and immature dendritic cells, but not in mature dendritic cells. *Mol Immunol.* (2000) 37:407–12. doi: 10.1016/S0161-5890(00)00067-5

12. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Köhl J. The role of the anaphylatoxins in health and disease. *Mol Immunol.* (2009) 46:2753–66. doi: 10.1016/j.molimm.2009.04.027

13. Li R, Coulthard LG, Wu MCL, Taylor SM, Woodruff TM. C5L2: a controversial receptor of complement anaphylatoxin, C5a. *FASEB J.* (2012) 27:855–64. doi: 10.1096/fj.12-220509

14. Li XX, Lee JD, Kemper C, Woodruff TM. The complement receptor C5aR2: A powerful modulator of innate and adaptive immunity. *J Immunol Baltim Md 1950.* (2019) 202:3339–48. doi: 10.4049/jimmunol.1900371

15. Bamberg CE, Mackay CR, Lee H, Zahra D, Jackson J, Lim YS, et al. The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. *J Biol Chem.* (2010) 285:7633–44. doi: 10.1074/jbc.M109.092106

16. Karsten CM, Laumonnier Y, Eurich B, Ender F, Bröker K, Roy S, et al. Monitoring and cell-specific deletion of C5aR1 using a novel floxed GFP-C5aR1 reporter knock-in mouse. *J Immunol Baltim Md 1950.* (2015) 194:1841–55. doi: 10.4049/jimmunol.1401401

17. Karsten CM, Wiese AV, Mey F, Figge J, Woodruff TM, Reuter T, et al. Monitoring C5aR2 expression using a floxed tdTomato-C5aR2 knock-in mouse. *J Immunol.* (2017) 199:3234–48. doi: 10.4049/jimmunol.1700710

18. Seiler DL, Kleingarn M, Kähler KH, Gruner C, Schanzenbacher J, Ehlers-Jeske E, et al. C5aR2 deficiency ameliorates inflammation in murine epidermolysis bullosa acquisita by regulating FcγRIIb expression on neutrophils. *J Invest Dermatol.* (2022) 142(10): S0022–202X(22)00001-X. doi: 10.1016/j.jid.2021.12.029

19. Zhang T, Wu KY, Ma N, Wei LL, Garstka M, Zhou W, et al. The C5a/C5aR2 axis promotes renal inflammation and tissue damage. *JCI Insight.* (2020) 5:134081. doi: 10.1172/jci.insight.134081

20. Mueller-Ortiz SL, Shivshankar P, Wetsel RA. The Second Receptor for C5a, C5aR2, Is Detrimental to Mice during Systemic Infection with Listeria monocytogenes. *J Immunol Baltim Md 1950.* (2019) 203:2701–11. doi: 10.4049/jimmunol.1900314

21. Moffett A, Loke C. Immunology of placentation in eutherian mammals. *Nat Rev Immunol.* (2006) 6:584–94. doi: 10.1038/nri1897

22. Crespo ÂC, Strominger JL, Tilburgs T. Expression of KIR2DS1 by decidual natural killer cells increases their ability to control placental HCMV infection. *Proc Natl Acad Sci.* (2016) 113:15072–7. doi: 10.1073/pnas.1617927114

23. Boulenouar S, Doisne JM, Sferruzzi-Perri A, Gaynor LM, Kieckbusch J, Balmas E, et al. The residual innate lymphoid cells in NFIL3-deficient mice support suboptimal maternal adaptations to pregnancy. *Front Immunol.* (2016) 7:43. doi: 10.3389/fimmu.2016.00043

24. Paffaro VA, Bizinotto MC, Joazeiro PP, Yamada AT. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. *Placenta.* (2003) 24:479–88. doi: 10.1053/plac.2002.0919

25. King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ Population. *Dev Immunol.* (1991) 1:169–90. doi: 10.1155/1991/83493

26. King A, Jokhi PP, Burrows TD, Gardner L, Sharkey AM, Lore YW. Functions of human decidual NK cells. *Am J Reprod Immunol.* (1996) 35:258–60. doi: 10.1111/j.1600-0897.1996.tb00041.x

27. Vacca P, Pietra G, Falco M, Romeo E, Bottino C, Bellora F, et al. Analysis of natural killer cells isolated from human decidua: evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood.* (2006) 108:4078–85. doi: 10.1182/blood-2006-04-017343

28. Stewart IJ, Peel S. Mouse metrial gland cells do not kill Yac-1 myeloma cells. *J Reprod Immunol.* (1993) 24:165–71. doi: 10.1016/0165-0378(93)90018-D

29. Gaynor LM, Colucci F. Uterine natural killer cells: functional distinctions and influence on pregnancy in humans and mice. *Front Immunol.* (2017) 8. doi: 10.3389/fimmu.2017.00467

30. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med.* (2006) 12:1065–74. doi: 10.1038/nm1452

31. King A, Birkby C, Loke YW. Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. *Cell Immunol.* (1989) 118:337–44. doi: 10.1016/0008-8749(89)90382-1

32. Yokoyama WM. The mother–child union: The case of missing-self and protection of the fetus. *Proc Natl Acad Sci U S A.* (1997) 94:5998–6000. doi: 10.1073/pnas.94.12.5998

33. Crespo ÂC, Mulik S, Dotiwala F, Ansara JA, Santara SS, Ingersoll K, et al. Decidual NK cells transfer granulysin to selectively kill bacteria in trophoblasts. *Cell.* (2020) 182:1125–1139.e18. doi: 10.1016/j.cell.2020.07.019

34. Ashkar AA, Di Santo JP, Croy BA. Interferon γ Contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell

maturation during normal murine pregnancy. *J Exp Med*. (2000) 192:259–70. doi: 10.1084/jem.192.2.259

35. Ashkar AA, Croy BA. Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy. *Semin Immunol*. (2001) 13:235–41. doi: 10.1006/smim.2000.0319

36. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: Dendritic cells and NK cells take centre stage. *Nat Rev Immunol*. (2005) 5:112–24. doi: 10.1038/nri1549

37. Karsten CM, Behrends J, Wagner AK, Fuchs F, Figge J, Schmudde I, et al. DC within the pregnant mouse uterus influence growth and functional properties of uterine NK cells. *Eur J Immunol*. (2009) 39:2203–14. doi: 10.1002/eji.200838844

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262

39. Senegas A, Villard O, Neuville A, Marcellin L, Pfaff AW, Steinmetz T, et al. Toxoplasma gondii-induced foetal resorption in mice involves interferon-gamma-induced apoptosis and spiral artery dilation at the maternofoetal interface. *Int J Parasitol*. (2009) 39:481–7. doi: 10.1016/j.ijpara.2008.08.009

40. Felker AM, Croy BA. Natural cytotoxicity receptor 1 in mouse uNK cell maturation and function. *Mucosal Immunol*. (2017) 10:1122–32. doi: 10.1038/mi.2016.126

41. Zhang JH, Yamada AT, Croy BA. DBA-lectin reactivity defines natural killer cells that have homed to mouse decidua. *Placenta*. (2009) 30:968–73. doi: 10.1016/j.placenta.2009.08.011

42. Chen Z, Zhang J, Hatta K, Lima PDA, Yadi H, Colucci F, et al. DBA-lectin reactivity defines mouse uterine natural killer cell subsets with biased gene expression. *Biol Reprod*. (2012) 87:81. doi: 10.1095/biolreprod.112.102293

43. Tagliani E, Erlebacher A. Dendritic cell function at the maternal–fetal interface. *Expert Rev Clin Immunol*. (2011) 7:593–602. doi: 10.1586/eci.11.52

44. Leno-Durán E, Muñoz-Fernández R, Olivares EG, Tirado-González I. Liaison between natural killer cells and dendritic cells in human gestation. *Cell Mol Immunol*. (2014) 11:449–55. doi: 10.1038/cmi.2014.36

45. Blois SM, Kammerer U, Soto CA, Tometten MC, Shaikly V, Barrientos G, et al. Dendritic cells: key to fetal tolerance? *Biol Reprod*. (2007) 77:590–8. doi: 10.1095/biolreprod.107.060632

46. Sojka DK, Yang L, Yokoyama WM. Uterine natural killer cells. *Front Immunol*. (2019) 10. doi: 10.3389/fimmu.2019.00960

47. Ashkar AA, Black GP, Wei Q, He H, Liang L, Head JR, et al. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. *J Immunol*. (2003) 171:2937–44. doi: 10.4049/jimmunol.171.6.2937

48. Tayade C, Hilchie D, He H, Fang Y, Moons L, Carmeliet P, et al. Genetic deletion of placenta growth factor in mice alters uterine NK cells. *J Immunol*. (2007) 178:4267–75. doi: 10.4049/jimmunol.178.7.4267

49. Peel S. Granulated metrial gland cells. *Adv Anat Embryol Cell Biol*. (1989) 115:1–112. doi: 10.1007/978-3-642-74170-8

50. Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, et al. Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J Immunol*. (2002) 168:22–8. doi: 10.4049/jimmunol.168.1.22

51. Male V, Hughes T, McClory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol*. (2010) 185:3913–8. doi: 10.4049/jimmunol.1001637

52. Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, et al. Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood*. (2008) 111:3108–15. doi: 10.1182/blood-2007-08-105965

53. Croy BA, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol Rev*. (2006) 214:161–85. doi: 10.1111/j.1600-065X.2006.00447.x

54. Zhang J, Chen Z, Smith GN, Croy BA. Natural killer cell-triggered vascular transformation: maternal care before birth? *Cell Mol Immunol*. (2011) 8:1–11. doi: 10.1038/cmi.2010.38

55. Min X, Liu C, Wei Y, Wang N, Yuan G, Liu D, et al. Expression and regulation of complement receptors by human natural killer cells. *Immunobiology*. (2014) 219:671–9. doi: 10.1016/j.imbio.2014.03.018

56. Goldman-Wohl D, Yagel S. NK cells and pre-eclampsia. *Reprod BioMed Online*. (2008) 16:227–31. doi: 10.1016/S1472-6483(10)60578-0

57. Williams PJ, Bulmer JN, Searle RF, Innes BA, Robson SC. Altered decidual leucocyte populations in the placental bed in pre-eclampsia and foetal growth restriction: a comparison with late normal pregnancy. *Reprod Camb Engl*. (2009) 138:177–84. doi: 10.1530/REP-09-0007

58. Sabau L, Terriou L, Provot F, Fourrier F, Roumier C, Caron C, et al. Are there any additional mechanisms for haemolysis in HELLP syndrome? *Thromb Res*. (2016) 142:40–3. doi: 10.1016/j.thromres.2016.03.014

59. Vaught AJ, Gavriilaki E, Hueppchen N, Blakemore K, Yuan X, Seifert SM, et al. Direct evidence of complement activation in HELLP syndrome: a link to atypical hemolytic uremic syndrome. *Exp Hematol*. (2016) 44:390–8. doi: 10.1016/j.exphem.2016.01.005

60. Ma Y, Kong L, Ge Q, Lu Y, Hong M, Zhang Y, et al. Complement 5a-mediated trophoblasts dysfunction is involved in the development of pre-eclampsia. *J Cell Mol Med*. (2018) 22:1034–46. doi: 10.1111/jcmm.13466

61. Fang CJ, Fremeaux-Bacchi V, Liszewski MK, Pianetti G, Noris M, Goodship THJ, et al. Membrane cofactor protein mutations in atypical hemolytic uremic syndrome (aHUS), fatal Stx-HUS, C3 glomerulonephritis, and the HELLP syndrome. *Blood*. (2008) 111:624–32. doi: 10.1182/blood-2007-04-084533

62. Burwick RM, Feinberg BB. Eculizumab for the treatment of preeclampsia/HELLP syndrome. *Placenta*. (2013) 34:201–3. doi: 10.1016/j.placenta.2012.11.014

63. Bosmann M, Grailer JJ, Ruemmler R, Russkamp NF, Zetoune FS, Sarma JV, et al. Extracellular histones are essential effectors of C5aR- and C5L2-mediated tissue damage and inflammation in acute lung injury. *FASEB J*. (2013) 27:5010–21. doi: 10.1096/fj.13-236380

64. Arbore G, Kemper C, Kolev M. Intracellular complement – the complosome – in immune cell regulation. *Mol Immunol*. (2017) 89:2–9. doi: 10.1016/j.molimm.2017.05.012

65. Heeger PS, Kemper C. Novel roles of complement in T effector cell regulation. *Immunobiology*. (2012) 217:216–24. doi: 10.1016/j.imbio.2011.06.004

66. Kolev M, Le Friec G, Kemper C. The role of complement in $CD4^+$ T cell homeostasis and effector functions. *Semin Immunol*. (2013) 25:12–9. doi: 10.1016/j.smim.2013.04.012

67. Liszewski MK, Kolev M, Le Friec G, Leung M, Bertram PG, Fara AF, et al. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity*. (2013) 39:1143–57. doi: 10.1016/j.immuni.2013.10.018

frontiers | Frontiers in Immunology

TYPE Systematic Review
PUBLISHED 16 August 2024
DOI 10.3389/fimmu.2024.1427454

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Hong Zeng,
Central South University, China
Chiara Tersigni,
Agostino Gemelli University Polyclinic (IRCCS), Italy

*CORRESPONDENCE
Carlo Ticconi
ticconi@med.uniroma2.it

†These authors have contributed equally to this work and share first authorship

RECEIVED 03 May 2024
ACCEPTED 30 July 2024
PUBLISHED 16 August 2024

CITATION
Ticconi C, Inversetti A, Marraffa S, Campagnolo L, Arthur J, Zambella E and Di Simone N (2024) Chronic endometritis and recurrent reproductive failure: a systematic review and meta-analysis. *Front. Immunol.* 15:1427454. doi: 10.3389/fimmu.2024.1427454

COPYRIGHT
© 2024 Ticconi, Inversetti, Marraffa, Campagnolo, Arthur, Zambella and Di Simone. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Chronic endometritis and recurrent reproductive failure: a systematic review and meta-analysis

Carlo Ticconi[1*†], Annalisa Inversetti[2,3†], Serena Marraffa[1], Luisa Campagnolo[4], Jephtah Arthur[2], Enrica Zambella[2] and Nicoletta Di Simone[2,3]

[1]Department of Surgical Sciences, Section of Gynecology and Obstetrics, University of Rome Tor Vergata, Rome, Italy, [2]Department of Biomedical Sciences, Humanitas University, Milan, Italy, [3]Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS) Humanitas Research Hospital, Milan, Italy, [4]Department of Biomedicine and Prevention, University of Rome Tor Vergata, Rome, Italy

Background: The endometrium holds a crucial role in reproduction by supporting blastocyst adhesion, cytotrophoblast invasion and fetal development. Among the various uterine disorders, endometritis, particularly chronic endometritis (CE), has gained attention due to its association with adverse reproductive outcomes (recurrent pregnancy loss (RPL), recurrent implantation failure (RIF), and infertility). The association between CE and adverse reproductive outcomes stresses the necessity for comprehensive diagnostic and therapeutic strategies to optimize fertility outcomes and support individuals in their journey towards parenthood.

Aim: To explore the relationship between CE and reproductive disorders.

Methods: Following PRISMA guidelines, a systematic review and meta-analysis using published data from 1990 to 2024 were carried out.

Results: A population of 1,038 women was included. Regarding CE-infertility association, a positive correlation was found, with 19.46% CE rate in infertile women compared to 7.7% in controls (OR: 2.96, 95% CI 1.53-5.72, p 0.001). No significant association was observed between RIF and CE (OR: 1.10, 95% CI 0.26-4.61, p 0.90), CE rates in both groups were relatively comparable, with 6.35% in women with RIF and 5.8% in controls. On the opposite, a strong association between CE and RPL was found, reporting a CE rate of 37.6% in RPL cases compared to 16.4% in controls (OR: 3.59, 95% CI 2.46-5.24, $p < 0.00001$).

Conclusions: CE appears to be associated to infertility and RPL, while no significant association was noted in cases of RIF.

10.3389/fimmu.2024.1427454

Systematic review registration: https://www.crd.york.ac.uk/prospero/#recordDetails PROSPERO, identifier CRD42024541879.

KEYWORDS

chronic endometritis, recurrent pregnancy loss, recurrent implantation failure, infertility, reproductive failure chronic endometritis, reproductive failure, systematic review, meta-analysis

1 Introduction

Chronic endometritis (CE) is a persistent and mild inflammation of the endometrial mucosa. Currently there is no standardized or accepted definition of chronic endometritis, but the presence of numerous plasma cells in the stroma is the most sensitive and specific finding for the definition and diagnosis of this disease (1). This immunological alteration is believed to be a consequence of a bacterial infection. CE has been found to be associated with reproductive failure. However, there are still no clear recommendations on whether its inclusion in the primary work-up of infertile couples is essential.

While acute endometritis typically occurs in response to infection following childbirth, miscarriage, or certain medical procedures, chronic endometritis involves persistent inflammation that may go unnoticed for extended periods. The diagnosis of chronic endometritis requires a thorough strategy that incorporates clinical assessment, imaging investigations, and histopathological examination. Recent progressions in diagnostic methodologies have enhanced detection precision, underscoring the importance of maintaining a vigilant stance, especially among individuals encountering infertility or recurrent pregnancy losses (2).

Previous studies have underscored the correlation between chronic endometritis and unfavorable reproductive outcomes, resulting in reduced pregnancy rates compared to individuals without the condition, following assisted reproductive technologies (ART) interventions (3).

Some investigators have shown possible adverse effects of CE on human reproduction. The frequency of CE is 2.8–56.8% in infertility, 14–67.5% in recurrent implantation failure (RIF), and 9.3–67.6% in recurrent pregnancy loss (RPL) (4). The current epidemiological data exhibit significant heterogeneity.

A recent study by Volodarsky-Perel et al. highlights a significant association between chronic endometritis (CE) and infertility, particularly in women with endometrial polyps (EP) and a history of infertility. Vascular changes observed in the endometrium of infertile women suggest a potential link between CE and infertility-related vascular pathology (5).

The success of *in vitro* fertilization (IVF) has improved dramatically since its inception. However, there are still transfers that do not result in implantation. RIF presents a challenging development in assisted reproductive technology (ART) where despite multiple transfers, successful implantation does not occur (6). With the increasing success of *in vitro* fertilization, the demand for better outcomes has grown among patients and providers, leading to a rise in literature exploring recurrent implantation failure. Yet, there remains a lack of consensus on its definition. It can therefore be described as three failed IVF or ICSI (intracytoplasmic sperm injection) treatments, each with at least one fresh good quality embryo per transfer, or failure to achieve pregnancy after transfer of 10 good quality embryos (7).

A study investigated the prevalence of chronic endometritis in women with failed implantation, and its impact on subsequent live birth rates (LBRs) after antibiotic treatment (8). Chronic endometritis was found in 9% of participants, suggesting the inclusion of endometrial biopsy in evaluations. Those with chronic endometritis had more failed implantations and showed greater improvement in subsequent LBR after treatment.

Furthermore, mounting evidence suggests that chronic endometrial inflammation may disrupt the delicate balance necessary for successful implantation and pregnancy maintenance, thereby predisposing women to recurrent miscarriages (9, 10). Despite growing interest in this area (11), a comprehensive synthesis of available evidence is warranted to elucidate the nature of the relationship between CE and RPL.

Overall, the findings stress on the significance of the endometrial environment in embryo implantation and fetal development, urging further research and attention to CE's role in reproductive health to optimize fertility outcomes and support individuals in their journey towards parenthood (12).

This systematic review and meta-analysis aimed to investigate the potential link between chronic endometritis (CE) and various clinically significant female reproductive disorders, such as infertility, RIF and RPL.

2 Materials and methods

This systematic review and meta-analysis were conducted and reported following the guidelines of the *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA) (13).

As the review was based on data already published in the literature, approval from the territorial ethics committee was not required. The study protocol is currently undergoing evaluation in the *International Prospective Register of Systematic Reviews (PROSPERO registry)* with the ID CRD42024541879.

2.1 Inclusion and exclusion criteria

All studies examining the correlation between chronic endometritis (CE) and recurrent pregnancy loss (RPL), infertility, and repeated implantation failure (RIF) were included in the review. However, due to the absence of standardized diagnostic criteria for chronic endometritis and the varying definitions across the included studies, CE was identified based on the specific parameters outlined in each article reviewed.

Randomized controlled trials, cohort studies, case-control studies, and cross-sectional studies were deemed suitable for scientific analysis. The research and selection criteria were restricted to articles published in English from 1990 onwards.

Systematic reviews, meta-analyses, reviews, case reports, notes or letters, book chapters, errata, and conference abstracts were excluded. Only studies involving human participants were included, while those involving animals were excluded.

2.2 Sources of information

We conducted a systematic literature search spanning from January 1, 1990, to February 2, 2024, across five electronic databases*: PubMed, Scopus, Web of Science, ScienceDirect, and the Cochrane Library.*

2.3 Research strategy

For our research, we devised combinations of relevant Medical Subject Headings (MeSH) terms and keywords pertaining to the exposure and event under scrutiny. We crafted two distinct search strings for each of the five databases considered: one concentrating on terms related to chronic endometritis and recurrent pregnancy losses/spontaneous abortions, and another aimed at locating articles concerning chronic endometritis in connection with infertility and repeated implantation failure. This approach was chosen to prevent the creation of overly complex search strings, which might compromise the overall effectiveness of the search. Moreover, certain databases, such as *ScienceDirect*, have a maximum limit of eight logical or Boolean operators per search. Therefore, dividing the search into multiple parts enabled us to manage this limitation more effectively and ensure comprehensive coverage of the topics of interest. Additionally, we meticulously scrutinized the bibliographic lists of all identified articles to prevent the omission of pertinent data.

The following are the two search strings used for each database:

2.3.1 Pubmed

1. ("Endometritis"[Mesh] OR endometritis*[tiab] OR endomyometritis OR endometrial inflammation [tiab] OR "CD138 antigen"[Mesh] OR CD138 antigen [tiab] OR "Plasma cells"[Mesh] OR plasma cell* [tiab] OR plasmacyte* [tiab]) AND ("Recurrent pregnancy loss"[Mesh] OR recurrent pregnancy loss*[tiab] OR recurrent abortion*[tiab] OR recurrent miscarriage* [tiab] OR recurrent early pregnancy loss*[tiab] OR "Abortion,habitual"[Mesh] OR abortion,habitual[tiab])
2. ("Endometritis"[Mesh] OR endometritis*[tiab] OR endomyometritis OR endometrial inflammation [tiab] OR "CD138 antigen"[Mesh] OR CD138 antigen [tiab] OR "Plasma cells"[Mesh] OR plasma cell* [tiab] OR plasmacyte* [tiab]) AND ("Infertility"[Mesh] OR infertility [tiab] OR sterility, reproductive[tiab] OR sterility[tiab] OR reproductive sterility[tiab] OR subfertility[tiab] OR sub-fertility[tiab] OR "Reproductive Techniques, Assisted"[Mesh] OR reproductive technique*, assisted[tiab] OR assisted reproductive technique*[tiab] OR technique*, assisted reproductive[tiab] OR assisted reproductive technic*[tiab] OR reproductive technic*, assisted[tiab] OR technic, assisted reproductive[tiab] OR reproductive technolog*, assisted[tiab] OR assisted reproductive technolog*[tiab] OR reproductive technolog*, assisted[tiab] OR technolog*, assisted reproductive[tiab] OR "Fertilization in Vitro"[Mesh] OR fertilization *in vitro* OR *in vitro* fertilization*[tiab] OR "Sperm Injection, Intracytoplasmic"[Mesh] OR sperm injection, intracytoplasmic[tiab] OR injection*, intracytoplasmic sperm [tiab] OR intracytoplasmic sperm injection*[tiab] OR ICSI [tiab] OR recurrent implantation failure[tiab] OR repeated implantation failure[tiab] OR implantation failure[tiab])

2.3.2 Scopus

1. ((TITLE-ABS-KEY ("recurrent miscarriage") OR TITLE-ABS-KEY (abortions) OR TITLE-ABS-KEY ("recurrent pregnancy loss"))) AND ((TITLE-ABS-KEY (endometritis) OR TITLE-ABS-KEY (endomyometritis) OR TITLE-ABS-KEY ("endometrial inflammation") OR TITLE-ABS-KEY ("cd138 antigen") OR TITLE-ABS-KEY ("plasma cells") OR TITLE-ABS-KEY ("chronic endometritis")))
2. ((TITLE-ABS-KEY (endometritis) OR TITLE-ABS-KEY (endomyometritis) OR TITLE-ABS-KEY ("endometrial inflammation") OR TITLE-ABS-KEY ("CD138 antigen") OR TITLE-ABS-KEY ("plasma cells") OR TITLE-ABS-KEY ("chronic endometritis")) AND ((TITLE-ABS-KEY (infertility) OR TITLE-ABS-KEY (sterility) OR TITLE-ABS-KEY ("reproductive sterility") OR TITLE-ABS-KEY ("sub-fertility") OR TITLE-ABS-KEY ("Reproductive Techniques Assisted") OR TITLE-ABS-KEY ("recurrent implantation failure") OR TITLE-ABS-KEY ("fertilization *in vitro*"))

2.3.3 Web of Science

1. ((((((TS=(endometritis)) OR TS=(endomyometritis)) OR TS=("endometrial inflammation")) OR TS=(CD138)) OR TS=("CD138 antigen")) OR TS=("chronic endometritis")) OR TS=("plasma cells") AND ((((TS=("recurrent pregnancy loss")) OR TS=("recurrent miscarriage")) OR TS=(abortions)) OR TS=("recurrent spontaneous abortion"))
2) ((((((TS=(endometritis)) OR TS=(endomyometritis)) OR TS=("endometrial inflammation")) OR TS=(CD138)) OR TS=("CD138 antigen")) OR TS=("chronic endometritis")) OR TS=("plasma cells") AND (((((((((TS=("recurrent implantation failure")) OR TS=("repeated implantation failure")) OR TS=(infertility)) OR TS=(sterility)) OR TS=("reproductive sterility")) OR TS=(sub-fertility)) OR TS=("Reproductive Techniques Assisted")) OR TS=("Fertilization in Vitro") OR TS=("reproductive failure")) OR TS= (RIF))

2.3.4 ScienceDirect

1) [("Chronic endometritis") OR ("CD138 antigen") OR (endometritis) OR ("plasma cells") OR ("endometrial inflammation")] AND [("recurrent pregnancy loss") OR ("recurrent miscarriage") OR ("recurrent spontaneous abortion") OR (abortions)]
2) [(Chronic endometritis)" OR ("CD138 antigen") OR ("plasma cells") OR (endometritis) OR ("endometrial inflammation")] AND [("recurrent implantation failure") OR ("fertilization *in vitro*")) OR (infertility) OR ("reproductive failure")]

2.3.5 Cochrane Library

1) ("Chronic endometritis" OR "CD138 antigen" OR "plasma cells" OR "endometritis" OR "endometrial inflammation") AND ("recurrent pregnancy loss" OR "recurrent miscarriage" OR "recurrent spontaneous abortion" OR "abortions")
2) ("Chronic endometritis" OR "CD138 antigen" OR "endometritis" OR "plasma cells" OR "endometrial inflammation") AND ("recurrent implantation failure" OR "fertilization *in vitro*" OR "infertility" OR "reproductive failure")

2.4 Inclusion criteria used for the selection of control patients

The general inclusion criteria used for the selection of control patients are summarized below:

1. Normal personal general and gynecologic history:
2. Regular menstrual cycles/Regular endocrine profile
3. Normal previous obstetric history

The detailed inclusion criteria for each selected study have been reported in the results section

2.5 Data selection and extraction process

Two authors (S.M. and C.T.) independently conducted electronic searches and analyzed bibliographic lists. Subsequently, they evaluated titles, abstracts, and full texts based on the pre-defined inclusion and exclusion criteria. Any discrepancies between the reviewers were resolved through mutual consensus or with the supervision of N.D.S.

The selected documents were then retrieved and thoroughly analyzed to extract the following information: first author's name, publication year, country of origin, study type, duration if specified, participant characteristics, chronic endometritis diagnosis method, chronic endometritis definition, and the primary findings of each study.

2.6 Study outcomes

The present meta-analysis was conducted to evaluate the association between chronic endometritis and three specific female reproductive diseases. In particular, the aspects investigated are:

- Association of chronic endometritis and infertility
- Association of chronic endometritis and recurrent implantation failure (RIF)
- Association of chronic endometritis and recurrent pregnancy losses (RPL)

2.7 Study bias risk assessment

Three authors (S.M., C.T. and J.A.) utilized the Newcastle-Ottawa Scale (NOS) to assess the quality of the included studies, specifically focusing on non-randomized trials (case-control and cohort) (14). The quality assessment covered three domains: study group selection, group comparability, and assessment of exposure or outcome of interest, for case-control or cohort studies, respectively. Any discrepancies between the reviewers were resolved through discussion with a third-party auditor (A.I.).

The overall score ranged from 0 to 9, with the study considered qualitatively adequate if the total score was greater than or equal to 5. Additionally, funnel plots were generated for each result to examine potential publication bias.

2.8 Statistical analysis

Quantitative analysis of the extracted data was performed using RevMan 5.4 software. Study outcomes were presented using odds ratios (OR) with 95% confidence intervals (95% CI). A p value <0.05 indicated a statistically significant difference in results.

Heterogeneity among studies was assessed using I^2 statistics. The degree of Heterogeneity was classified as low if I^2 was less than 30%, moderate if between 30% and 70%, and high if greater than 70%. These criteria were set according to Higgins et al. (14) and Ioannidis (15).

The subsequent step involved determining the appropriate statistical model for the meta-analysis. If the heterogeneity index (I^2) exceeded 70%, suggesting substantial variability among studies, the data were not combined for meta-analysis. If I^2 fell between 30% and 70%, indicating moderate heterogeneity, a random-effects model was chosen. Conversely, in cases where heterogeneity was less than 30%, a fixed-effects model was employed.

3 Results

3.1 Selection of studies

Following the research strategy, a total of 3,004 relevant publications were identified (*PubMed*: 522, *Scopus*: 1,447, *Web of Science*: 692, *ScienceDirect*: 230, *Cochrane Library*: 113). Upon removal of duplicates, the titles and abstracts of the remaining 1,511 documents were reviewed. No automated software was utilized for duplicate removal. Out of these 1,511 documents, 1,495 were excluded based on the imposed exclusion criteria, while 16 studies were initially selected for inclusion. Subsequently, after a thorough examination of the full texts, seven studies were excluded for various reasons: three lacked a control group consisting of healthy fertile women, in two, the presence or absence of chronic endometritis was a prerequisite for group selection, one did not report the prevalence of chronic endometritis in controls, and one did not aim to detect the prevalence of chronic endometritis.

Ultimately, nine studies were incorporated into the current systematic review and meta-analysis (5, 12, 16–23). Figure 1 displays the PRISMA 2020 flowchart for systematic reviews.

It is important to highlight that despite generating two search strings for each database, a singular overall flowchart was devised. This was essential to ensure the exclusion of all duplicate.

3.2 Features of included studies

Table 1 provides comprehensive details on the characteristics of all included studies. In terms of study design, the selection comprised four prospective cohort studies, one prospective and retrospective cohort study, two retrospective cohort studies, and two case-control studies.

3.2.1 Population

In total, the study encompassed a population of 1,038 women, consisting of 185 experiencing infertility, 63 facing repeated implantation failures, 489 diagnosed with recurrent miscarriages, and 486 healthy fertile women serving as controls.

While the definition of infertility was not explicitly outlined, all sources aligned with the interpretation of the inability to achieve pregnancy after twelve months of regular, unprotected sexual intercourse. Repeated implantation failure was characterized as the inability to achieve a clinical pregnancy after two failed embryo transfer cycles according to Takimoto et al. (20), while Liu Y et al. (18) considered three or more failed transfers. Regarding recurrent miscarriage, definitions varied among the selected studies. Four studies defined it as two or more miscarriages (19–21, 23), while four others considered three or more miscarriages (12, 17, 18, 22).

The results of the inclusion criteria used for the selection of control patients in each specific study are the following:

1. Normal personal general and gynecologic history:
   a. no known medical conditions (12)
   b. no history of gynecological conditions (uterine myoma, adenomyosis, endometriosis, malignancy, or surgery requiring intrauterine manipulation after the last delivery (20), polyps, pelvic inflammatory disease, or retained pregnancy tissue (21) or absence of abnormal ovarian and endometrial ultrasonographic features (22)
   c. no autoimmune disease (21)
2. Regular menstrual cycles/regular endocrine profile:
   regular menstrual cycles for ≥ 1 year (20) or regular endocrine profile/normal FSH serum levels on day 3 of the menstrual cycle (22)
3. Normal previous obstetric history:
   a. no previous history or treatment of infertility or RPL (5, 12, 20, 23)
   b. spontaneous pregnancy within the previous 3 years (5) or ≥ 2 pregnancies whose last child was conceived within the previous 1 year (12) or ≥ 2 pregnancies whose last delivery was ≥ 1 year before the study (17) or ≥1 live birth within the previous 2 years (18) or ≥1 normal delivery (20)
   c. no history of preeclampsia or intrauterine growth retardation (12)

In both studies by Chiokadze and McQueen, endometrial samples were collected in patients undergoing oocyte cryopreservation for elective fertility preservation or egg donation (21, 23).

Obstetric inclusion criteria for selection controls patients were assessed in every studies.

3.2.2 Diagnosis of CE

Out of the nine selected studies, four exclusively employ immunohistochemistry (IHC) for diagnosing chronic

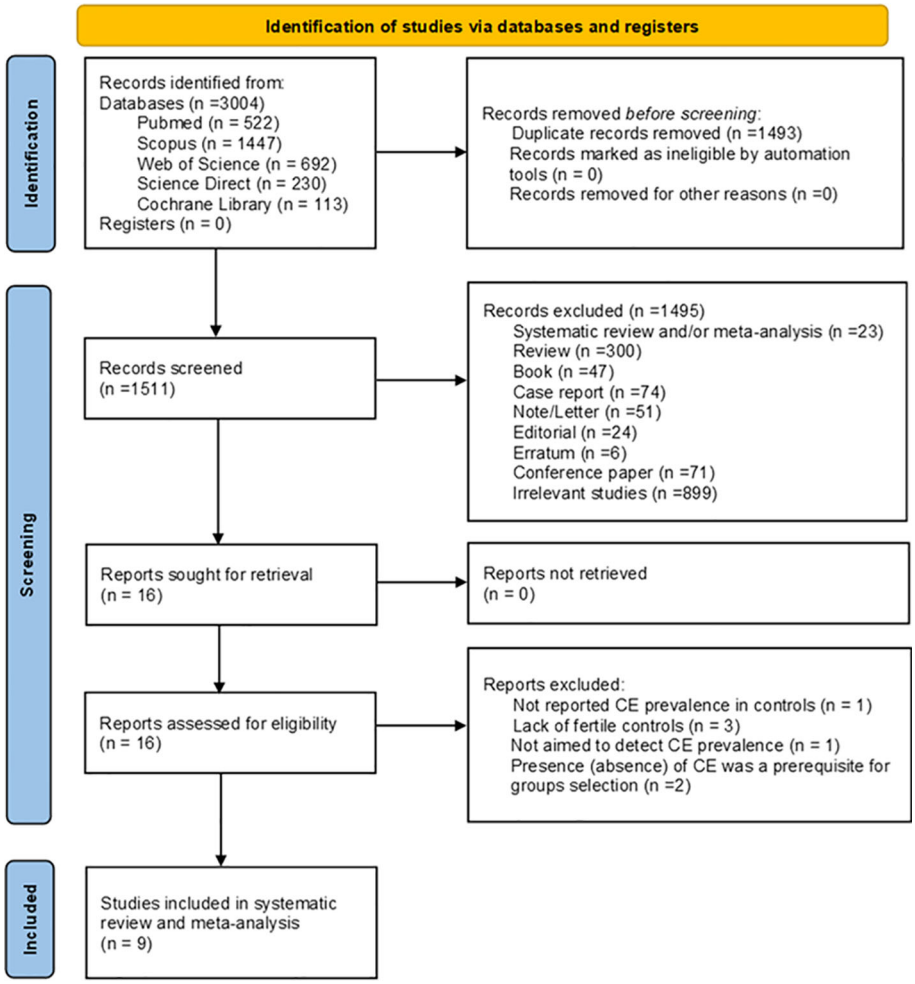


FIGURE 1
PRISMA 2020 Flowchart for bibliographic research.

endometritis, utilizing the CD138 marker (syndecan-1) (5, 19–21). Meanwhile, three studies solely rely on conventional hematoxylin and eosin (H&E) staining (12, 17, 22). Two studies employ both diagnostic methodologies (18, 23). Furthermore, criteria for plasma cell counts vary among the studies due to the lack of international consensus on this matter.

3.3 Risk of bias in studies

Among the cohort studies, five scored 7 and two scored 8. Both case-control studies scored 5.

NOS assessment scores are shown in Tables 2, 3.

3.4 Summary of results

Funnel plots were created for each outcome to assess potential publication bias. Inspection of the funnel charts visually indicated no asymmetry (Figures 2, 3).

3.4.1 Chronic endometritis and infertility

A quantitative analysis of chronic endometritis prevalence in women with infertility was conducted based on two studies (5, 18). The study encompassed a total of 185 infertile women, including 36 with chronic endometritis, and 180 controls, including 14 with chronic endometritis.

Combined results from the two studies (5, 18) revealed a positive association between infertility and chronic endometritis: a higher prevalence of chronic endometritis was observed in infertile women (36/185 [19.46%]) compared to controls (14/180 [7.7%]). Utilizing a fixed-effects model, the odds ratio (OR) was calculated to be 2.96, with a 95% confidence interval (CI) of 1.53-5.72 and a p-value of 0.001. Heterogeneity was minimal (I^2 0%) (Figure 4).

3.4.2 Chronic endometritis and recurrent implantation failure

A quantitative analysis of chronic endometritis prevalence in patients with RIF was conducted based on two studies (18, 20). The study involved a total of 63 women with RIF, including 4 with

TABLE 1 Main features of the included studies.

Study ID years and country	Study design and time of realization	Participant information		Diagnosis method of CE	CE definition	Main findings
		Group	Main characteristics			
Takimoto K et al. (20), Japan	Prospective cohort, from March 2021 to January 2023	Group A:24 RIF Group B: 27 RPL Group C: 29 fertile control women	RIF group: women with failure to achieve a clinical pregnancy after 2 or more transfer cycles, mean age 40 y/o, one had endometriosis, 2 PCOS, none had uterine myoma and received probiotics/prebiotics,4 received antibiotic treatment RPL group: women with 2 or more recurrent pregnancy losses, mean age 37 y/o, none had uterine myoma and received probiotics/prebiotics Fertile control group: women <44 y/o, regular menstrual cycles, without history of infertility, RPL, myoma, endometriosis, adenomyosis	IHC staining of the plasma cell marker CD138	Liu's method: Plasma cell count >5,15/10 mm² Mc Queen DB score: 0: none/ <1plasma cell/ HPF(x40) 1: 1-5/ HPF or cluster < 20cells 2:6-20/ HPF or cluster>20 cells 3: ≥ 20/HPF or sheets of cells	The frequency of CE (plasma cells > 5.15/10 mm2) was higher in women with RPL (29.6%) than in fertile controls (6.8%, $p < 0.05$) The plasma cell count/10 mm2 in women with RPL (median 1.53, range 0–252.6, $p < 0.01$) and women with RIF (median 0.6, range 0–6.98, $p < 0.05$) was higher than in fertile controls (median 0, range 0–29) The relative dominance rate of *Lactobacillus iners* (median 4.7% vs. median 0%) and the positive rates of *Ureaplasma* species (36.3% vs. 8.6%) were higher in women with CE than in women without CE
Goto T et al. (19), Japan	Retrospective cohort, from January 2011 to March 2019	Group A: 49 RPL Group B: 17 controls	RPL group: women with 2 or more unexplained pregnancy losses, divided in euploid (n=22, mean age 31,40 y/o) and aneuploid miscarriage (n=27, mean age 33,70 y/o) Fertile control group: women with mean age 33,3 y/o, women who underwent an artificial abortion in the first trimester of pregnancy	IHC staining of the plasma cell marker CD138	Grade 1: 1 plasma cell/ HPF Grade 2: ≥ 2 plasma cells/HPF	The prevalence of Grade 1 was 18.2% (4/22) in patients with euploid miscarriage, 37.0% (10/27) in patients with aneuploid miscarriage and 23.5% (4/17) in control women. The prevalence of Grade 2 was 45.5% (10/22) in patients with euploid miscarriage, 55.6% (15/27) in patients with aneuploid miscarriage and 23.5% (4/17) in control women. There was a significant difference in the prevalence of CD ($p = 0.015$). The LBR of patients with CD was similar to that of patients without CD
McQueen DB et al. (23), USA	Prospective and retrospective cohort, from February 2016 to February 2020	Group A: 50 RPL Group B: 26 controls	RPL group: women with 2 or more pregnancy losses, mean age 35,2 y/o, TSH<4mU/L, negative antiphospholipid antibodies, normal uterine anatomy Fertile control group: women with mean age 33,2 y/o, no history of RPL, infertility, uterine fibroids, polyps, PID or retained pregnancy tissue	H&E staining and IHC staining of the plasma cell marker CD138	H&E/CD138: ≥ 1, ≥2 and ≥5 plasma cells/ 10HPF with and without endometrial stromal changes	The results confirm increased sensitivity of CD138 staining compared to H&E. With CD138 staining, the prevalence of CE was 31% in controls and 56% in women with RPL No control had both plasma cells and endometrial stromal changes in biopsy samples compared to women with RPL (0% vs 30%)
Chiokadze M et al. (21), Germany, Georgia	Case-control, no data reported	Group A: 61 RPL Group B: 10 controls	RPL group: women with 2 or more consecutive pregnancy losses, mean age 33,5 y/o Control fertile group: women with mean age 27 y/ o, no miscarriage, least one live birth, no autoimmune disease, antibiotic therapy, hormonal treatment or vaccination for at least 3 months	IHC staining of the plasma cell marker CD138	<3 CD138+ plasma cells/10 mm² Plasma cell count>5,15/10 mm² (Liu's method)	The prevalence of CE (CD138+ <3 plasma cells/ 10mm²) was 20 % in controls and 22 % in women with RPL. With Liu's method the potential influence of this condition was excluded The mean number of CD16+ cells was significantly increased in the endometrium of uRPL patients compared to controls ($p < 0.001$). No differences were observed in the mean values of CD45 ($p = 0.06$), CD56 ($p = 0.99$), and CD57 ($p = 0.14$). By additional analysis of these markers showed their different distributions in uRPL patients ($p < 0.001$ for CD45, CD56, and CD16; $p = 0.003$ for CD57) compared to controls

(Continued)

TABLE 1 Continued

Study ID years and country	Study design and time of realization	Participant information		Diagnosis method of CE	CE definition	Main findings
		Group	Main characteristics			
Volodarsky-Perel A et al. (2019), Canada (5)	Retrospective cohort, from 2015 to 2018	Group A: 137 infertile women Group B: 140 controls	Patients undergoing hysteroscopic polipectomy Infertile group: women with mean age 38 y/o, fallopian tubes patency, regular ovulatory cycles and normal partner's sperm parameters Control fertile group: women with mean age 40 y/o no history of infertility, no hormone treatment in the previous 3 months before hysteroscopy, no spontaneous pregnancy in the previous 3 years before the procedure	IHC staining of the plasma cell marker CD138	Plasma cells >1/10HPF	The prevalence of CE in the group of infertile women was significantly higher than that in the control group (22.6% vs. 8.6%; p = 001) Women with primary infertility and those with secondary infertility showed no difference in CE prevalence Pregnancy outcome in infertile women with treated CE was like those who were infertile and without CE
Barath SH et al. (2019), (17)	Case-control, no data reported	Group A: 40 RPL Group B: 60 controls	Patients undergoing hysteroscopic RPL group: women with 3 or more unexplained pregnancy losses, mean age 28,8 y/o Control fertile group: women with mean age 32,8 y/o, vaginal bleeding or other causes except RPL and infertility, at least two non-aborted pregnancies whose last delivery was at least one year before study	H&E staining	Localized or diffuse endometrial bleeding symptoms and, in some cases, polyps<1mm in the endometrium	Endometritis rate was 8% in all patients. Patients with RPL had increased incidence of CE both hysteroscopically (30% vs. 6.7%; p<0.005) and pathologically (27.5% vs. 6.7%; p < 0.005)
Liu Y et al. (18), China	Prospective cohort, from December 2014 to June 2017	Group A: 39 RIF Group B: 93 RPL Group C: 48 infertility Group D: 40 controls	Mean age of all women 34,4 y/o RIF group: women with failure to achieve a clinical pregnancy after 3 or more transfer cycles RPL group: women with 3 or more consecutive unexplained pregnancy losses Infertile group: women undergoing endometrial scratch in a natural cycle preceding frozen-thawed embryo transfer Fertile control group: women who at least one live birth	H&E staining and IHC staining of plasma cell marker CD138	Methods: 1) CD138+ cell count/10HPF 2) CD138+/whole section 3) CD138+/unit area (cell density) Definition CE: presence CD138+ plasma cell count or density >95th percentile	The use of CD138 staining was higher sensitive than H&E staining. The plasma cell count per unit area showed the least observer variability among the three methods and the prevalence of CE in women with RM, RIF, and Infertility were 10.8%, 7.7%, and 10.4%, respectively, in the controls was 5%
D'Ippolito S et al. (22), Italy	Prospective cohort, no data reported	Group A:27 RPL Group B:10 controls	Inclusion criteria for both groups: age ≤39 years, healthy, regular ovulatory cycles, normal endocrine profile, absence of abnormal ultrasonographic	H&E staining	Endometrial inflammatory component (weak or absent)	The expression of NALP-3 inflammasome and ASC protein is greater in the endometrium of women with RPL compared to controls, as is the activation of caspase-1 and levels of IL-1b and IL-18 A significant statistical difference in the

(Continued)

TABLE 1 Continued

Study ID years and country	Study design and time of realization	Participant information		Diagnosis method of CE	CE definition	Main findings
		Group	Main characteristics			
			features, no use of any contraceptive drugs or intrauterine device (IUD) in the past 6 months, no vaginal infections. RPL group: women with 3 or more consecutive unexplained pregnancy losses, mean age 36,3 y/o			inflammatory component of the endometrium was highlighted between the two groups (P<0.0001)
Zolghadri J et al. (12), Iran	Prospective cohort, from January 2006 to December 2008	Group A:142 RPL Group B: 154 controls	Patients undergoing hysteroscopic RPL group: women with mean age 30,1 y/o, divided in primary (≥ 3 loss pregnancies before 20 week of gestation) and secondary unexplained RPL (≥3 with at least one pregnancy beyond 20 week of gestation) Control fertile group: mean age 30,8 y/o, with two or more pregnancies with no history of pregnancy loss	H&E staining	Presence of plasma cells in endometrial stroma >1/HPF	RPL group had higher incidence of CE compared to controls both hysteroscopically (67.6% vs. 27.3%; $p < 0.0001$) and pathologically (42.9% vs. 18.2%; $p < 0.0001$) Patients with secondary RPL demonstrated a higher prevalence of CE both pathologically (83.9% vs. 45.9%; $p < 0.0001$) and hysteroscopically (58.1% vs. 24.6%; $p < 0.0001$)

chronic endometritis, and 69 controls, including 4 with chronic endometritis.

Combined results from the two studies (18, 20) revealed no significant association between recurrent implantation failure and chronic endometritis. Utilizing a fixed-effects model, the odds ratio (OR) was calculated to be 1.10, with a 95% confidence interval (CI) of 0.26-4.61 and a p-value of 0.90. Heterogeneity was negligible (I^2 = 0%) (Figure 5).

The prevalence of chronic endometritis in both groups was quite similar, with 6.35% in women with RIF and 5.8% in controls.

3.4.3 Chronic endometritis and recurrent pregnancy loss

A quantitative analysis of chronic endometritis prevalence in women with recurrent pregnancy loss was conducted based on eight studies (12, 17–23). The study encompassed a total of 489 women with RPL, including 184 with chronic endometritis, and 346 controls, including 57 with chronic endometritis.

Combined results from the eight studies revealed an association between recurrent miscarriage and chronic endometritis: a higher proportion of chronic endometritis was observed among women with RPL (184 out of 489 [37.6%]) compared to controls (57 out of 346 [16.4%]). Utilizing a fixed-effects model, the odds ratio (OR) was calculated to be 3.59, with a 95% confidence interval of 2.46-5.24 and a p-value of less than 0.00001. Heterogeneity was minimal (I^2= 0%), as depicted in Figure 6.

3.5 Sensitivity analysis

Three sensitivity analyses were conducted, all pertaining to RPL. In five out of the nine included studies (Table 1), immunohistochemistry (IHC) employing CD138 was utilized for chronic endometritis diagnosis. Consequently, we performed a sensitivity analysis incorporating only these five studies. As depicted in the forest plot in Figure 7, the findings closely resembled those of the previous analysis: a higher prevalence of chronic endometritis was observed among women with recurrent miscarriage (98/280 [35%]) compared to controls (22/122 [18%]). A fixed-effects model was consistently applied, given the absence of heterogeneity (I^2=0%). The odds ratio (OR) was calculated to be 2.96, with a 95% confidence interval (CI) of 1.63-5.38 and a p-value of 0.0004.

The remaining two sensitivity analyses were performed based on the definition of recurrent miscarriage provided in the various included studies (Table 1).

Firstly, considering only those studies (17–19, 21) in which recurrent miscarriage was defined as two or more spontaneous pregnancy losses, a higher prevalence of chronic endometritis was observed among women with RPL (100/187 [53.4%]) compared to controls (23/82 [28%]). Utilizing a fixed-effects model, the odds ratio (OR) was calculated to be 3.95, with a 95% confidence interval (CI) of 2.04-7.64 and a p-value of less than 0.00001. Heterogeneity was absent (I^2 = 0%), as illustrated in Figure 8.

TABLE 2 NOS scores in cohort studies.

Study	Selection				Comparability	Outcome			Total quality score
	Representativeness of the exposed cohort	Selection of the non exposed cohort	Ascertaiment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Follow-up long enough for outcomes to occur	Adequacy of follow up of cohorts	
Takimoto K et al. (20)	★	★	★	★	★★	–	★	★	8
Goto et al. (19)	–	★	★	★	★	★	★	★	7
McQueen DB et al. (23)	★	★		★	★	★	–	★	7
Volodarsky-Perel A et al. (2019)	★	★	★	★	★★	★	–	–	7
Liu Y et al. (18)	★	★	★	★	★	★	★	★	8
D'Ippolito S et al. (22)	★	★	★	★	★	★	–	★	7
Zolghadri J et al. (12)	★	★	★	★	★	★	–	★	7

The stars are the symbols commonly used in the evaluation of NOS scale.

10.3389/fimmu.2024.1427454

10.3389/fimmu.2024.1427454

TABLE 3 NOS score in a case-control study.

Study	Selection				Comparability	Exposure			Total quality score
	Adequacy of case definition	Representativeness of the cases	Selection of Controls	Definition of Controls	Comparability of cases and controls on the basis of the design or analysis	Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-Response Rate	
Chiokadze M et al. (21)	★	★	-	★	★	★	-	-	5
Barath SH et al. (2019) (17)	-	-	★	★	★	★	★	-	5

The stars are the symbols commonly used in the evaluation of NOS scale.

Similarly, restricting the sensitivity analysis to only those studies (12, 17, 18, 22) that defined RPL as three or more losses, a higher prevalence of chronic endometritis was observed among women with RPL (84/302 [27.8%]) compared to controls (34/264 [12.1%]). Utilizing a fixed-effects model, the odds ratio (OR) was calculated to be 3.43, with a 95% confidence interval (CI) of 2.16-5.43 and a p-value of less than 0.00001. Heterogeneity was also absent ($I^2 = 0\%$), as depicted in Figure 9.

4 Discussion

Chronic endometritis, characterized by persistent inflammation of the endometrium (24), commonly arises from intrauterine infections attributed to bacteria such as *Escherichia coli, Enterococcus faecalis, Streptococcus, Staphylococcus*, as well as *Mycoplasma* and *Ureaplasma* species (3). Over the past two decades, there has been a burgeoning interest in researching endometrial diseases and their impact on reproductive health. However, determining the prevalence of CE among women of reproductive age remains challenging, primarily due to the difficulties associated with obtaining endometrial tissue samples from healthy women for biopsy. Moreover, chronic endometritis is asymptomatic in approximately 25% of cases, or it may present with nonspecific symptoms that may go unnoticed for extended periods (1).

Further complicating research in this area is the absence of a universally agreed-upon definition and standardized diagnostic criteria of CE. Nevertheless, experts concur that the presence of endometrial plasma stromal cells endometrial stromal plasma cells (ESPCs) represents the most specific and sensitive indicator of this disorder (25). Presently, the gold standard for diagnosis involves identifying plasma cells through immunohistochemical (IHC) staining targeting the CD138 marker (syndecan-1) (26), a method demonstrated to be more sensitive and precise than conventional hematoxylin-eosin (H&E) staining. However, there remains no consensus regarding the specific threshold of plasma cells necessary for definitively diagnosing chronic endometritis (27), which may impede the interpretation and comparability of findings across studies.

An alternative diagnostic approach involves hysteroscopic examination to identify endometrial characteristics indicative of chronic endometritis, including a strawberry appearance, focal hyperemia, micropolyps, stromal edema, and hemorrhagic spots (28). *McQueen et al.* suggested defining CE as the detection of one or more plasma cells per ten high-magnification fields (HPF), particularly in the presence of endometrial stromal alterations (29).

Recent research has highlighted the emergence of additional immunohistochemical markers like MUM-1 and advanced molecular biology techniques for identifying microbial species undetectable through classical microbiological culture (30, 31).

The aforementioned challenges, coupled with the lack of comprehensive clinical evidence in this area, render the assessment of chronic endometritis (CE) uncertain regarding its role in various critical reproductive pathologies, such as infertility, RIF and RPL. Consequently, given these uncertainties, current

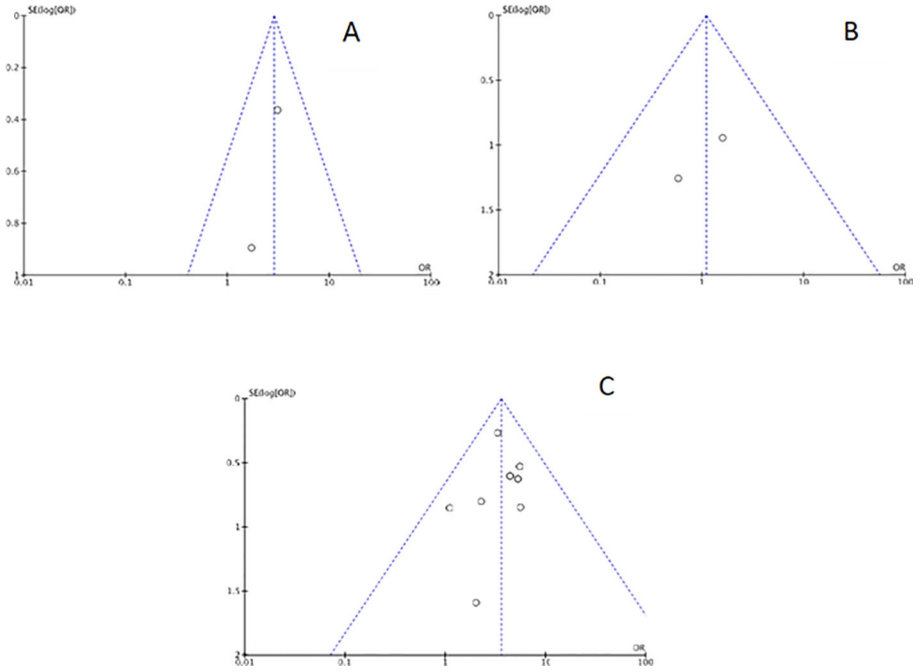


FIGURE 2
Funnel plot for each main outcome: **(A)** Chronic endometritis and infertility; **(B)** Chronic endometritis and Recurrent Implantation Failure; **(C)** Chronic endometritis and Recurrent Pregnancy Loss.

guidelines do not recommend routine endometrial biopsy for CE investigation (31, 32).

The current epidemiological data exhibit significant heterogeneity: the prevalence of chronic endometritis (CE) among women with infertility varies widely from 2.8% to 56.8%, while among those with repeated implantation failures (RIF) it ranges from 14% to 67.5%, and in cases of recurrent miscarriage, it spans from 9.3% to 67.6% (4).

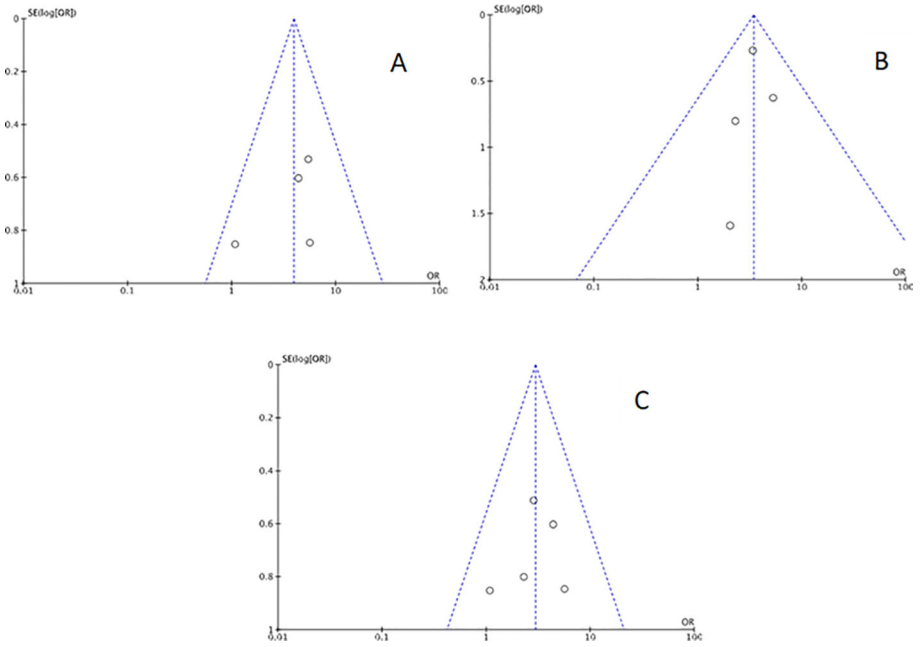


FIGURE 3
Funnel plot for sensitivity analysis: **(A)** studies with Chronic endometritis and Recurrent Pregnancy Loss defined as two or more abortions. **(B)** studies with Chronic endometritis and Recurrent Pregnancy Loss defined as three or more abortions. **(C)** studies with Chronic endometritis diagnosed with IHC for CD138 and RPL.

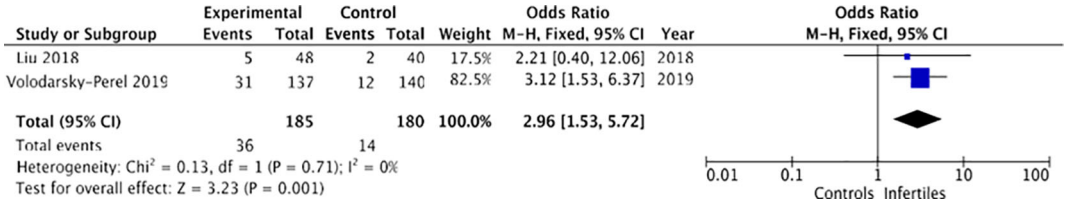


FIGURE 4
Forest plot for chronic endometritis and infertility.

Furthermore, recent systematic reviews and/or meta-analyses have primarily focused on assessing the impact of antibiotic therapy for CE on reproductive outcomes. Hence, this systematic review and meta-analysis were undertaken to address these gaps.

The findings from this study reveal that chronic endometritis (CE) is more prevalent in women experiencing infertility and recurrent miscarriages (RPL) compared to the control group, with rates of 19.46% versus 7.7% for infertility and 37.6% versus 16.4% for RPL. Conversely, no significant disparities in CE prevalence were observed between women with RIF and the control group. However, since the overall number of women included in the analysis of the effect of CE on RIF was limited to 132 subjects (63 women with RIF and 69 control women), further studies are needed before a definitive conclusion regarding the lack of association of CE with RIF can be drawn. Moreover, CE patients with RIF usually are given antibiotic treatment before embryo transfer. The therapy could affect the final outcome and the results.

One of the limitations of this study pertains to the small sample size of subjects available for comparison across each category of reproductive pathologies investigated, despite the extensive duration of the research (1990–2024) and the utilization of five electronic databases.

This underscores the aforementioned challenge of obtaining healthy endometrial tissue samples.

Moreover, the presence of divergent definitions of RPL, coupled with the absence of a universally accepted criterion for plasma cell counts and the utilization of varied diagnostic methods for chronic endometritis (CE), further complicates the harmonization of the overall data. In an effort to mitigate these challenges, sensitivity analyses were conducted, yielding results akin to those obtained through the primary analysis.

Specifically, in the sensitivity analysis incorporating only studies employing immunohistochemical (IHC) staining for CD138 in diagnosing chronic endometritis (CE), a higher proportion of CE was observed among women with recurrent pregnancy loss (RPL) compared to controls (35% vs. 18%). In the other two sensitivity analyses, studies were categorized based on the definition of recurrent miscarriage: either as two or more miscarriages or as

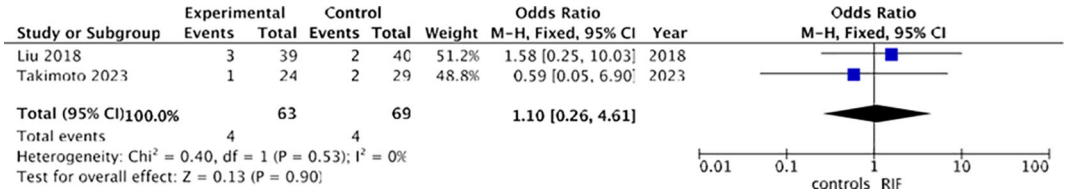


FIGURE 5
Forest plot for chronic endometritis and repeated implantation failure (RIF).

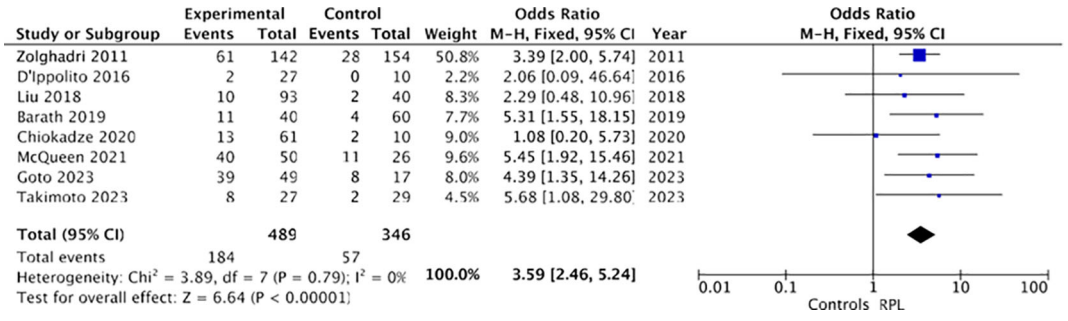


FIGURE 6
Forest plot for chronic endometritis and recurrent pregnancy loss (RPL).

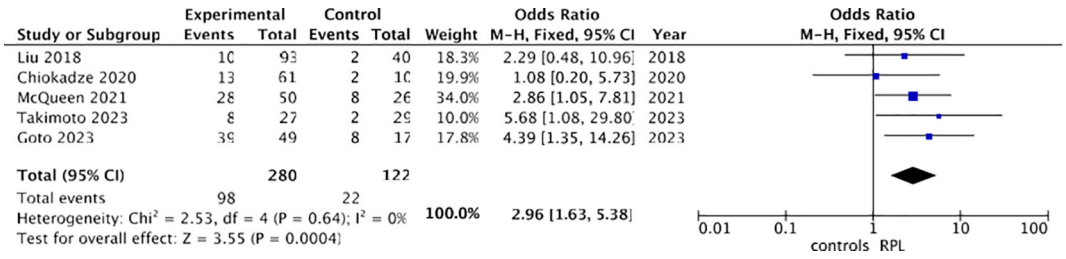


FIGURE 7
Forest plot for sensitivity analysis: CE diagnosed with IHC CD138 and RPL.

three or more miscarriages. In both scenarios, a greater prevalence of CE was noted in women with RPL compared to controls (53.4% vs. 28% for RPL defined as two or more miscarriages, 27.8% vs. 12.1% for RPL with three or more pregnancy losses). The hypothesis that the presence of chronic endometritis (CE) may correlate with infertility and RPL holds biological plausibility. Chronic inflammation can disrupt the delicate immunological equilibrium within the endometrium during implantation and early pregnancy stages. Since it is reported that the expression of proinflammatory cytokines in the endometrium of women with history of RPL is upregulated compared with controls, D'Ippolito et al. (22) hypothesized a role for an abnormal aspecific activation of the proteic system infammosome. In detail, they showed that NALP-3/ASC inflammosome is expressed in human endometrium and, furthermore, it is increased in the endometrium obtained from women with history of RPL. Due to the lack of specificity of the innate immune system, several stimuli might be responsible of the inflammosome activation.

While previous studies have explored the pathophysiological mechanisms underlying this interference (9, 10), ongoing advancements in knowledge may unveil novel pathways. This area of research remains dynamic and continuously evolving. Moreover, this association finds support in studies investigating the positive effects of treatment rather than directly assessing the prevalence of chronic endometritis.

Due to the limited sample size, we are unable to provide precise percentages for both healthy individuals and those affected by these diseases. Further studies may yield significant variations in results.

Concerning RIF, insufficient available data hinder us from drawing definitive conclusions regarding the potential impact of chronic endometritis on this condition.

In summary, chronic endometritis may play a role in the development of certain significant reproductive failures, particularly infertility and RPL.

It is worth noting that, to the best of our knowledge, this study is the only meta-analysis addressing this issue by employing healthy

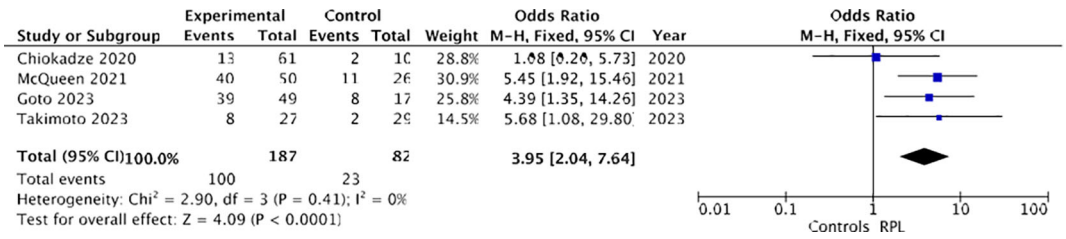


FIGURE 8
Forest plot for sensitivity analysis: CE and recurrent pregnancy loss defined as two or more spontaneous pregnancy losses.

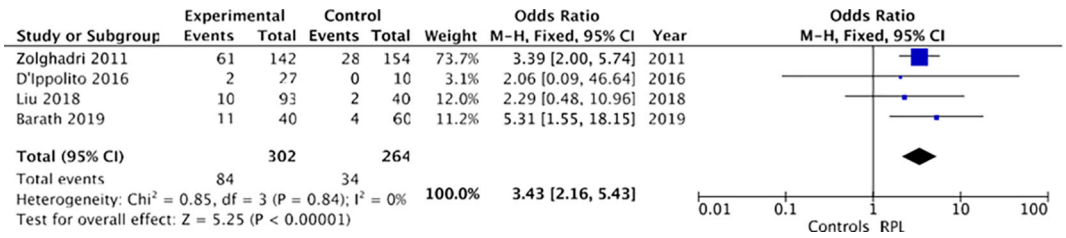


FIGURE 9
Forest plot for sensitivity analysis: CE and Recurrent pregnancy loss defined as three or more spontaneous pregnancy losses.

and fertile women as a control group. In contrast, other meta-analyses in the literature have predominantly, if not exclusively, focused on comparing cases of RPL with cases of RIF.

5 Conclusions

The gathered data reaffirmed the existing scientific literature. It was observed that women experiencing infertility exhibited a notably higher prevalence of chronic endometritis compared to controls, with rates of 19.46% versus 7.7%, respectively (OR 2.96, p 0.001, I^2=0%). Similarly, among women with RPL, a comparable pattern emerged, with a prevalence of CE at 37.6% versus 16.4% in controls (OR 3.59, p<0.00001, I^2= 0%). Conversely, no significant association was found between CE and RIF, potentially due to the limited sample size analyzed. These findings contribute further evidence to support a potential correlation between CE and adverse reproductive outcomes. Nonetheless, definitive conclusions regarding the role of CE in women with reproductive disorders necessitate additional clinical investigations to elucidate its true impact on female reproductive health. Establishing an international consensus on diagnostic criteria is imperative to establish clear clinical guidelines for the diagnosis and management of CE in patients with reproductive disorders.

Ultimately, this review underscores the significance of CE within the spectrum of female reproductive pathologies and emphasizes the importance of its thorough assessment and management in clinical settings.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CT: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing, Project administration. AI: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing, Conceptualization, Project administration, Supervision. SM: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft. LC: Data curation, Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. JA: Software, Validation, Visualization, Writing – review & editing. EZ: Data curation, Methodology, Visualization, Writing – review & editing. NS: Conceptualization, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work has been supported by Progetto Bio-MARI (PorFesr LazioInnova 2020; Codice progetto A0375-2020-36775) (Recipient: LC).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Espinós JJ, Fabregues F, Fontes J, García-Velasco JA, Llácer J, Requena A, et al. Impact of chronic endometritis in infertility: a SWOT analysis. *Reprod BioMedicine Online*. (2021) 42:939–51. doi: 10.1016/j.rbmo.2021.02.003

2. Singh N, Sethi A. Endometritis - Diagnosis,Treatment and its impact on fertility - A Scoping Review. *JBRA Assist Reprod*. (2022) 26:538–46. doi: 10.5935/1518-0557.20220015

3. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. *Reprod Sci*. (2014) 21:640–7. doi: 10.1177/1933719113508817

4. Kimura F, Takebayashi A, Ishida M, Nakamura A, Kitazawa J, Morimune A, et al. Review: Chronic endometritis and its effect on reproduction. *J Obstetrics Gynaecology Res*. (2019) 45:951–60. doi: 10.1111/jog.13937

5. Volodarsky-Perel A, Badeghiesh A, Shrem G, Steiner N, Tulandi T. Chronic endometritis in fertile and infertile women who underwent hysteroscopic polypectomy. *J Minim Invasive Gynecol*. (2020) 27:1112–8. doi: 10.1016/j.jmig.2019.08.017

6. Franasiak JM, Alecsandru D, Forman EJ, Gemmell LC, Goldberg JM, Llarena N, et al. A review of the pathophysiology of recurrent implantation failure. *Fertility Sterility*. (2021) 116:1436–48. doi: 10.1016/j.fertnstert.2021.09.014

7. Koot YEM, Hviid Saxtorph M, Goddijn M, de Bever S, Eijkemans MJC, Wely MV, et al. What is the prognosis for a live birth after unexplained recurrent implantation failure following IVF/ICSI? *Hum Reprod*. (2019) 34:2044–52. doi: 10.1093/humrep/dez120

8. McQueen DB, Bernardi LA, Stephenson MD. Chronic endometritis in women with recurrent early pregnancy loss and/or fetal demise. *Fertil Steril*. (2014) 101:1026–30. doi: 10.1016/j.fertnstert.2013.12.031

9. Tersigni C, D'Ippolito S, Di Nicuolo F, Marana R, Valenza V, Masciullo V, et al. Recurrent pregnancy loss is associated to leaky gut: a novel pathogenic model of endometrium inflammation? *J Transl Med*. (2018) 16:102. doi: 10.1186/s12967-018-1482-y

10. Di Simone N, Caliandro D, Castellani R, Ferrazzani S, Caruso A. Interleukin-3 and human trophoblast: *in vitro* explanations for the effect of interleukin in patients with antiphospholipid antibody syndrome. *Fertil Steril*. (2000) 73:1194–200. doi: 10.1016/s0015-0282(00)00533-1

11. Ticconi C, Di Simone N, Campagnolo L, Fazleabas A. Clinical consequences of defective decidualization. *Tissue Cell*. (2021) 72:101586. doi: 10.1016/j.tice.2021.101586

12. Zolghadri J, Momtahan M, Aminian K, Ghaffarpasand F, Tavana Z. The value of hysteroscopy in diagnosis of chronic endometritis in patients with unexplained recurrent spontaneous abortion. *Eur J Obstetrics Gynecology Reprod Biol*. (2011) 155:217–20. doi: 10.1016/j.ejogrb.2010.12.010

13. Moher D, Liberati A, Tetzlaff J, Altman DGPRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PloS Med*. (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097

14. Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analysis. *BMJ*. (2003) 327:557–60. doi: 10.1136/bmj.327.7414.557

15. Ioannidis JPA. Interpretation of tests of heterogeneity and bias in meta-analysis. *J Eval Clin Pract*. (2008) 14:951–7. doi: 10.1111/j.1365-2753.2008.00986.x

16. Wells GA, Shea B, O'Connell D, Peterson J, Welch V, Losos M, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. (2012). doi: 10.2307/632432

17. Barat S, Adnani M, Basirat Z, Ranaei M. Hysteroscopic and Pathologic evaluation of Chronic Endometritis in patients with Unexplained Recurrent Spontaneous Abortion. *J Babol Univ Med Sci*. (2019) 21:99–104. doi: 10.22088/jbums.21.1.99

18. Liu Y, Chen X, Huang J, Wang CC, Yu MY, Laird S, et al. Comparison of the prevalence of chronic endometritis as determined by means of different diagnostic methods in women with and without reproductive failure. *Fertil Steril*. (2018) 109:832–9. doi: 10.1016/j.fertnstert.2018.01.022

19. Goto T, Goto S, Ozawa F, Yoshihara H, Kitaori T, Komura M, et al. The association between chronic deciduitis and recurrent pregnancy loss. *J Reprod Immunol*. (2023) 156:103824. doi: 10.1016/j.jri.2023.103824

20. Takimoto K, Yamada H, Shimada S, Fukushi Y, Wada S, Takimoto K, et al. Chronic endometritis and uterine endometrium microbiota in recurrent implantation failure and recurrent pregnancy loss. *Biomedicines*. (2023) 11:2391. doi: 10.3390/biomedicines11092391

21. Chiokadze M, Bär C, Pastuschek J, Dons'koi BV, Khazhylenko KG, Schleußner E. Beyond uterine natural killer cell numbers in unexplained recurrent pregnancy loss: Combined analysis of CD45, CD56, CD16, CD57, and CD138. *Diagnostics*. (2020) 10:650. doi: 10.3390/diagnostics10090650

22. D'Ippolito S, Tersigni C, Marana R, Di Nicuolo F, Gaglione R, Rossi ED, et al. Inflammosome in the human endometrium: Further step in the evaluation of the 'maternal side'. *Fertil Steril*. (2016) 105:111–118.e4. doi: 10.1016/j.fertnstert.2015.09.027

23. McQueen DB, Maniar KP, Hutchinson A, Confino R, Bernardi L, Pavone ME. Redefining chronic endometritis: the importance of endometrial stromal changes. *Fertil Steril*. (2021) 116:855–61. doi: 10.1016/j.fertnstert.2021.04.036

24. Song D, Feng X, Zhang Q, Xia E, Xiao Y, Xie W, et al. Prevalence and confounders of chronic endometritis in premenopausal women with abnormal bleeding or reproductive failure. *Reprod BioMed Online*. (2018) 36:78–83. doi: 10.1016/j.rbmo.2017.09.008

25. Kitaya K, Takeuchi T, Mizuta S, Matsubayashi H, Ishikawa T. Endometritis: new time, new concepts. *Fertil Steril*. (2018) 110:344–50. doi: 10.1016/j.fertnstert.2018.04.012

26. Klimaszyk K, Svarre Nielsen H, Wender-Ozegowska E, Kedzia M. Chronic endometritis – is it time to clarify diagnostic criteria? *Ginekol Pol*. (2023) 94:152–7. doi: 10.5603/GP.a2022.0147

27. Li Y, Xu S, Yu S, Huang C, Lin S, Chen W, et al. Diagnosis of chronic endometritis: How many CD138+ cells/HPF in endometrial stroma affect pregnancy outcome of infertile women? *Am J Reprod Immunol*. (2021) 85:e13369. doi: 10.1111/aji.13369

28. Cicinelli E, Vitagliano A, Kumar A, Lasmar RB, Bettocchi S, Haimovich S, et al. Unified diagnostic criteria for chronic endometritis at fluid hysteroscopy: proposal and reliability evaluation through an international randomized-controlled observer study. *Fertil Steril*. (2019) 112:162–173.e2. doi: 10.1016/j.fertnstert.2019.03.004

29. McQueen DB, Perfetto CO, Hazard FK, Lathi RB. Pregnancy outcomes in women with chronic endometritis and recurrent pregnancy loss. *Fertil Steril*. (2015) 104:927–31. doi: 10.1016/j.fertnstert.2015.06.044

30. Moreno I, Cicinelli E, Garcia-Grau I, Gonzalez-Monfort M, Bau D, Vilella F, et al. The diagnosis of chronic endometritis in infertile asymptomatic women: a comparative study of histology, microbial cultures, hysteroscopy, and molecular microbiology. *Am J Obstet Gynecol*. (2018) 218:602.e1–602.e16. doi: 10.1016/j.ajog.2018.02.012

31. Klimaszyk K, Bednarek-Rajewska K, Svarrre Nielsen H, Wender Ozegowska E, Kedzia M. Significance of multiple myeloma oncogene immunohistochemistry in chronic endometritis detection in patients with recurrent pregnancy losses: an observational study. *J Physiol Pharmacol*. (2023) 74:647–53. doi: 10.26402/jpp.2023.6.05

32. ESHRE Guideline Group on RPL, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, et al. ESHRE guidelines: recurrent pregnancy loss. An update in 2022. *Hum Reprod Open*. (2023) 1):hoad002. doi: 10.1093/hropen/hoad002

10.3389/fimmu.2024.1427454

Glossary

Medically Assisted Reproduction (MAR)	Reproduction brought about through various interventions, procedures, surgeries, and technologies to treat different forms of fertility impairment and infertility. These include ovulation induction, ovarian stimulation, all ART procedures, uterine transplantation, and intra-uterine, intracervical, and intravaginal insemination with semen of husband/partner or donor.
Assisted Reproductive Technologies (ART)	All interventions that include the *in vitro* handling of both human oocytes and sperm or of embryos for the purpose of reproduction, such as *In Vitro* Fertilization (IVF). Fertility care Interventions that include support and fertility management with an intention to assist patients to realize their parenthood goals.
In Vitro Fertilization (IVF)	A sequence of procedures that involves extracorporeal fertilization of gametes. It includes conventional IVF and intracytoplasmic sperm injection (ICSI), a procedure in which a single spermatozoon is injected into the oocyte cytoplasm.

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 16 September 2024
DOI 10.3389/fimmu.2024.1400977

Check for updates

OPEN ACCESS

EDITED BY
Bhesh Raj Sharma,
St. Jude Children's Research Hospital,
United States

REVIEWED BY
Balamurugan Sundaram,
St. Jude Children's Research Hospital,
United States
Rima Irwinda,
University of Indonesia, Indonesia
Ellen Menkhorst,
The University of Melbourne, Australia

*CORRESPONDENCE
Shuli Yang
yangsl@jlu.edu.cn

RECEIVED 14 March 2024
ACCEPTED 28 August 2024
PUBLISHED 16 September 2024

CITATION
Li J, Wang M, Zhou H, Jin Z, Yin H and Yang S (2024) The role of pyroptosis in the occurrence and development of pregnancy-related diseases.
Front. Immunol. 15:1400977.
doi: 10.3389/fimmu.2024.1400977

COPYRIGHT
© 2024 Li, Wang, Zhou, Jin, Yin and Yang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The role of pyroptosis in the occurrence and development of pregnancy-related diseases

Jiahui Li, Min Wang, Haiyan Zhou, Zhong Jin, Haonan Yin and Shuli Yang*

Department of Gynecology and Obstetrics, The Second Hospital of Jilin University, Changchun, Jilin, China

Pyroptosis is a form of programmed cell death that is crucial in the development of various diseases, including autoimmune diseases, atherosclerotic diseases, cancer, and pregnancy complications. In recent years, it has gained significant attention in national and international research due to its association with inflammatory immune overactivation and its involvement in pregnancy complications such as miscarriage and preeclampsia (PE). The mechanisms discussed include the canonical pyroptosis pathway of gasdermin activation and pore formation (caspase-1-dependent pyroptosis) and the non-canonical pyroptosis pathway (cysteoaspartic enzymes other than caspase-1). These pathways work on various cellular and factorial levels to influence normal pregnancy. This review aims to summarize and analyze the pyroptosis pathways associated with abnormal pregnancies and pregnancy complications. The objective is to enhance pregnancy outcomes by identifying various targets to prevent the onset of pyroptosis.

KEYWORDS

cell death, pyroptosis, pregnancy complications, preeclampsia, GDM

1 Introduction

Cell death is typically classified as either non-programmed or programmed. Pyroptosis is a lytic form of programmed cell death (1). Pyroptotic cells undergo membrane blistering, swelling, and flattening before lysis, as opposed to the explosive rupture observed in necroptotic apoptotic cells or the shrinking and wilting of apoptotic cells (2). This process results in the formation of cellular pores, membrane ruptures, cellular swelling, and the release of cellular contents. It also leads to DNA fragmentation, cellular vesiculation, and the formation of pyknotic vesicles (3, 4). Pyroptosis can expose microorganisms, eliminate pathogens, or retain pathogens within the burnt carcass to promote a local inflammatory response (5, 6). Pyroptosis can have both beneficial and detrimental effects on the host, depending on the degree and context of activation (1). In normal physiology, pyroptosis plays a crucial role in defending the host against pathogen infections. However, excessive

pyroptosis can lead to maladaptive and prolonged inflammatory responses that are implicated in the development of diseases (3). Emerging evidence suggests that pyroptosis contributes to the pathogenesis of a wide range of non-infectious diseases, including aseptic inflammatory diseases, autoimmune disorders, neurological disorders, cancer, atherosclerosis, acute injury, and adverse pregnancy complications (7–10) (see Figure 1).

2 The model of pyroptosis

In 2015, the discovery and characterization of GSDMD proteins (14) led to the identification of a fundamental mechanism underlying cellular pyroptosis (11). GSDMD is encoded by a gene on chromosome 8q24.3 and is the primary execution gene for inflammatory vesicle-driven pyroptosis (12). It is widely expressed in various tissues (colon, liver, brain and so on) and immune cells (13–15). Pyroptosis is carried out by pore-forming proteins called gasdermins (1), which are triggered by pyroptotic caspases (caspase-1/4/5/11) (4, 11). The initiating event of pyroptosis is the activation of the inflammasome, a multi-protein complex that activates caspase-1 (16). Due to the involvement of various cysteoaspartic enzymes, pyroptosis has recently been defined as "gasdermin-mediated programmed necrotic cell death", as gasdermin activation and pore formation in cell membranes are common features of both canonical (caspase-1-dependent pyroptosis) and non-canonical (cysteoaspartic enzymes other than caspase-1) pyroptosis (17). In the non-canonical pathway, caspase-4/5 (in human) and caspase-11 (in mouse) are directly activated by their lipopolysaccharide (LPS) ligands (11), and upon oligomerization of the LPS-cysteaspase complex, cysteaspase-4/5/11 act as effector proteins, cleaving full-length GSDMD and triggering pyroptosis (18). Other proteins have also been proposed to cleave GSDMD, including cysteinyl asparagine-8, neutrophil elastase (NE) and histone G (19–23). Activated caspase-4/5/11 cleaves the junctional region of GSDMD to release its N-terminal pore-forming structural domain (GSDMD-NT), which oligomerizes in the plasma membrane to form a pore that allows the release of DAMPs, such as the mature form of interleukin-1β (IL-1β), which recruits immune cells and induces inflammation (24), alters the intracellular environment and ultimately leads to cell death (11, 25–27) (see Figure 2). Since then, the GSDMD has been considered to be a central factor in the execution of cellular cell death (11).

GSDMs are expressed in a wide range of cell types and tissues (17, 28–30), and GSDMD is only one member of the GSDM family, which is evolutionarily and structurally conserved (16). Human GSDMs include GSDMA (31, 32), GSDMB (32), GSDMC (32, 33), GSDMD (34), GSDME and pejvakin(PJVK), also known as DFNB59 or GSDMF (35), a GSDM-related gene common to both humans and mice (16, 36). Mice lack GSDMB but have three homologs encoding GSDMA (GSDMA 1-3), four homologs of GSMDC (GSDMC 1-4) and GSDMD, GSDME (37) and PJVK. Except PJVK, the gasdermin family of proteins consists of two conserved structural domains, the N-terminal pore-forming

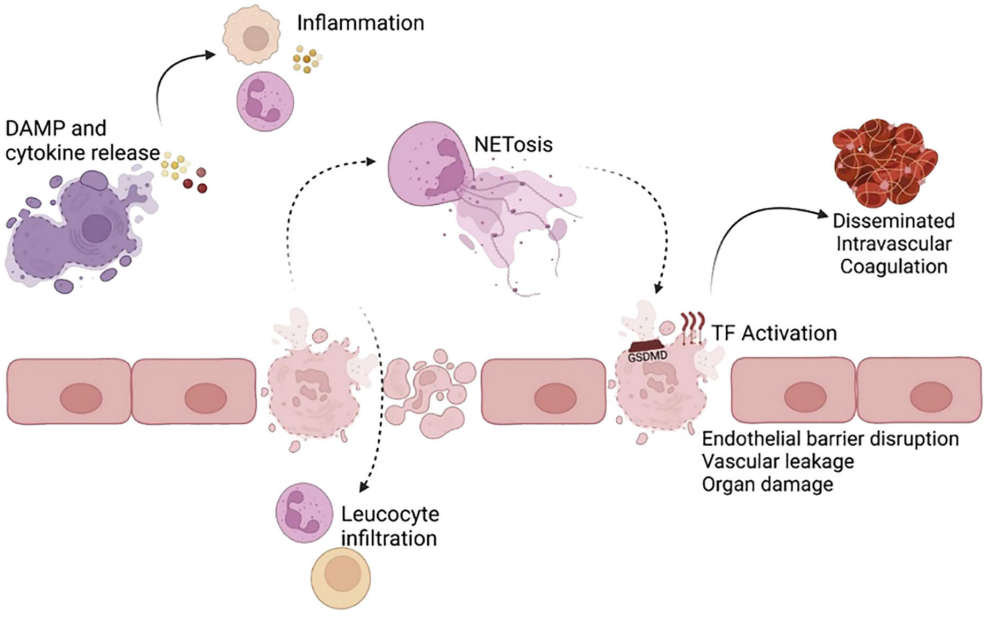


FIGURE 1
Inflammatory and pathological consequences of pyroptosis (1). Pyroptosis, induced by Gasdermin D (GSDMD) pore formation, results in the release of proinflammatory cytokines, alarmins, and damage-associated molecular patterns (DAMPs). These inflammatory molecules act on bystander cells (e.g., endothelial cells, lymphocytes) to promote an inflammatory response. The resulting cell death and inflammation can disrupt the endothelial barrier in blood vessels and vital organs, such as the lungs, leading to leucocyte infiltration. GSDMD pore formation activates the coagulation cascade and can contribute to lethality from disseminated intravascular coagulation. Pyroptosis in neutrophils and other cells triggers NETosis. Improper neutrophil extracellular trap (NET) and aberrant NETosis and DAMP removal can further induce pyroptosis and tissue damage. Reproduced with permission from [Vasudevan SO, Behl B, Rathinam VA], [Pyroptosis-induced inflammation and tissue damage.]; published by [Semin Immunol], [2023].

10.3389/fimmu.2024.1400977

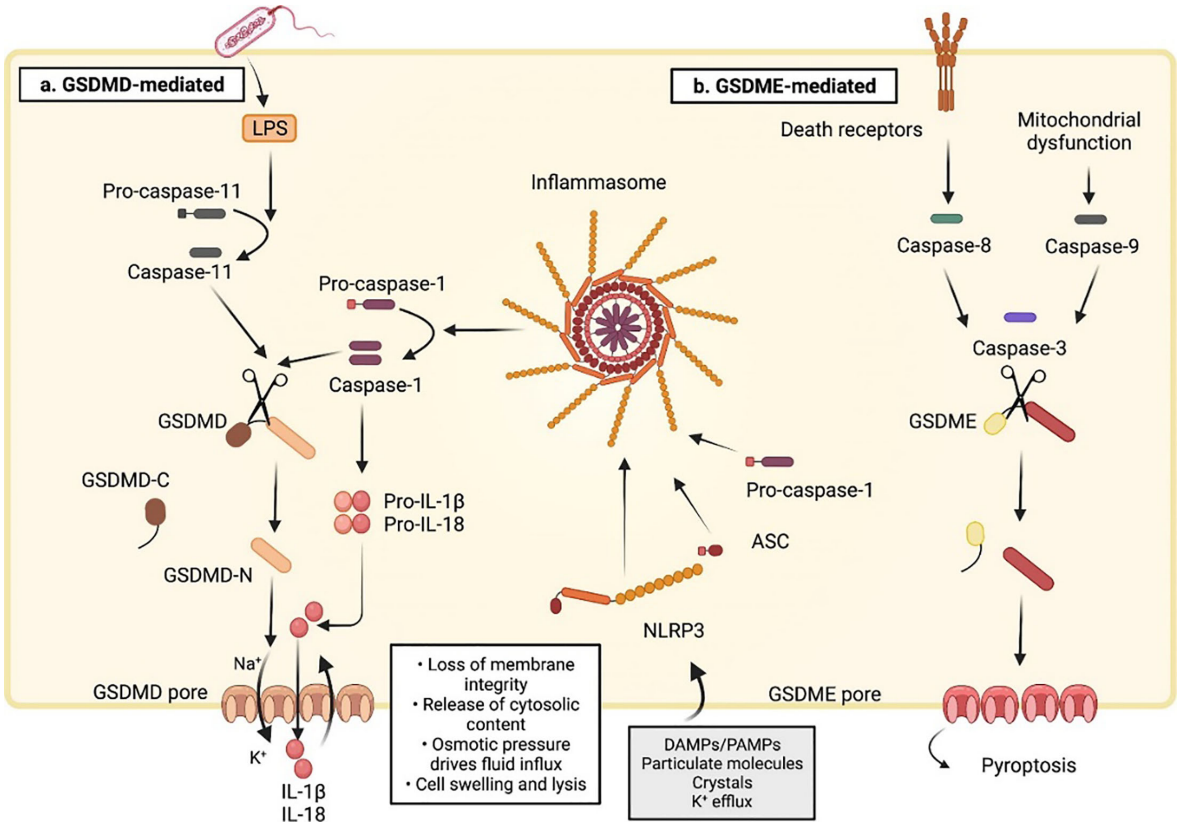


FIGURE 2
Molecular mechanisms of pyroptotic cell death (16). **(A)** The sensing of cytosolic disturbances by NLRP3 (NLR family pyrin domain-containing 3) receptor recruits the adaptor protein apoptosis-associated speck-like protein containing (ASC) to a large aggregate platform (called the inflammasome) that serves as a site of caspase-1 activation. Active caspase-1 cleaves the 53-kDa GSDMD and generates a 31-kDa N-terminal pore-forming fragment that controls pyroptosis(Canonical model). GSDMD can also be processed via inflammasome-independent activation of caspase-11(Non-canonical model). In this pathway, caspase-11 in mice and caspase-4 and 5 in humans bind to and are activated by LPS released into the cytoplasm after gram-negative bacterial infection. Unlike cleaved caspase-1, cleaved caspase-11 does not convert pro-IL-1β and pro-IL-18 to their mature forms. Instead, the process of pyroptosis promotes K+ efflux to activate caspase-1-dependent maturation of the pro-inflammatory cytokines IL-1β and IL-18 after NLRP3 inflammasome activation, leading to cellular pyroptosis. **(B)** Besides GSDMD, there is also a gasdermin E (GSDME)-dependent pyroptosis. GSDME is cleaved by caspase-3 upon mitochondrial dysfunction or death receptor activation. The GSDME N-fragments promote cell swelling and lysis by forming pores in the plasma membrane. ASC, apoptosis-associated speck-like protein containing; LPS: lipopolysaccharide. Reproduced with permission from [Ketelut-Carneiro N, Fitzgerald KA. Apoptosis], [Pyroptosis, and Necroptosis-Oh My! The Many Ways a Cell Can Die.]; published by [J Mol Biol], [2022].

structural domain, and the C-terminal deterrent protein structural domain (38, 39), which are linked by a variable linkage region (36). The C-terminal structural domain has an autoinhibitory function and is removed by proteolytic cleavage of the linker region, mediated by enzymes such as cysteinyl asparaginase (36). PJVK is a structurally unique exception to this rule, lacking a cleavable linker and containing only a truncated C-terminal structural domain (40, 41).

Gasdermin D cleavage has both positive and negative regulators of pore formation (42). These pores act as conduits for inward and outward flow across the cell membrane. Living cells employ active transport to uphold an ion gradient across the plasma membrane. This gradient collapses upon pore formation (42). Gasdermins exhibit structural similarity, and it has been hypothesized that pores formed by other members of the Gasdermin family may result in comparable intracellular consequences. However, there is currently insufficient data to directly support this hypothesis (42).

Pathogen-associated molecular patterns (PAMPs), present on the surface of toxins, viruses, and bacteria, or DAMPs produced following tissue or cellular injury, can be recognized by intracellular pattern recognition receptors (PRRs) (43, 44). Nucleotide-binding oligomerization domain-like receptors (NLRs), leucine-rich repeat (LRR) receptors, and NOD-like receptor pyrin-containing 3 (NLRP3) inflammasomes are key mediators of sterile inflammation induced by various types of DAMPs (45). Unlike other modes of cell death, pyroptosis is characterized by caspase-1-dependent plasma membrane rupture, as well as the facilitation of NLRP3 inflammatory vesicles, and the release of DAMPs and cytokines. It is important to note that all evaluations presented are objective and supported by evidence. The cytokines such as IL-1β and interleukin-18(IL-18) are released into the extracellular environment, leading to sterile inflammation (46–49). This can induce a range of diseases. Pyroptosis mediated by the NLRP3 inflammasome can also be stimulated by pathogen-associated molecules such as LPS, flagellin, or DNA fragments, as well as by

hazard-associated molecules such as reactive oxygen species (ROS) (50). These are elements of the atypical pathway.

Disruption of immune function can lead to infertility, placental inflammation, and pregnancy complications such as PE, maternal obesity, gestational diabetes mellitus (GDM), spontaneous abortion, and recurrent miscarriage (RM) (51). Excessive inflammation has been shown to be a potential cause of pregnancy complications such as PE and miscarriage (52). In this paper, we review the mechanisms and pathophysiology of pyroptosis leading to various pregnancy complications.

Furthermore, cellular death occurs via sensor proteins that detect pathogens and serve as a platform for the recruitment and activation of Caspase-1. Caspase-1 then cleaves the cytokines IL-1β and -18, as well as the pore-forming protein GSDMD. In comparison, apoptosis inhibitory protein (NAIP) and NLR family, CARD domain-containing protein 4 (NLRC4) function as a sensor (NAIP) and junctional protein (NLRC4), respectively, to form individual inflammasomes in a synergistic manner. It seems that NAIP/NLRC4 inflammasomes serve to safeguard mucosal barriers, including the lungs, stomach, and intestines, from bacterial pathogen invasion. Upon systemic activation, NAIP/NLRC4 results in a robust autoinflammatory response that is deleterious to the host (53). For example, in response to Salmonella, human intestinal epithelial cells undergo caspase-4-dependent pyroptosis, IL-18 cytokine release, restriction of bacterial replication, and extrusion of infected cells (54). The intrinsic expression of NAIP or NLRC4 in intestinal epithelial cells is both necessary and sufficient for limiting the intraepithelial Salmonella load, indicating an intestinal epithelial-intrinsic role for the NAIP/NLRC4 inflammasome in Salmonella limitation (55). Furthermore, immortalized human intestinal epithelial cells (IECs) and primary small intestinal enteroids express minimal levels of NAIP and NLRC4 in comparison to human peripheral blood mononuclear cells (56). This may explain the absence of functional NAIP/NLRC4 inflammasomes in human IECs in these *in vitro* models. The role of NAIP/NLRC4 inflammasomes in the human intestinal epithelium *in vivo* remains unknown. *In vivo*, host or microbial signaling may lead to the upregulation of NAIP/NLRC4 expression in human IECs. Alternatively, NAIP/NLRC4 may be expressed in rare IEC subpopulations not represented in *in vitro* models (57). The question of whether this interesting tissue specificity occurs in the specific group of pregnant women will be the focus of subsequent studies.

3 Effects of maternal obesity on inflammation and pyroptosis

Maternal obesity is associated with adverse perinatal outcomes and increased maternal morbidity and mortality. It is a major risk factor for a wide range of antenatal, intrapartum, postpartum, and neonatal complications (58). The release of pro-inflammatory cytokines in the placenta has been linked to maternal obesity and adverse pregnancy complications (59). Both human and animal studies have demonstrated that offspring born to obese mothers are at a higher risk of developing chronic diseases. However, the mechanisms that lead to developmental abnormalities and how different pathways are activated in the offspring of obese mothers remain unclear. Recent evidence suggests that early changes in inflammatory markers may predict the onset of various diseases later in life (60). A study in China was carried out on rats. It found that a high-fat maternal (MHF) diet led to a decrease in the number of peroxisomes in the fetal kidneys. This decrease subsequently activated oxidative stress and inflammasomes, leading to pyroptosis and apoptosis. Downregulation of the peroxisome markers peroxisomes (PEX) 3 and 14 was observed in fetal kidneys, along with a decrease in the antioxidant enzymes Superoxide Dismutase (SOD) 2 and catalase and an increase in the oxidative stress marker Ephx2 (61).Several studies have reported increased levels of free fatty acids (FFA) in the plasma of obese individuals, including palmitic acid (PA), a major saturated fatty acid. PA has been shown to promote inflammatory responses (62). Shirasuna et al. found that PA activates the NLRP3 inflammasome, leading to significant caspase-1 activation and IL-1β secretion. The authors also demonstrated that pyroptosis is involved in this process, as disruption of caspase-1 activity reduced PA-induced IL-1β release (62).

4 Pyroptosis and pregnancy-related diseases

4.1 Inflammation, toxicity, virus, and miscarriage

Miscarriage represents one of the most prevalent and severe complications of pregnancy. Miscarriage occurs in approximately 15% of recognized pregnancies, and it is associated with substantial costs in terms of physical, psychological, and economic consequences (63). Its occurrence is attributed to a multitude of identified multifactorial etiologies, encompassing fetal chromosomal abnormalities, infections, immunization, thrombosis, endocrine dysfunction, and structural anomalies of the reproductive system (64). Approximately 50% of miscarriages are of unknown etiology (65), and the clinical diagnosis is referred to as recurrent unexplained spontaneous abortion (URSA) (66).

HMGB1 is a non-histone DNA-binding protein that is highly conserved. It is a typical DAMP molecule (67). During pregnancy, high levels of HMGB1 may cause excessive or persistent inflammation, which can lead to unfavorable pregnancy outcomes at critical stages (68). A study conducted in China explored the mechanism by which HMGB1 enters cells through its receptor and activates the necrosis factor kappa B (NF-κB) signaling pathway. This, in turn, activates pyroptosis and NLRP-3 inflammatory vesicles assemble, activating caspase-1 proteins and releasing inflammatory factors, such as HMGB1, inducing aseptic inflammation. Ultimately, this leads to the disruption of the maternal-fetal interface and the development of URSA (68). Furthermore, the activation of NLRP-3 inflammatory vesicles and increased expression of caspase-1 proteins have also been observed in moult and chorionic villus tissues of induced abortion (69).

Environmental toxins can also play a role in causing miscarriage. According to recent studies, exposure to Benzo(a) pyrene (BaP) and its ultimate metabolite benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) can lead to trophoblast cell pyroptosis and ultimately result in miscarriage. Lnc-HZ14 is highly expressed in human trophoblast cells and RM vs healthy control (HC) chorionic tissues exposed to BPDE, which induces trophoblast cell pyroptosis leading to miscarriage (70).

Maternal viral infections have been a challenging issue for obstetricians for a long time. The Zika virus (ZIKV) is a flavivirus that is transmitted by mosquitoes and infects the female reproductive system (71). Unfortunately, placental infections and vertical transmission of the virus can lead to adverse effects on the fetus, such as congenital Zika virus syndrome (CZS). CZS is a serious condition that can cause microcephaly, intrauterine growth restriction, spontaneous abortions, and developmental abnormalities (72). It has been established that apoptosis may occur in the placenta during ZIKV infection (72). The involvement of pyroptosis, another form of programmed cell death, in this process is still being explored. Researchers have found that ZIKV infection induces pyroptosis of placental trophoblasts through caspase-8-mediated activation of caspase-3 via the exogenous apoptotic pathway. This, in turn, activates GSDME (but not GSDMD), by adding different caspase inhibitors. A study showed that when pregnant mice were infected with ZIKV, those without GSDME had less placental damage and better fetal outcomes (73). In summary, this study has identified a new mechanism of placental damage and congenital CZS caused by ZIKV infection. The virus triggers placental cell pyroptosis through RIG-I recognition of the viral genome, leading to the release of TNF-α. This cytokine activates the cleavage of caspase-8 and caspase-3, which in turn activates the pyroptosis execution factor, GSDME, in placental cells.

In their experiments studying the pathogenesis of silicosis, Kang et al. found that GSDME cutting via Caspases-3/8 was required in addition to Caspase-1/GSDMD. This revealed another important pathway, alongside GSDMD, as a central factor triggering pyroptosis (see Figure 2). This mechanism is similar to the one by which ZIKV induces placental damage and CZS (74).

4.2 Preterm and full-term labor

Inflammation is a crucial factor in embryo implantation, development, and endometrial metamorphosis (75). Any abnormalities during these stages may result in miscarriage or preterm labor. Preterm labor, which is caused mainly by inflammation leading to premature rupture of membranes (PROM), is a common cause of early birth due to various factors. IL-1 was the first cytokine associated with the mechanism of preterm birth (PTB) linked to infection or acute inflammation in humans, as well as with full-term spontaneous labor (76). In particular, IL-1β induces the expression of cyclooxygenase-2 (COX-2), an enzyme that catalyzes the synthesis of prostaglandins derived from arachidonic acid. These prostaglandins are known to be induced in human pregnancy tissues to promote cervical ripening and myometrial contractions (77), thereby initiating labor. Animal studies have supported the human data, and recent evidence has highlighted the significance of IL-1 in labor for both humans and animals. Inflammatory vesicles are cytoplasmic protein complexes that, once assembled, activate caspase-1 (78). This leads to the release of mature forms of the inflammatory cytokines IL-1β and/or IL-18 (79) and ultimately results in pyroptosis (80). A cross-sectional study conducted in the United States discovered that gasdermin D, the effector molecule of pyroptosis, was detectable in amniotic fluid from patients with spontaneous preterm labor and intra-amniotic infections or aseptic amniotic inflammation (80). However, further experiments are required to investigate the origin of gasdermin D in amniotic fluid. A cross-sectional study found that amniotic fluid and chorionic amnion levels were higher in women who had full-term spontaneous deliveries compared to those who had not delivered. The up-regulation of gasdermin D expression was associated with a corresponding increase in caspase-1 and IL-1β. These findings suggest that pyroptosis is the mechanism responsible for the aseptic inflammatory process in full-term labor (81).

4.3 GDM

GDM is a common pregnancy complication (82). It is associated with various adverse pregnancy outcomes, such as an increased risk of gestational infections, amniotic fluid overload, preterm labor, birth injuries, and post-partum infections. Additionally, it can lead to fetal hypoxia, higher-than-normal fetal weights, neonatal hypoglycemia, and macrosomia (83, 84). In GDM, certain alterations result in reduced insulin sensitivity, impaired insulin secretion, and the development of carbohydrate intolerance (85). The molecular mechanisms responsible for these changes remain unclear. Prior research has demonstrated that type 2 diabetes is typified by sustained low-grade inflammation. Furthermore, it has been demonstrated that NLRP3 inflammatory vesicles can contribute to insulin resistance (IR) through the downstream signaling of IL-1β (86). As reported by Ning and colleagues (87), pregnant women with GDM exhibited elevated serum levels of NLRP3, caspase-1, IL-1, and IL-18 in comparison to those with uncomplicated pregnancies. This finding suggests that pyroptosis factors may play roles in the pathogenesis of GDM by promoting chronic inflammation. Hu et al. (88) verified the changes of NLRP3 inflammatory vesicles in GDM placentas. The expression of NLRP3 and Caspase-1 was significantly elevated in GDM placentas relative to healthy placentas. Furthermore, there was a positive correlation between the expression of these proteins and maternal IR, indicating that placental NLRP3 activation is associated with the pathogenesis of GDM. The analysis of potential mechanisms indicates that the activation of NLRP3 inflammatory vesicles may increase the release of IL-1β and IL-18, thereby activating the maternal inflammatory response, IR, and glucose metabolism. Conversely, the irregularities in maternal glucose metabolism may incite the activation of NLRP3 inflammatory vesicles in the placenta, the discharge of IL-1β and IL-18, and exacerbate maternal IR. It can thus be surmised that

there is a close relationship between pyroptosis and GDM, which may well represent one of the pathogenic mechanisms of GDM.

It has been demonstrated that there is a direct correlation between GDM and dysfunction of glucose and lipid metabolism involving multiple genes. ROS are harmful mediators of inflammation. There are two primary sources of cellular ROS: phagocytic triphosphopyridine nucleotide (NADPH) oxidase, which produces superoxide anion, and mitochondria, which produce reactive oxidants (62). ROS have a dual role in disease development, acting as both signaling molecules in the cell and unavoidable toxic by-products of aerobic metabolism (89, 90). Recent studies have shown that ROS significantly inhibits NLRP3 inflammasome activation and inflammatory responses both *in vivo* and *in vitro* (91). TP53-induced glycolysis regulatory phosphatase Gene (TIGAR), a novel p53-inducible protein first identified in 2006 (92, 93), limits ROS and has been demonstrated to contribute to the resolution of ischemia/reperfusion and prevent pathologies such as ischemic diseases (94). The study investigated the impact of TIGAR on placental injury in GDM. The results showed that ATP-induced pyroptosis increased the expression of GSDMD at both the RNA and protein levels. However, cleaved GSDMD proteins were increased after TIGAR knockdown. Based on this evidence, it can be concluded that TIGAR has a mild regulatory effect on the pyroptosis construct compared to ATP (95). Inflammation and oxidative stress in the placenta are crucial factors in GDM. During pyroptosis, oxidative damage and inflammatory cytokines are elevated. The GSDMD pore releases IL-18, IL-1β, and IL-6 upon TIGAR loss, which is often considered the terminal event of pyroptosis (95).

Hyperglycemia induces apoptosis and pyroptosis in vascular endothelial cells, leading to endothelial damage or dysfunction and inflammation (96). The Wnt/β-catenin pathway is considered an important local cell-regulatory signal that determines cell fate and plays a key role in endothelial cell injury (97). β-catenin inhibits macrophage pyroptosis induced by NLRP3 inflammatory vesicles (98). A study conducted in China found that overexpression of spen paralog and direct homolog C-terminal domain containing 1 (SPOCD1) induced by chorionic mesenchymal stem cells (VMSCs) activated the β-catenin pathway, ultimately inhibiting high levels of glucose-induced apoptosis, pyroptosis, and senescence in human umbilical vein endothelial cells (HUVECs) (99).

4.4 PE

In healthy pregnancies, regulatory mechanisms at the maternal-fetal interface prevent excessive local and systemic inflammation. However, in cases of PE, these regulatory mechanisms are disrupted due to local hypoxia/ischemia, innate immune activation, hormonal imbalances, and regulatory T-cell abnormalities. This leads to a cytotoxic microenvironment that causes placental inflammation and insufficiency (100). The normal function of trophoblast cells is essential for placental development. Extravillous trophoblasts (EVT) migrate from the trophoblast column and invade the spiral arteries directly through the metaplastic stroma, partially replacing endothelial cells. This process remodels the uterine spiral arteries to increase maternal blood flow to the placenta (101). The pathophysiology of PE is not yet fully understood, but it is believed to involve placental dysplasia, oxidative stress, and altered local and systemic immunomodulation (100). Studies have shown that the trophoblast is a convergence of various pathways (102). The death of trophoblasts and the release of inflammatory factors induced by various stimuli may contribute to the development of PE (102, 103).

A recent study identified a novel protein associated with PE, transmembrane BAX inhibitor motif containing 4 (TMBIM4), which was significantly downregulated in the placentas of women with PE. TMBIM4 is mainly expressed in the human placental trophoblast. Lack of TMBIM4 in the trophoblast cell line significantly enhances NLRP3 inflammatory vesicle activity, promotes subsequent pyroptosis, and ultimately disrupts trophoblast viability, migration, and invasion. This may contribute to the PE pathological process (104). Exposure of trophoblasts to pregnancy-incompatible factors, such as LPS, suppresses TMBIM4 expression. This exacerbates NLRP3 inflammasome-mediated inflammation, leading to trophoblast dysfunction and maternal system syndrome (see Figure 3) (104).

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNA molecules that regulate mRNA expression by complementary base pairing with the 3-untranslated region (3-UTR) (105). Certain miRNAs play a crucial role in biological pathways associated with PE, such as the MAPK and TGF-β pathways, and are regulated in PE patients (106). A recent study has observed that miR-124-3p induces apoptosis and reduces cell migration and invasion in trophoblast cells when transfected with HTR8-S/Vneo cells. Additionally, miR-124-3p mimics increased the expression of NLRP3, caspase1 and IL-1β. These results suggest that miR-124-3p plays an important role in preeclampsia by mediating trophoblast cell viability, migration, invasion, and pyroptosis (107).

The clinical manifestations of PE are indicative of systemic inflammation and endothelial dysfunction, resulting in vasoconstriction, end-organ ischemia, and increased vascular permeability (108). According to a study conducted in Pittsburgh, placental cell pyroptosis is the primary sterile inflammatory pathway in e-PE. This may result in the production of virulence factors, such as IL-1β and IL-18, which cause inflammation and enhance the syndrome's systemic manifestations (100). Therefore, placental cell pyroptosis has been identified as a significant event leading to sterile inflammation in preeclampsia. Upon activation, NLRP3 inflammatory vesicles induce a significant release of mature IL-1β, initiating a positive feedback loop that results in the accumulation of other immune cells such as neutrophils and macrophages, as well as an increase in cytokines and chemokines that pose a risk (45).

Mitochondrial autophagy has been a significant signaling hub for regulating inflammatory cytokine secretion (109). The classical ubiquitin-dependent mitochondrial autophagy pathway in mammals, which is mediated by PTEN-induced putative kinase 1 (PINK1), plays a crucial role in various immune and inflammatory diseases (109). PINK1-mediated mitochondrial autophagy may have a protective role in PE by reducing ROS and trophectodermal pyroptosis (109). A Chinese controlled trial of 20

10.3389/fimmu.2024.1400977

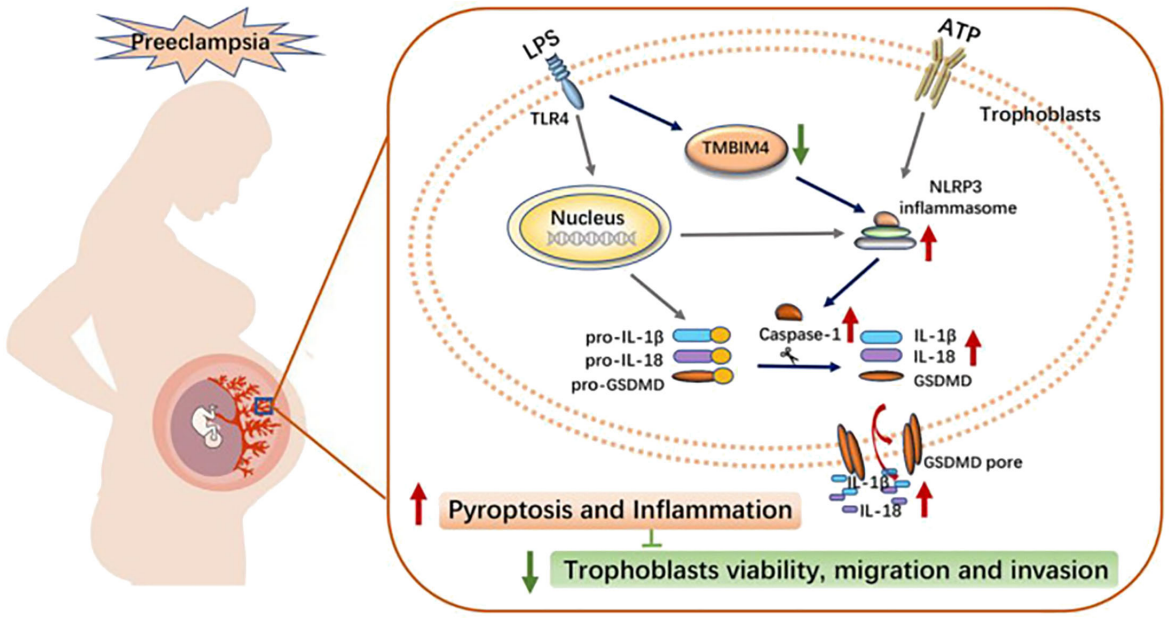


FIGURE 3
Schematic diagram illustrating the potential mechanism of action of TMBIM4 on PE pathogenesis (104). LPS induced the downregulation of TMBIM4 in the trophoblasts. TMBIM4 deficiency in the trophoblasts markedly enhanced the NLRP3 inflammasome activity and promoted subsequent pyroptosis, thereby disrupting trophoblast viability, migration, and invasion, and might be involved in the pathogenesis of PE. TMBIM4, transmembrane BAX inhibitor motif containing 4. Reproduced with permission from [Liu C], [TMBIM4 Deficiency Facilitates NLRP3 Inflammasome Activation-Induced Pyroptosis of Trophoblasts: A Potential Pathogenesis of Preeclampsia.)]; published by [Biology (Basel), [2023].

pregnant women with pre-eclampsia versus 20 healthy pregnant women found that PINK1-mediated mitochondrial autophagy was down-regulated in PE placentas (109), which is consistent with the findings of Chen et al. in a mouse model of PE (110). However, these findings contradict those of Ausman et al. (111), who reported elevated levels of full-length PINK1 relative to inactive PINK1 levels in cleaved PE placentas.

IL11 is a pleiotropic cytokine (112, 113) and is elevated in maternal serum, placenta, and meconium in early preeclamptic pregnancies (114, 115). A large animal study from Australia demonstrated that IL11 drives activation of ASC/NLRP3 inflammatory vesicles, leading to chorioamnionitis, placental and renal fibrosis, and maternal pre-eclampsia syndromes, including chronic postnatal hypertension, in mice. It has also been shown that IL11 has inflammatory vesicle-independent effects leading to dysregulation of trophoblastic differentiation, placental injury, and possibly impaired placental function, resulting in fetal growth restriction and perinatal death (116). It is well established that IL-11 activates many pathways known to be altered in pre-eclampsia, but the exact mechanism by which IL-11 causes placental injury and induces pre-eclampsia is unknown (114), and this finding fills this gap.

4.5 Intrauterine growth restriction

Intrauterine growth restriction (IUGR) is defined as the failure of the fetus to reach its genetically determined growth potential *in utero* due to a complex interaction of maternal, placental, fetal, and genetic factors (117).Excessive ROS have been identified as a detrimental factor, with evidence indicating that ROS play a critical role in the onset of cellular death (118, 119). Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is a transcription factor that plays a pivotal role in the activation of downstream anti-oxidative stress genes (120). and is a crucial mediator of antioxidant pathways (121). An increase in expression has been observed in the cytoplasm of invasive extravillous trophoblast cells, which have been linked to severe early-onset IUGR and preeclampsia (122). Despite evidence indicating a correlation between focal death and IUGR, the underlying molecular mechanisms remain unclear. It was demonstrated that Nrf2 inhibits GSDMD transcription, whereas Nrf2 deficiency upregulates GSDMD expression, thereby exacerbating maternal hypoxia-induced pyroptosis in IUGR offspring. Furthermore, Nrf2 deficiency exacerbates maternal hypoxia-induced Lung dysplasia has been observed in IUGR progeny (123). Additionally, a cross-sectional study by Berna et al. found that GSDMD-mediated cellular pyroptosis was increased in placental tissues in IUGR cases (124). In the rat study, IUGR rats exhibited increased quantification of GSDMD immunofluorescence staining of the hippocampus, increased mRNA and protein expression of NLRP1, caspase-1, and GSDMD, as well as increased quantification of IL-1β and IL-18 in the hippocampus (117). Further clarification is required to determine whether this breakthrough in animal modeling is applicable to humans. The aforementioned studies substantiated the involvement of cellular pyroptosis in the pathogenesis of IUGR, and offered insights into potential avenues for the prevention and management of IUGR.

10.3389/fimmu.2024.1400977

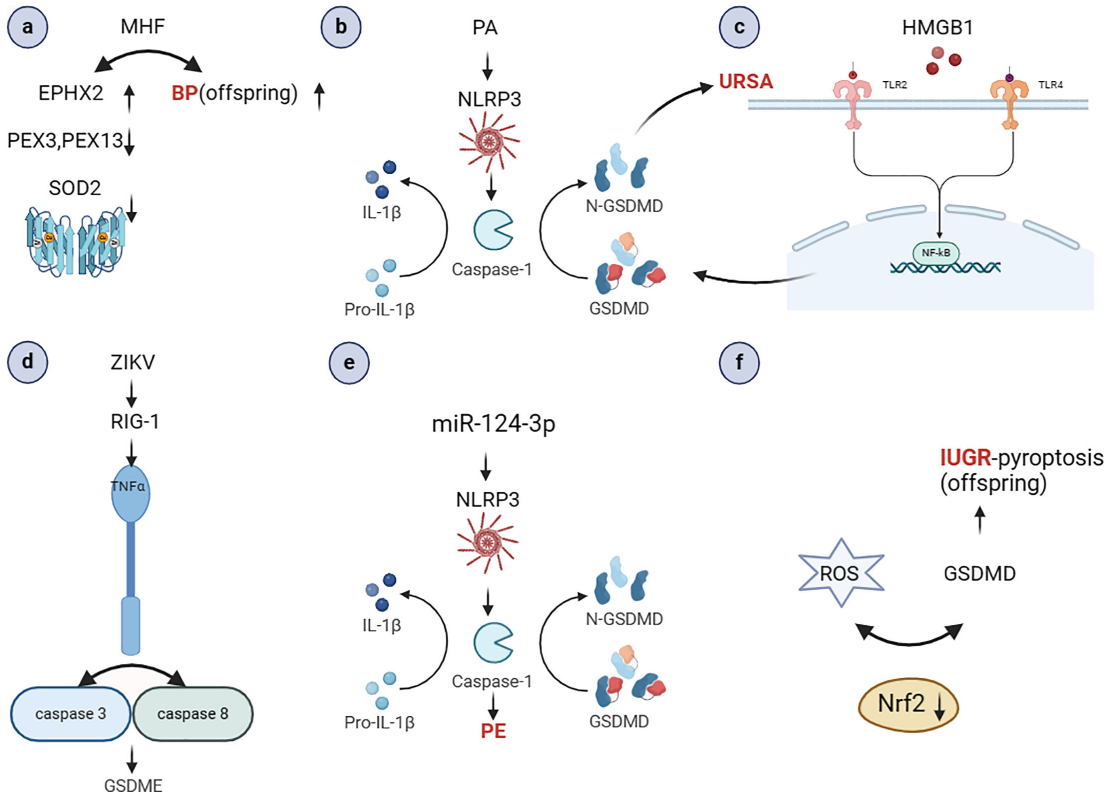


FIGURE 4
The role of pyroptosis in the occurrence and development of pregnancy-related diseases (Created with BioRender.com). **(A)** MHF may cause high blood pressure in adult offspring. It also lead to the down-regulation of fetal renal peroxisomal markers PEX3 and 14, decreased antioxidant SOD2 and catalase, and elevated oxidative stress marker Ephx2 (61); **(B)** PA can activate NLRP3 inflammatory vesicles, resulting in significant caspase-1 activation and IL-1β secretion (62); **(C)** HMGB1 activates the NF-κB signaling pathway, NLRP-3 inflammatory vesicle assembly, caspase-1 protein activation, and release of inflammatory factors, ultimately inducing aseptic inflammation. This leads to the disruption of the maternal-fetal interface and the development of URSA (68); **(D)** ZIKV infection can activate RIG-I, which recognizes the viral genome and causes placental cell pyroptosis. This leads to the release of TNF-α, which activates caspase-8 and caspase-3, resulting in the cleavage of GSDME in placental cells (73); **(E)** miR-124-3p mimics increased the expression of NLRP3, caspase1 and IL-1β (107); **(F)** Nrf2 deficiency upregulates GSDMD expression, thereby exacerbating maternal hypoxia-induced pyroptosis in IUGR offspring (123). MHF, high-fat maternal; PEX, peroxisomes; SOD2, Superoxide Dismutase 2; HMGB1, high mobility group box-1; ZIKV, Zika virus; Nrf2, Nuclear factor erythroid-derived 2-related factor 2.

5 Conclusion

Cellular pyroptosis has been extensively documented in the pathogenesis of numerous pathological conditions, including autoimmune disorders, neoplastic growths, and atherosclerosis. Our findings indicate that pregnancy-associated disorders are also associated with pyroptosis to varying aspects (see Figure 4). In recent years, the discovery of various pathways has contributed significantly to the development of the field of obstetrics and gynecology. Although the role of pyroptosis in the pathogenesis of abnormal pregnancies has not been fully elucidated, studies have shown that the release of interleukins triggers a series of cascading and causal effects that may lead to an increased prevalence of certain diseases in the offspring, inflammatory effects, labor initiation, and GDM. An integrated analysis of inflammation, immunity and genetics is also essential to gain insight into the role of cellular pyroptosis in the development of pregnancy-related diseases. It is our contention that inflammasome, which play a pivotal role in pyroptosis, represent a promising avenue for the development of novel therapeutic strategies for pregnancy-related disorders. Target exploration has the potential to impede disease progression and enhance human health and well-being.

Author contributions

JL: Writing – original draft, Methodology, Conceptualization. MW: Writing – original draft, Investigation. HZ: Writing – original draft, Investigation. JZ: Writing – original draft, Investigation. HY: Writing – original draft, Investigation. SY: Writing – review & editing, Supervision, Funding acquisition.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Funding for this work was provided by the Jilin Provincial Department of

Science and Technology (grant numbers 20240305067YY, 3R219H273429 and 3R219G283429).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Vasudevan SO, Behl B, Rathinam VA. Pyroptosis-induced inflammation and tissue damage. *Semin Immunol*. (2023) 69:101781. doi: 10.1016/j.smim.2023.101781

2. Chen X, He WT, Hu L, Li J, Fang Y, Wang X, et al. Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. *Cell Res*. (2016) 26:1007–20. doi: 10.1038/cr.2016.100

3. Rao Z, Zhu Y, Yang P, Chen Z, Xia Y, Qiao C, et al. Pyroptosis in inflammatory diseases and cancer. *Theranostics*. (2022) 12:4310–29. doi: 10.7150/thno.71086

4. Li L, Dickinson MS, Coers J, Miao EA. Pyroptosis in defense against intracellular bacteria. *Semin Immunol*. (2023) 69:101805. doi: 10.1016/j.smim.2023.101805

5. Davis MA, Fairgrieve MR, Den Hartigh A, Yakovenko O, Duvvuri B, Lood C, et al. Calpain drives pyroptotic vimentin cleavage, intermediate filament loss, and cell rupture that mediates immunostimulation. *Proc Natl Acad Sci U.S.A*. (2019) 116:5061–70. doi: 10.1073/pnas.1818598116

6. Place DE, Lee S, Kanneganti TD. PANoptosis in microbial infection. *Curr Opin Microbiol*. (2021) 59:42–9. doi: 10.1016/j.mib.2020.07.012

7. Xu YJ, Zheng L, Hu YW, Wang Q. Pyroptosis and its relationship to atherosclerosis. *Clin Chim Acta*. (2018) 476:28–37. doi: 10.1016/j.cca.2017.11.005

8. Wu J, Lin S, Wan B, Velani B, Zhu Y. Pyroptosis in liver disease: new insights into disease mechanisms. *Aging Dis*. (2019) 10:1094–108. doi: 10.14336/AD.2019.0116

9. McKenzie BA, Dixit VM, Power C. Fiery cell death: pyroptosis in the central nervous system. *Trends Neurosci*. (2020) 43:55–73. doi: 10.1016/j.tins.2019.11.005

10. Xia X, Wang X, Cheng Z, Qin W, Lei L, Jiang J, et al. The role of pyroptosis in cancer: pro-cancer or pro-"host"? *Cell Death Dis*. (2019) 10:650. doi: 10.1038/s41419-019-1883-8

11. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature*. (2015) 526:660–5. doi: 10.1038/nature15514

12. He WT, Wan H, Hu L, Chen P, Wang X, Huang Z, et al. Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. *Cell Res*. (2015) 25:1285–98. doi: 10.1038/cr.2015.139

13. Broz P, Pelegrín P, Shao F. The gasdermins, a protein family executing cell death and inflammation. *Nat Rev Immunol*. (2020) 20:143–57. doi: 10.1038/s41577-019-0228-2

14. Angosto-Bazarra D, Alarcón-Vila C, Hurtado-Navarro L, Baños MC, Rivers-Auty J, Pelegrín P. Evolutionary analyses of the gasdermin family suggest conserved roles in infection response despite loss of pore-forming functionality. *BMC Biol*. (2022) 20:9. doi: 10.1186/s12915-021-01220-z

15. Schmiedel BJ, Singh D, Madrigal A, Valdovino-Gonzalez AG, White BM, Zapardiel-Gonzalo J, et al. Impact of genetic polymorphisms on human immune cell gene expression. *Cell*. (2018) 175:1701–15.e16. doi: 10.1016/j.cell.2018.10.022

16. Ketelut-Carneiro N, Fitzgerald KA. Apoptosis, pyroptosis, and necroptosis—Oh my! The many ways a cell can die. *J Mol Biol*. (2022) 434:167378. doi: 10.1016/j.jmb.2021.167378

17. Shi J, Gao W, Shao F. Pyroptosis: gasdermin-mediated programmed necrotic cell death. *Trends Biochem Sci*. (2017) 42:245–54. doi: 10.1016/j.tibs.2016.10.004

18. Downs KP, Nguyen H, Dorfleutner A, Stehlik C. An overview of the non-canonical inflammasome. *Mol Aspects Med*. (2020) 76:100924. doi: 10.1016/j.mam.2020.100924

19. Orning P, Weng D, Starheim K, Ratner D, Best Z, Lee B, et al. Pathogen blockade of TAK1 triggers caspase-8-dependent cleavage of gasdermin D and cell death. *Science*. (2018) 362:1064–9. doi: 10.1126/science.aau2818

20. Sarhan J, Liu BC, Muendlein HI, Li P, Nilson R, Tang AY, et al. Caspase-8 induces cleavage of gasdermin D to elicit pyroptosis during Yersinia infection. *Proc Natl Acad Sci U.S.A*. (2018) 115:E10888–97. doi: 10.1073/pnas.1809548115

21. Chen KW, Demarco B, Heilig R, Shkarina K, Boettcher A, Farady CJ, et al. Extrinsic and intrinsic apoptosis activate pannexin-1 to drive NLRP3 inflammasome assembly. *EMBO J*. (2019) 38:e101638. doi: 10.15252/embj.2019101638

22. Kambara H, Liu F, Zhang X, Liu P, Bajrami B, Teng Y, et al. Gasdermin D exerts anti-inflammatory effects by promoting neutrophil death. *Cell Rep*. (2018) 22:2924–36. doi: 10.1016/j.celrep.2018.02.067

23. Burgener SS, Leborgne NGF, Snipas SJ, Salvesen GS, Bird PI, Benarafa C. Cathepsin G inhibition by serpinb1 and serpinb6 prevents programmed necrosis in neutrophils and monocytes and reduces GSDMD-driven inflammation. *Cell Rep*. (2019) 27:3646–3656.e5. doi: 10.1016/j.celrep.2019.05.065

24. Van Opdenbosch N, Lamkanfi M. Caspases in cell death, inflammation, and disease. *Immunity*. (2019) 50:1352–64. doi: 10.1016/j.immuni.2019.05.020

25. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*. (2015) 526:666–71. doi: 10.1038/nature15541

26. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, et al. Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature*. (2016) 535:111–6. doi: 10.1038/nature18590

27. Liu X, Zhang Z, Ruan J, Pan Y, Magupalli VG, Wu H, et al. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature*. (2016) 535:153–8. doi: 10.1038/nature18629

28. Qiu S, Liu J, Xing F. [amp]]lsquo;Hints' in the killer protein gasdermin D: unveiling the secrets of gasdermins driving cell death. *Cell Death Differ*. (2017) 24:588–96. doi: 10.1038/cdd.2017.24

29. Aglietti RA, Dueber EC. Recent insights into the molecular mechanisms underlying pyroptosis and gasdermin family functions. *Trends Immunol*. (2017) 38:261–71. doi: 10.1016/j.it.2017.01.003

30. Kovacs SB, Miao EA. Gasdermins: effectors of pyroptosis. *Trends Cell Biol*. (2017) 27:673–84. doi: 10.1016/j.tcb.2017.05.005

31. Saeki N, Kuwahara Y, Sasaki H, Satoh H, Shiroishi T. Gasdermin (Gsdm) localizing to mouse Chromosome 11 is predominantly expressed in upper gastrointestinal tract but significantly suppressed in human gastric cancer cells. *Mamm Genome*. (2000) 11:718–24. doi: 10.1007/s003350010138

32. Tamura M, Tanaka S, Fujii T, Aoki A, Komiyama H, Ezawa K, et al. Members of a novel gene family, Gsdm, are expressed exclusively in the epithelium of the skin and gastrointestinal tract in a highly tissue-specific manner. *Genomics*. (2007) 89:618–29. doi: 10.1016/j.ygeno.2007.01.003

33. Watabe K, Ito A, Asada H, Endo Y, Kobayashi T, Nakamoto K, et al. Structure, expression and chromosome mapping of MLZE, a novel gene which is preferentially expressed in metastatic melanoma cells. *Jpn J Cancer Res*. (2001) 92:140–51. doi: 10.1111/j.1349-7006.2001.tb01076.x

34. Katoh M, Katoh M. Identification and characterization of human DFNA5L, mouse Dfna5l, and rat Dfna5l genes in silico. *Int J Oncol*. (2004) 25:765–70. doi: 10.3892/ijo

35. Delmaghani S, del Castillo FJ, Michel V, Leibovici M, Aghaie A, Ron U, et al. Mutations in the gene encoding pejvakin, a newly identified protein of the afferent auditory pathway, cause DFNB59 auditory neuropathy. *Nat Genet*. (2006) 38:770–8. doi: 10.1038/ng1829

36. Zou J, Zheng Y, Huang Y, Tang D, Kang R, Chen R. The versatile gasdermin family: their function and roles in diseases. *Front Immunol*. (2021) 12:751533. doi: 10.3389/fimmu.2021.751533

37. Van Laer L, Huizing EH, Verstreken M, van Zuijlen D, Wauters JG, Bossuyt PJ, et al. Nonsyndromic hearing impairment is associated with a mutation in DFNA5. *Nat Genet*. (1998) 20:194–7. doi: 10.1038/2503

38. Rogers C, Erkes DA, Nardone A, Aplin AE, Fernandes-Alnemri T, Alnemri ES. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. *Nat Commun*. (2019) 10:1689. doi: 10.1038/s41467-019-09397-2

39. Liu Z, Wang C, Yang J, Zhou B, Yang R, Ramachandran R, et al. Crystal structures of the full-length murine and human gasdermin D reveal mechanisms of autoinhibition, lipid binding, and oligomerization. *Immunity*. (2019) 51:43–49.e4. doi: 10.1016/j.immuni.2019.04.017

40. Schwander M, Sczaniecka A, Grillet N, Bailey JS, Avenarius M, Najmabadi H, et al. A forward genetics screen in mice identifies recessive deafness traits and reveals that pejvakin is essential for outer hair cell function. *J Neurosci*. (2007) 27:2163–75. doi: 10.1523/JNEUROSCI.4975-06.2007

41. Op de Beeck K, Van Camp G, Thys S, Cools N, Callebaut I, Vrijens K, et al. The DFNA5 gene, responsible for hearing loss and involved in cancer, encodes a novel apoptosis-inducing protein. *Eur J Hum Genet*. (2011) 19:965–73. doi: 10.1038/ejhg.2011.63

42. Huston HC, Anderson MJ, Fink SL. Pyroptosis and the cellular consequences of gasdermin pores. *Semin Immunol*. (2023) 69:101803. doi: 10.1016/j.smim.2023.101803

43. Zhao Y, Yang J, Shi J, Gong YN, Lu Q, Xu H, et al. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature*. (2011) 477:596–600. doi: 10.1038/nature10510

44. Kim RY, Rae B, Neal R, Donovan C, Pinkerton J, Balachandran L, et al. Elucidating novel disease mechanisms in severe asthma. *Clin Transl Immunol*. (2016) 5:e91. doi: 10.1038/cti.2016.37

45. Shirasuna K, Karasawa T, Takahashi M. Role of the NLRP3 inflammasome in preeclampsia. *Front Endocrinol (Lausanne)*. (2020) 11:80. doi: 10.3389/fendo.2020.00080

46. Chen A, Chen Z, Xia Y, Lu D, Yang X, Sun A, et al. Liraglutide attenuates NLRP3 inflammasome-dependent pyroptosis via regulating SIRT1/NOX4/ROS pathway in H9c2 cells. *Biochem Biophys Res Commun*. (2018) 499:267–72. doi: 10.1016/j.bbrc.2018.03.142

47. Sun N, Zhang H. Pyroptosis in pterygium pathogenesis. *Biosci Rep*. (2018) 38: BSR20180282. doi: 10.1042/BSR20180282

48. Vakrakou AG, Boiu S, Ziakas PD, Xingi E, Boleti H, Manoussakis MN. Systemic activation of NLRP3 inflammasome in patients with severe primary Sjögren's syndrome fueled by inflammagenic DNA accumulations. *J Autoimmun*. (2018) 91:23–33. doi: 10.1016/j.jaut.2018.02.010

49. Heo MJ, Kim TH, You JS, Blaya D, Sancho-Bru P, Kim SG. Alcohol dysregulates miR-148a in hepatocytes through FoxO1, facilitating pyroptosis via TXNIP overexpression. *Gut*. (2019) 68:708–20. doi: 10.1136/gutjnl-2017-315123

50. Yu P, Zhang X, Liu N, Tang L, Peng C, Chen X. Pyroptosis: mechanisms and diseases. *Signal Transduct Target Ther*. (2021) 6:128. doi: 10.1038/s41392-021-00507-5

51. Kalagiri RR, Carder T, Choudhury S, Vora N, Ballard AR, Govande V, et al. Inflammation in complicated pregnancy and its outcome. *Am J Perinatol*. (2016) 33:1337–56. doi: 10.1055/s-0036-1582397

52. Christiansen OB, Nielsen HS, Kolte AM. Inflammation and miscarriage. *Semin Fetal Neonatal Med*. (2006) 11:302–8. doi: 10.1016/j.siny.2006.03.001

53. Bauer R, Rauch I. The NAIP/NLRC4 inflammasome in infection and pathology. *Mol Aspects Med*. (2020) 76:100863. doi: 10.1016/j.mam.2020.100863

54. Holly MK, Han X, Zhao EJ, Crowley SM, Allaire JM, Knodler LA, et al. Salmonella enterica infection of murine and human enteroid-derived monolayers elicits differential activation of epithelium-intrinsic inflammasomes. *Infect Immun*. (2020) 88:e00017-20. doi: 10.1128/IAI.00017-20

55. Rauch I, Deets KA, Ji DX, von Moltke J, Tenthorey JL, Lee AY, et al. NAIP-NLRC4 inflammasomes coordinate intestinal epithelial cell expulsion with eicosanoid and IL-18 release via activation of caspase-1 and -8. *Immunity*. (2017) 46:649–59. doi: 10.1016/j.immuni.2017.03.016

56. Naseer N, Zhang J, Bauer R, Constant DA, Nice TJ, Brodsky IE, et al. Salmonella enterica serovar typhimurium induces NAIP/NLRC4- and NLRP3/ASC-independent, caspase-4-dependent inflammasome activation in human intestinal epithelial cells. *Infect Immun*. (2022) 90:e0066321. doi: 10.1128/iai.00663-21

57. Egan MS, Zhang J, Shin S. Human and mouse NAIP/NLRC4 inflammasome responses to bacterial infection. *Curr Opin Microbiol*. (2023) 73:102298. doi: 10.1016/j.mib.2023.102298

58. Zehravi M, Maqbool M, Ara I. Correlation between obesity, gestational diabetes mellitus, and pregnancy outcomes: an overview. *Int J Adolesc Med Health*. (2021) 33:339–45. doi: 10.1515/ijamh-2021-0058

59. Marchi J, Berg M, Dencker A, Olander EK, Begley C. Risks associated with obesity in pregnancy, for the mother and baby: a systematic review of reviews. *Obes Rev*. (2015) 16:621–38. doi: 10.1111/obr.12288

60. Denizli M, Capitano ML, Kua KL. Maternal obesity and the impact of associated early-life inflammation on long-term health of offspring. *Front Cell Infect Microbiol*. (2022) 12:940937. doi: 10.3389/fcimb.2022.940937

61. Zhou P, Guan H, Guo Y, Zhu L, Liu X. Maternal high-fat diet programs renal peroxisomes and activates NLRP3 inflammasome-mediated pyroptosis in the rat fetus. *J Inflammation Res*. (2021) 14:5095–110. doi: 10.2147/JIR.S329972

62. Shirasuna K, Takano H, Seno K, Ohtsu A, Karasawa T, Takahashi M, et al. Palmitic acid induces interleukin-1β secretion via NLRP3 inflammasomes and inflammatory responses through ROS production in human placental cells. *J Reprod Immunol*. (2016) 116:104–12. doi: 10.1016/j.jri.2016.06.001

63. Quenby S, Gallos ID, Dhillon-Smith RK, Podesek M, Stephenson MD, Fisher J, et al. Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet*. (2021) 397:1658–67. doi: 10.1016/S0140-6736(21)00682-6

64. La X, Wang W, Zhang M, Liang L. Definition and multiple factors of recurrent spontaneous abortion. *Adv Exp Med Biol*. (2021) 1300:231–57. doi: 10.1007/978-981-33-4187-6_11

65. Rull K, Nagirnaja L, Laan M. Genetics of recurrent miscarriage: challenges, current knowledge, future directions. *Front Genet*. (2012) 3:34. doi: 10.3389/fgene.2012.00034

66. Gao Y, Wang PL. Increased CD56(+) NK cells and enhanced Th1 responses in human unexplained recurrent spontaneous abortion. *Genet Mol Res*. (2015) 14:18103–9. doi: 10.4238/2015.December.22.36

67. Yuan S, Liu Z, Xu Z, Liu J, Zhang J. High mobility group box 1 (HMGB1): a pivotal regulator of hematopoietic Malignancies. *J Hematol Oncol*. (2020) 13:91. doi: 10.1186/s13045-020-00920-3

68. Zhu D, Zou H, Liu J, Wang J, Ma C, Yin J, et al. Inhibition of HMGB1 ameliorates the maternal-fetal interface destruction in unexplained recurrent spontaneous abortion by suppressing pyroptosis activation. *Front Immunol*. (2021) 12:782792. doi: 10.3389/fimmu.2021.782792

69. Zhang X, Zhang K, Zhang Y. Pigment epithelium–derived factor facilitates NLRP3 inflammasome activation through downregulating cytidine monophosphate kinase 2: A potential treatment strategy for missed abortion. *Int J Mol Med*. (2020) 45:1436–46. doi: 10.3892/ijmm

70. Wang R, Xu X, Yang J, Chen W, Zhao J, Wang M, et al. BPDE exposure promotes trophoblast cell pyroptosis and induces miscarriage by up-regulating lnc-HZ14/ZBP1/NLRP3 axis. *J Hazardous Materials*. (2023) 455:131543. doi: 10.1016/j.jhazmat.2023.131543

71. Zimmerman MG, Wrammert J, Suthar MS. Cross-reactive antibodies during zika virus infection: protection, pathogenesis, and placental seeding. *Cell Host Microbe*. (2020) 27:14–24. doi: 10.1016/j.chom.2019.12.003

72. Miner JJ, Cao B, Govero J, Smith AM, Fernandez E, Cabrera OH, et al. Zika virus infection during pregnancy in mice causes placental damage and fetal demise. *Cell*. (2016) 165:1081–91. doi: 10.1016/j.cell.2016.05.008

73. Zhao Z, Li Q, Ashraf U, Yang M, Zhu W, Gu J, et al. Zika virus causes placental pyroptosis and associated adverse fetal outcomes by activating GSDME. *Elife*. (2022) 11:e73792. doi: 10.7554/eLife.73792.sa2

74. Kang L, Dai J, Wang Y, Shi P, Zou Y, Pei J, et al. Blocking Caspase-1/Gsdmd and Caspase-3/-8/Gsdme pyroptotic pathways rescues silicosis in mice. *PloS Genet*. (2022) 18:e1010515. doi: 10.1371/journal.pgen.1010515

75. Saito Reis CA, Padron JG, Norman Ing ND, Kendal-Wright CE. High-mobility group box 1 is a driver of inflammation throughout pregnancy. *Am J Reprod Immunol*. (2021) 85:e13328. doi: 10.1111/aji.13328

76. Romero R, Parvizi ST, Oyarzun E, Mazor M, Wu YK, Avila C, et al. Amniotic fluid interleukin-1 in spontaneous labor at term. *J Reprod Med*. (1990) 35:235–8.

77. Nadeau-Vallée M, Obari D, Quiniou C, Lubell WD, Olson DM, Girard S, et al. A critical role of interleukin-1 in preterm labor. *Cytokine Growth Factor Rev*. (2016) 28:37–51. doi: 10.1016/j.cytogfr.2015.11.001

78. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med*. (2015) 21:677–87. doi: 10.1038/nm.3893

79. Black RA, Kronheim SR, Merriam JE, March CJ, Hopp TP. A pre-aspartate-specific protease from human leukocytes that cleaves pro-interleukin-1 beta. *J Biol Chem*. (1989) 264:5323–6. doi: 10.1016/S0021-9258(18)83546-3

80. Gomez-Lopez N, Romero R, Tarca AL, Miller D, Panaitescu B, Schwenkel G, et al. Gasdermin D: Evidence of pyroptosis in spontaneous preterm labor with sterile intra-amniotic inflammation or intra-amniotic infection. *Am J Reprod Immunol*. (2019) 82:e13184. doi: 10.1111/aji.13184

81. Gomez-Lopez N, Romero R, Panaitescu B, Miller D, Zou C, Gudicha DW, et al. Gasdermin D: *in vivo* evidence of pyroptosis in spontaneous labor at term. *J Matern Fetal Neonatal Med*. (2021) 34:569–79. doi: 10.1080/14767058.2019.1610740

82. Pervjakova N, Moen GH, Borges MC, Ferreira T, Cook JP, Allard C, et al. Multi-ancestry genome-wide association study of gestational diabetes mellitus highlights genetic links with type 2 diabetes. *Hum Mol Genet*. (2022) 31:3377–91. doi: 10.1093/hmg/ddac050

83. Giannakou K, Evangelou E, Yiallouros P, Christophi CA, Middleton N, Papatheodorou E, et al. Risk factors for gestational diabetes: An umbrella review of meta-analyses of observational studies. *PloS One*. (2019) 14:e0215372. doi: 10.1371/journal.pone.0215372

84. Sweeting A, Wong J, Murphy HR, Ross GP. A clinical update on gestational diabetes mellitus. *Endocr Rev*. (2022) 43:763–93. doi: 10.1210/endrev/bnac003

85. Fakhrul-Alam M, Sharmin-Jahan, Mashfiqul-Hasan, Nusrat-Sultana, Mohona-Zaman, Rakibul-Hasan M, et al. Insulin secretory defect may be the major determinant of GDM in lean mothers. *J Clin Transl Endocrinol*. (2020) 20:100226. doi: 10.1016/j.jcte.2020.100226

86. Lu S, Li Y, Qian Z, Zhao T, Feng Z, Weng X, et al. Role of the inflammasome in insulin resistance and type 2 diabetes mellitus. *Front IN Immunol*. (2023) 14:1052756. doi: 10.3389/fimmu.2023.1052756

87. Han N, Chang XY, Yuan ZL, Wang YZ. Expression and correlation analysis of silent information regulator 1 (SIRT1), sterol regulatory element-binding protein-1 (SREBP1), and pyroptosis factor in gestational diabetes mellitus. *J Matern Fetal Neonatal Med*. (2024) 37:2311809. doi: 10.1080/14767058.2024.2311809

88. Hu T, Wang X, Ruan Y, Yao J, Wang F, Xu Y, et al. High glucose promotes the release of IL-1β and IL-18 from placental trophoblast by activating NLRP3 inflammasome. *Chin J Endocrinol Metab*. (2022) 38:36–41. doi: 10.3760/cma.j.cn311282-20210312-00158

89. Mittler R. ROS are good. *Trends Plant Sci*. (2017) 22:11–9. doi: 10.1016/j.tplants.2016.08.002

90. Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD. ROS and the DNA damage response in cancer. *Redox Biol*. (2019) 25:101084. doi: 10.1016/j.redox.2018.101084

91. Shirasuna K, Usui F, Karasawa T, Kimura H, Kawashima A, Mizukami H, et al. Nanosilica-induced placental inflammation and pregnancy complications: Different roles of the inflammasome components NLRP3 and ASC. *Nanotoxicology*. (2015) 9:554–67. doi: 10.3109/17435390.2014.956156

92. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell*. (2006) 126:107–20. doi: 10.1016/j.cell.2006.05.036

93. Green DR, Chipuk JE. p53 and metabolism: inside the TIGAR. *Cell*. (2006) 126:30–2. doi: 10.1016/j.cell.2006.06.032

94. Zhang D-M, Zhang T, Wang MM, Wang XX, Qin YY, Wu J, et al. TIGAR alleviates ischemia/reperfusion-induced autophagy and ischemic brain injury. *Free Radical Biol Med*. (2019) 137:13–23. doi: 10.1016/j.freeradbiomed.2019.04.002

95. Guo J, Zhou M, Zhao M, Li S, Fang Z, Li A, et al. TIGAR deficiency induces caspase-1-dependent trophoblasts pyroptosis through NLRP3-ASC inflammasome. *Front Immunol*. (2023) 14:1114620. doi: 10.3389/fimmu.2023.1114620

96. Meng X, Li Z, Li Y. CircSHOC2 Knockdown Alleviates High Glucose-Induced Vascular Endothelial Cell Pyroptosis via Targeting miR-145/FOXO1 Axis *In Vitro* Condition. *Mol Biotechnol*. (2023) 65:384–93. doi: 10.1007/s12033-022-00544-7

97. Pećina-Slaus N. Wnt signal transduction pathway and apoptosis: a review. *Cancer Cell Int*. (2010) 10:22. doi: 10.1186/1475-2867-10-22

98. Wang Y, Cao C, Zhu Y, Fan H, Liu Q, Liu Y, et al. TREM2/β-catenin attenuates NLRP3 inflammasome-mediated macrophage pyroptosis to promote bacterial clearance of pyogenic bacteria. *Cell Death Dis*. (2022) 13:771. doi: 10.1038/s41419-022-05193-x

99. Wang D, Wei Z, Lin F, Wang Y, Liu X, Li Q, et al. Protective effects of villi mesenchymal stem cells on human umbilical vein endothelial cells by inducing SPOCD1 expression in cases of gestational diabetes mellitus. *Biochem Biophys Res Commun*. (2023) 686:149177. doi: 10.1016/j.bbrc.2023.149177

100. Cheng SB, Nakashima A, Huber WJ, Davis S, Banerjee S, Huang Z, et al. Pyroptosis is a critical inflammatory pathway in the placenta from early onset preeclampsia and in human trophoblasts exposed to hypoxia and endoplasmic reticulum stressors. *Cell Death Dis*. (2019) 10:927. doi: 10.1038/s41419-019-2162-4

101. Staff AC, Fjeldstad HE, Fosheim IK, Moe K, Turowski G, Johnsen GM, et al. Failure of physiological transformation and spiral artery atherosis: their roles in preeclampsia. *Am J Obstet Gynecol*. (2022) 226:S895–s906. doi: 10.1016/j.ajog.2020.09.026

102. Redman CWG, Staff AC, Roberts JM. Syncytiotrophoblast stress in preeclampsia: the convergence point for multiple pathways. *Am J Obstet Gynecol*. (2022) 226:S907–s927. doi: 10.1016/j.ajog.2020.09.047

103. Staff AC. The two-stage placental model of preeclampsia: An update. *J Reprod Immunol*. (2019) 134-135:1–10. doi: 10.1016/j.jri.2019.07.004

104. Chen Y, Xiao L, Sun G, Li M, Yang H, Ming Z, et al. TMBIM4 deficiency facilitates NLRP3 inflammasome activation-induced pyroptosis of trophoblasts: A potential pathogenesis of preeclampsia. *Biol (Basel)*. (2023) 12:208. doi: 10.3390/biology12020208

105. Lui JC. Regulation of body growth by microRNAs. *Mol Cell Endocrinol*. (2017) 456:2–8. doi: 10.1016/j.mce.2016.10.024

106. Lykoudi A, Kolialexi A, Lambrou GI, Braoudaki M, Siristatidis C, Papaioanou GK, et al. Dysregulated placental microRNAs in Early and Late onset Preeclampsia. *Placenta*. (2018) 61:24–32. doi: 10.1016/j.placenta.2017.11.005

107. Tao J, Xia LZ, Liang L, Chen Y, Wei D, Meng J, et al. MiR-124–3p promotes trophoblast cell HTR-8/SVneo pyroptosis by targeting placental growth factor. *Placenta*. (2020) 101:176–84. doi: 10.1016/j.placenta.2020.08.011

108. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. *Lancet*. (2005) 365:785–99. doi: 10.1016/S0140-6736(05)17987-2

109. Sun Y, Lv D, Xie Y, Xu H, Li X, Li F, et al. PINK1-mediated mitophagy induction protects against preeclampsia by decreasing ROS and trophoblast pyroptosis. *Placenta*. (2023) 143:1–11. doi: 10.1016/j.placenta.2023.09.010

110. Chen G, Lin Y, Chen L, Zeng F, Zhang L, Huang Y, et al. Role of DRAM1 in mitophagy contributes to preeclampsia regulation in mice. *Mol Med Rep*. (2020) 22:1847–58. doi: 10.3892/mmr

111. Ausman J, Abbade J, Ermini L, Farrell A, Tagliaferro A, Post M, et al. Ceramide-induced BOK promotes mitochondrial fission in preeclampsia. *Cell Death Dis*. (2018) 9:298. doi: 10.1038/s41419-018-0360-0

112. Lim WW, Corden B, Ng B, Vanezis K, D'Agostino G, Widjaja AA, et al. Interleukin-11 is important for vascular smooth muscle phenotypic switching and aortic inflammation, fibrosis and remodeling in mouse models. *Sci Rep*. (2020) 10:17853. doi: 10.1038/s41598-020-74944-7

113. Ng B, Cook SA, Schafer S. Interleukin-11 signaling underlies fibrosis, parenchymal dysfunction, and chronic inflammation of the airway. *Exp Mol Med*. (2020) 52:1871–8. doi: 10.1038/s12276-020-00531-5

114. Winship AL, Koga K, Menkhorst E, Van Sinderen M, Rainczuk K, Nagai M, et al. Interleukin-11 alters placentation and causes preeclampsia features in mice. *Proc Natl Acad Sci U.S.A*. (2015) 112:15928–33. doi: 10.1073/pnas.1515076112

115. Basar M, Yen CF, Buchwalder LF, Murk W, Huang SJ, Godlewski K, et al. Preeclampsia-related increase of interleukin-11 expression in human decidual cells. *Reproduction*. (2010) 140:605–12. doi: 10.1530/REP-10-0263

116. Menkhorst E, Santos LL, Zhou W, Yang G, Winship AL, Rainczuk KE, et al. IL11 activates the placental inflammasome to drive preeclampsia. *Front Immunol*. (2023) 14:1175926. doi: 10.3389/fimmu.2023.1175926

117. Wan L, He X, He M, Yu Y, Jiang W, Liang C, et al. Docosahexaenoic acid improves cognition and hippocampal pyroptosis in rats with intrauterine growth restriction. *Heliyon*. (2023) 9:e12920. doi: 10.1016/j.heliyon.2023.e12920

118. Tian K, Yang Y, Zhou K, Deng N, Tian Z, Wu Z, et al. The role of ROS-induced pyroptosis in CVD. *Front Cardiovasc Med*. (2023) 10:1116509. doi: 10.3389/fcvm.2023.1116509

119. Yin Z, Wan B, Gong G, Yin J. ROS: Executioner of regulating cell death in spinal cord injury. *Front Immunol*. (2024) 15:1330678. doi: 10.3389/fimmu.2024.1330678

120. Grilc NK, Sova M, Kristl J. Drug delivery strategies for curcumin and other natural nrf2 modulators of oxidative stress-related diseases. *Pharmaceutics*. (2021) 13:2137. doi: 10.3390/pharmaceutics13122137

121. Suzuki T, Motohashi H, Yamamoto M. Toward clinical application of the Keap1-Nrf2 pathway. *Trends Pharmacol Sci*. (2013) 34:340–6. doi: 10.1016/j.tips.2013.04.005

122. Kweider N, Huppertz B, Wruck CJ, Beckmann R, Rath W, Pufe T, et al. A role for Nrf2 in redox signalling of the invasive extravillous trophoblast in severe early onset IUGR associated with preeclampsia. *PloS One*. (2012) 7:e47055. doi: 10.1371/journal.pone.0047055

123. Chen D, Man LY, Wang YY, Zhu WY, Zhao HM, Li SP, et al. Nrf2 deficiency exacerbated pulmonary pyroptosis in maternal hypoxia-induced intrauterine growth restriction offspring mice. *Reprod Toxicol*. (2024) 129:108671. doi: 10.1016/j.reprotox.2024.108671

124. Kobal BB, Kale İ, Pektaş AM, Ozel A, Muhcu M. Maternal serum gasdermin D concentrations in pregnancies complicated by isolated intrauterine growth restriction. *Z Geburtshilfe Neonatol*. (2023) 227:197–203. doi: 10.1055/a-1967-2274

Glossary

PE	preeclampsia
GSDMD	Gasdermin D
DAMPs	damage-associated molecular patterns
NET	neutrophil extracellular trap
LPS	lipopolysaccharide
NE	neutrophil elastase
IL-1β	interleukin-1β
PJVK	pejvakin
PAMPs	Pathogen-associated molecular patterns
PRRs	pattern recognition receptors
NLRs	Nucleotide-binding oligomerization domain-like receptors
LRR	leucine-rich repeat
NLRP3	NOD-like receptor pyrin-containing 3
IL-18	interleukin-18
ROS	reactive oxygen species
ASC	apoptosis-associated speck-like protein containing
GSDME	gasdermin E
TF	tissue factor
NINJ-1	ninjurin-1
HMGB1	high mobility group box-1
LDH	lactate dehydrogenase 1
SQSTM1	sequestosome
GDM	gestational diabetes mellitus
RM	recurrent miscarriage
NAIP	apoptosis inhibitory protein
NLRC4	CARD domain-containingprotein 4
IECs	intestinal epithelial cells
MHF	high-fat maternal
PEX	peroxisomes
SOD	Superoxide Dismutase
FFA	free fatty acids
PA	palmitic acid
URSA	unexplained spontaneous abortion
NF-κB	necrosis factor kappa B
BaP	Benzo(a)pyrene
BPDE	benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide
RM	recurrent miscarriage
HC	healthy control
ZIKV	Zika virus
CZS	Zika virus syndrome
PROM	premature rupture of membranes
PTB	preterm birth
COX-2	cyclooxygenase-2
IR	insulin resistance
NADPH	triphosphopyridine nucleotide
SPOCD1	spen paralog and direct homolog C-terminal domain containing 1
HUVECs	human umbilical vein endothelial cells
EVT	Extravillous trophoblasts
TMBIM4	transmembrane BAX inhibitor motif containing 4
miRNAs	MicroRNAs
3-UTR	3-untranslated region
PINK1	PTEN-induced putative kinase 1
IUGR	Intrauterine growth restriction
Nrf2	Nuclear factor erythroid-derived 2-related factor 2

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 23 September 2024
DOI 10.3389/fimmu.2024.1440388

Check for updates

OPEN ACCESS

EDITED BY
Abdel Rahim A. Hamad,
Johns Hopkins University, United States

REVIEWED BY
Amy Winship,
Monash University, Australia
Soledad Gori,
Universidad de Buenos Aires, Argentina

*CORRESPONDENCE
Amir-Hassan Zarnani
zarnania@sina.tums.ac.ir;
zarnania@gmail.com
Mahmood Jeddi-Tehrani
mahjed@avicenna.ac.ir;
mahjed@yahoo.com

†These authors have contributed equally to this work

RECEIVED 29 May 2024
ACCEPTED 30 August 2024
PUBLISHED 23 September 2024

CITATION
Zarnani K, Zarnani K, Maslehat-Lay N, Zeynali B, Vafaei S, Shokri M-R, Vanaki N, Soltanghoraee H, Mirzadegan E, Edalatkhah H, Naderi M-M, Sarvari A, Attari F, Jeddi-Tehrani M and Zarnani A-H (2024) *In-utero* transfer of decidualized endometrial stromal cells increases the frequency of regulatory T cells and normalizes the abortion rate in the CBA/J × DBA/2 abortion model. *Front. Immunol.* 15:1440388. doi: 10.3389/fimmu.2024.1440388

COPYRIGHT
© 2024 Zarnani, Zarnani, Maslehat-Lay, Zeynali, Vafaei, Shokri, Vanaki, Soltanghoraee, Mirzadegan, Edalatkhah, Naderi, Sarvari, Attari, Jeddi-Tehrani and Zarnani. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

In-utero transfer of decidualized endometrial stromal cells increases the frequency of regulatory T cells and normalizes the abortion rate in the CBA/J × DBA/2 abortion model

Kayhan Zarnani[1,2,3†], Kimia Zarnani[1,2†], Nasim Maslehat-Lay[2], Bahman Zeynali[4], Sedigheh Vafaei[2], Mohammad-Reza Shokri[3,5], Negar Vanaki[3], Haleh Soltanghoraee[6], Ebrahim Mirzadegan[7], Haleh Edalatkhah[6], Mohammad-Mehdi Naderi[6], Ali Sarvari[6], Farnoosh Attari[8], Mahmood Jeddi-Tehrani[9*] and Amir-Hassan Zarnani[2,3*]

[1]School of Biology, College of Sciences, University of Tehran, Tehran, Iran, [2]Reproductive Immunology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran, [3]Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, [4]Developmental Biology Lab., School of Biology, College of Sciences, University of Tehran, Tehran, Iran, [5]Department of Immunology, H. Lee Moffitt Cancer Center, Tampa, FL, United States, [6]Reproductive Biotechnology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran, [7]Nanobiotechnology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran, [8]Department of Animal Biology, School of Biology, College of Sciences, University of Tehran, Tehran, Iran, [9]Monoclonal Antibody Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran

Introduction: Failure to adequate decidualization leads to adverse pregnancy outcomes including pregnancy loss. Although there are plenty of reports underscoring immune dysfunction as the main cause of abortion in CBA/J females mated with DBA/2 males (CBA/J × DBA/2), little is known about the potential role of impaired endometrial decidualization.

Methods: Endometrial stromal cells (ESCs) from CBA/J mice were *in-vitro* decidualized, and the proteome profile of the secretome was investigated by membrane-based array. CBA/J mice were perfused *In-utero* with either decidualized ESCs (C×D/D), undecidualized ESCs (C×D/ND), or PBS (C×D/P) 12 days before mating with DBA/2 males. Control mice were not manipulated and were mated with male DBA/2 (C×D) or Balb/c (C×B) mice. On day 13.5 of pregnancy, reproductive parameters were measured. *In-vivo* tracking of EdU-labeled ESCs was performed using fluorescence microscopy. The frequency of regulatory T cells (Tregs) in paraaortic/renal and inguinal lymph nodes was measured by flow cytometry. The proliferation of pregnant CBA/J splenocytes in response to stimulation with DBA/2 splenocytes was assessed by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) flow cytometry.

10.3389/fimmu.2024.1440388

Results: In C×D/D mice, the resorption rate was reduced to match that seen in the C×B group. Intrauterine perfused ESCs appeared in uterine stroma after 2 days, which remained there for at least 12 days. There was no difference in the number of implantation sites and embryo weight across all groups. The frequency of Tregs in the inguinal lymph nodes was similar across all groups, but it increased in the paraaortic/renal lymph nodes of C×D/D mice to the level found in C×B mice. No significant changes were observed in the proliferation of splenocytes from pregnant C×D/D compared to those of the C×D group in response to stimulation with DBA/2 splenocytes. Decidualization of ESCs was associated with a profound alteration in ESC secretome exemplified by alteration in proteins involved in extracellular matrix (ECM) remodeling, response to inflammation, senescence, and immune cell trafficking.

Discussion: Our results showed that the deficiency of Tregs is not the primary driver of abortion in the CBA/J × DBA/2 model and provided evidence that impaired endometrial decidualization probably triggers endometrial immune dysfunction and abortion in this model.

KEYWORDS

abortion, CBA/J x DBA/2, decidualization, regulatory T cells, proteome, immune system

Introduction

Pregnancy and reproduction are complex processes and involve different stages that act in a harmonious manner. Most Eutheria use more or less the same stages for reproduction. During this process, different systems such as hormones and the immune system take action in concert with each other to control blastocyst implantation and development. The first step of a successful pregnancy is the implantation of a competent embryo in a receptive endometrium. Impairments in this critical step can cause miscarriage (1). Implantation is associated with considerable modifications in the endometrium and endometrial immune cells. Endometrial remodeling, which is known as decidualization, is associated with extensive structural and functional modification. This process is controlled by ovarian steroid hormones during the menstrual cycle, leading to a transient window of implantation, in which endometrial luminal epithelium adheres to embryonic trophectoderm (2). Decidualization results from a complex interplay of transcription factors, chemokines, growth factors, and their associated binding proteins, morphogens, cytokines, cell cycle regulators, and signaling pathways (3).

Compared with the original endometrial stromal cells, fully differentiated decidual cells have anti-inflammatory capacity and resist stress signals mediated by endometrial remodeling following embryo implantation (4, 5). Decidualized endometrial stromal cells surround the implanting embryo (6, 7) and form a cellular matrix around the fetus to protect it against maternal immune responses (8).

Human decidualization initiates at the beginning of a secretory phase of the menstrual cycle in response to steroid hormones, while decidualization in mouse endometrium commences after embryo implantation (9). The window of implantation also coincides with the accumulation of innate immune cells in the endometrium, primarily uterine natural killer (uNK) (4, 5) highlighting a link between decidualization and endometrial immune cell adaptation, a process which is needed for the establishment of maternal tolerance for a successful pregnancy.

There are plenty of reports from preclinical animal models suggesting that an impaired decidual response is associated with pregnancy loss (10, 11). However, the precise mechanisms remain to be defined. Loss of a conspicuous epigenetic stem cell signature has been demonstrated in endometrial stromal cells from endometrial biopsies of women with recurrent pregnancy loss (12) leading to a failure of endometrium plasticity to accommodate pregnancy. Decidualization also triggers uNK to eliminate senescent decidual cells (5, 13), which could otherwise induce pregnancy loss through the induction of senescence-associated secretory phenotype (SASP) in early pregnancy.

Immune cells residing in the endometrium comprise ~40% of all cells in first-trimester decidua (14–16) and undergo extensive modulation before the establishment of pregnancy and after implantation to ensure maternal tolerance to the fetal antigens (15). The eminent role of immune tolerance during pregnancy has been the basis of decades of research into immune-related basis for recurrent pregnancy loss (15). To address the classical immune theory of maternal tolerance to non-self fetus, the CBA/J × DBA/2 abortion model arose decades ago to delineate adverse pregnancy outcomes (resorption) in the face of deficiency of immune tolerance to fetal antigens (17). The value of mean resorptions (abortions)/total number of implantations

(R/T) for CBA/J × DBA/2 and CBA/J × Balb/c matings is approximately 33.32% and 5.11%, respectively (18). In CBA/J × DBA/2 mating, there is a loss of cellular contact between decidual cells and cells of the ectoplacental cone beginning approximately day 7 of gestation leading to embryonic loss by days 10–12 (17).

A number of mechanisms of immune dysregulation have been described in the CBA/J × DBA/2 model to mainly strengthen the classical theory for which this abortion model first drove (17). In this context, various immunotherapeutic approaches for the prevention of abortion in this model have also been tested, some with successful results. The most intriguing experiments suggesting immune dysfunction fingerprint in the CBA/J × DBA/2 abortion model are those focused on the suboptimal generation of regulatory T cells (Tregs). These cells are produced during pregnancy under the action of ovarian steroid hormones (19). Administration of induced Tregs or $CD4^+CD25^+$ spleen and thymus cells isolated from Balb/c-mated CBA/J female normal pregnant mice to a DBA/2-mated CBA/J female in early pregnancy decreased the resorption rate (20–22), while depletion of Tregs with anti-CD25 antibody administration (23) increased the resorption rate (21) and abolished the protective effect of Treg administration.

Although all these studies turn around the original putative role of maternal immune tolerance in the establishment of a successful pregnancy, it is still unclear how and to what extent the endometrium and specifically decidualization contribute to uterine immune regulation during pregnancy.

Early (24) and later studies (25) suggest that abnormalities existing in the CBA/J decidua and its interaction with the developing embryo are the prime movers of the pathology related to these pregnancies, and this idea is supported by the fact that "reverse" mating between DBA/2 females and CBA/J males are considered normal (26). Indeed, this mating combination suffers from a fundamental disorganization of DNA methylation (25). Collectively, these studies assume that the impaired decidual reaction could be viewed as one potential cause of abortion in this mouse model. We recently provided compelling evidence showing that human endometrial stromal cells modulate the functional features of NK (27), Treg (28), and TH17 (29, 30) cells in a pregnancy-friendly manner. Indeed, decidualized endometrial stromal cells exert potent immunomodulatory capacity (30). Based on this information, we hypothesized that *in-utero* perfusion of decidualized ESCs could rescue pregnancy loss and normalize immune dysfunction in this mouse model. Our results showed that *in-utero* perfusion of *in-vitro* decidualized, but not undecidualized, ESCs could normalize the resorption rate in DBA2-mated CBAJ female mice and upregulate Tregs in uterus-draining lymph nodes. Our results suggest that impaired decidual reaction in female CBA/J mice following mating with DBA/2 males is the main driver of immune dysregulation and abortion in this model.

Materials and methods

Animals

Mice ranging from 8 to 12 weeks of age (female CBA/J, male DBA/2, and male Balb/c) were obtained from Pasteur Institute, Tehran, Iran. All experimental procedures done in this study were approved by the Ethics Committee of Animal Experiments of Avicenna Research Institute (approval and grant no. IR.ACECR.AVICENNA.REC.1397.020).

Isolation of endometrial stromal cells

Isolation of mouse endometrial stromal cells was performed according to the protocol published elsewhere with some minor modifications (31). To induce estrus in non-pregnant CBA/J female mice, a subcutaneous injection of 100 µL of 17β-estradiol (E2) (Sigma, USA) solution (100 ng/100 µL) was administered for three consecutive days. The stage of the estrous cycle was determined in female CBA/J mice by conducting wet smear preparation of vaginal secretion and Papanicolaou staining (32). In some instances, uterine horn sections were prepared and stained with H&E to confirm the estrous phase. Following euthanasia, the uterine horns were carefully dissected, removing adipose and connective tissue, and washed multiple times with Ca^{2+} Mg^{2+}-free Hanks' Balanced Salt Solution (HBSS). The uterine horns were then opened longitudinally and dissected into small pieces. These pieces underwent digestion for 1 h at 37°C with 3 mL of digestion buffer (0.075 mg/mL of pancreatin and 2.5 mg/mL of trypsin). The supernatants were discarded, and the remaining tissues were washed with HBSS before undergoing a second digestion for 30 min in 3 mL of 0.5 mg/mL collagenase. The resulting cell suspension was passed through a 70-µm nylon mesh, and the collected cells were washed with HBSS plus 10% fetal bovine serum (FBS) and then resuspended in a complete DMEM medium. After 24 h, non-adherent cells were removed, and stromal cells were allowed to propagate until reaching 75% confluency. A total of 15 mice were used for the isolation of an adequate number of endometrial stromal cells needed to perform all experiments yielding an average of approximately 8×10^6 ESC/mouse.

Immunofluorescent staining

Cultured cells were detached through trypsinization and applied to adhesive slides. After air drying for 30 min, the cells were fixed with ice-cold acetone for 2 min and washed in PBS three times. Slides underwent blocking with 5% sheep serum for 30 min at 37°C, followed by incubation with mouse anti-vimentin (Santa Cruz, USA) at a concentration of 5 µg/mL at room temperature for 1 h. Following three washes in PBS-1.5% BSA, sheep anti-mouse-FITC (Sina Biotech, Iran) was added and incubated for 45 min. Nuclei were stained with DAPI (2 µg/mL) for 2 min. After three washes in PBS, the signals were detected using an epifluorescence microscope. Intracellular cytokeratin was stained with the recommended dilution of anti-cytokeratin-FITC antibody (BD Biosciences, USA). As a positive control for cytokeratin staining, human amniotic epithelial cells (hAECs) were isolated from a human amniotic membrane following a described protocol (33) and stained as mentioned above.

In-vitro decidualization

Induction of *in-vitro* decidualization was performed according to the protocol published elsewhere (31). For *in-vitro* decidualization (IVD), decidualization was induced in phenol red-free DMEM/F12 supplemented with 5% charcoal-stripped (CS)-FBS. After an overnight rest incubation period in a CO_2 incubator, the medium was changed to phenol red-free DMEM/F12 supplemented with 2% CS-FBS and 0.5 mM of 8-Br-cAMP and 1 µM of medroxyprogesterone acetate (MPA) (both from Sigma), and the culture continued for an additional 6 days. On day 3, the medium was semi-changed. Control culture wells received the same culture medium, excluding 8-Br-cAMP and MPA. At the end of the culture period, cell culture supernatants and cells were collected for proteome and RNA expression analyses, respectively.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was conducted on total RNA extracted from cell homogenates using an RNA extraction kit according to the manufacturer's instructions (ROJE Technologies, Iran). RNA quantification and integrity were assessed using a NanoDrop and agarose gel electrophoresis, respectively. cDNA was synthesized using a kit (ROJE Technologies). For each specimen, three PCR reactions were set up using primers for progesterone receptor (Pgr) (F: CTCCGGGACCGAACAGAGT, R: ACAACAA CCCTTTGGTAGCAG), prolactin (Prl) (F: AGCCAGAAATCACTG CCACTCT, R: CAGGAGTGATCCATGCACCCATA), and β2-microglobulin (β2m) (F: ACTGACCGGCCTGTATGCTA, R: AATGTGAGGCGGGTGGTAC) as housekeeping genes. Real-time qRT-PCR kit from Takara (Japan) was used for gene amplification. The thermal profile of the Rotor-Gene thermal cycler included an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C (5 s), annealing at 60°C (30 s), and extension at 72°C (20 s) with a final extension at 72°C for 5 min. Primers for Pgr and Prl were designated to amplify 122-bp and 125-bp fragments, respectively. The third set of primers amplified a 124-bp segment of the β2m gene as internal control.

Secretome protein profiling

The protein profile of endometrial stromal cells was analyzed using a mouse XL cytokine array kit, following the manufacturer's instructions (R&D Systems, USA). Each membrane contained captured antibodies against 111 cytokines, chemokines, growth factors, and other proteins. The pool of three supernatants from cultured *in-vitro* decidualized and undecidualized CBA/J endometrial cells was applied to the membranes in two independent preparations for each condition, and spots were developed using a biotin-streptavidin-enhanced chemiluminescence (ECL) system. Signal intensities were quantified using AlphaEase software. Integrated densities of each spot were adjusted for background signal and normalized to positive control spots on each membrane, and the resulting figures were plotted in GraphPad Prism version 9.2.

Bioinformatics analyses

All analyses were performed with R software (version 4.3.3). Before each analysis, quantified proteins were normalized and auto-scaled. For pathway enrichment, the Clusterprofiler package version 4.10.1 and ReactomePA package version 1.46.0 were used, and for obtaining GSVA scores, the GSVA package version 1.50.1 was used. The Annotationhub package version 3.10.0 was used to annotate mouse protein IDs.

Adoptive transfer of endometrial stromal cells

For the adoptive transfer of endometrial stromal cells, five experimental groups, each consisting of five mice, were employed. In the C×D/D group, female CBA/J mice underwent surgical *in-utero* perfusion of decidualized ESCs, while mice in the C×D/ND and C×D/P groups received undecidualized ESCs or PBS, respectively, before mating with DBA/2 males. Two groups of female CBA/J mice were not manipulated and were mated with either male DBA/2 (C×D) (abortion-prone group) or Balb/c mice (C×B) (non-abortion group). After confirming the estrous stage, mice were intraperitoneally injected with 10% ketamine (50 mg/kg) and xylazine 2% (5 mg/kg). Mice were placed on their abdomen, and after shaving back hair and disinfection, a surgical incision was made approximately 1.5 cm long and 1 cm away from the spine and parallel to it in the middle of the abdomen. Intrauterine perfusion of endometrial stromal cells was performed according to the protocol we published elsewhere (34). Accordingly, 5×10^5 primary culture ESCs from non-pregnant CBA/J mice (passages 2–4) suspended in 10 µL PBS was loaded in a glass micropipette (Straight, Grinded- with spike; i.d. 100 µm) attached to an Eppendorf Cell Tram® Vario micromanipulation pump and injected into the top of the right uterine horn at the utero-tubal junction (UTJ), with the aid of a ×10 stereomicroscope (Nikon, SMZ 800N). For the evaluation of leakage and tracing of the medium flow in the uterus, two mice were perfused as above with 10 µL of trypan blue solution (Supplementary Figure S1). Mice were then treated with tramadol (10–12 mg/kg) and gentamicin at a dose of 10 mg/kg subcutaneously for 3 days after surgery. After a 12-day recovery period, female mice were placed in the cages of male mice, and the day of vaginal plug detection was considered day 0.5 of pregnancy. On day 13.5 of pregnancy, mice in each group were sacrificed, and pregnancy parameters, including the number of implantation sites, number of live embryos, abortion percentage, and fetal weight, were assessed. Indeed, the morphology of the implantation sites in all groups was assessed by periodic acid-Schiff (PAS) and H&E stainings.

In-vivo tracking of intrauterine-perfused mesenchymal stem cells

Endometrial stromal cells were isolated from the non-pregnant CBA/J females as above and cultured in 75-cm flasks to reach 80% confluency. The cells were then labeled with 10 µm of EdU for 24 h

according to the manufacturer's instruction (Click-iT® EdU Imaging Kit, Invitrogen, USA). EdU-labeled cells were harvested by using a solution of trypsin–EDTA and washed in PBS, and then approximately 5×10^5 labeled cells in a total volume of 10 µL were perfused in the right horn of the uterus of three CBA/J female mice. The upper one-third of the right and left uterine horns were then collected after 2, 5, and 12 days post-perfusion for histological examination. Tissues were then processed as described elsewhere (35). In brief, tissues were fixed in a cold solution containing 2% formaldehyde and 0.002% picric acid in a 0.1 M phosphate buffer (pH 7.2) for 4 h. They were then immersed overnight in 30% sucrose prepared in 0.1 M of phosphate buffer. The specimens were embedded in Killik OCT compound embedding medium (Bio-Optica, Milan, Italy) and stored at −80°C until needed. The fixed frozen tissue specimens were sectioned at a thickness of 5 µm, mounted onto Polysine-coated slides (Hangzhou, China), and air-dried for 1 h. Following PBS rinses and tissue permeabilization with 0.5% Triton X-100, the tissues were washed and underwent EdU staining according to the manufacturer's protocol. The sections were treated with a freshly prepared Click-iT reaction cocktail for 30 min at room temperature in the dark, followed by staining with Hoechst 33342 for nuclear staining. Slides were then mounted with 50:50 PBS–glycerol, and images were taken with an Olympus BX51 fluorescent microscope (Olympus, Tokyo, Japan).

Quantification of regulatory T cells

On day 13.5 of pregnancy, the inguinal, renal, and paraaortic lymph nodes of pregnant CBA/J mice were extracted, and the frequency of Tregs in the mononuclear fraction of the lymph nodes was determined using a mouse regulatory T-cell kit from Thermo Fisher Scientific, USA. Due to the small size of paraaortic and renal lymph nodes, cell suspensions from these nodes were combined. To block FC receptors, cells were initially incubated with antibodies against CD16/32 for 15 min at room temperature. Subsequently, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and phycoerythrin (PE)-conjugated anti-mouse CD25, followed by a 30-min incubation in the dark. The lymphocytes were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set and stained with allophycocyanin (APC)-conjugated anti-mouse/rat Foxp3 antibody. Lymphocytes were gated based on forward and side scatter parameters, and stained cells were analyzed using flow cytometry (PAS System; Partec) with data interpretation performed using FlowJo, version 10 software.

One-way lymphocyte transformation assay

For the one-way lymphocyte transformation assay, pregnant CBA/J spleens were collected on day 13.5 of pregnancy, along with the spleens from male DBA/2 mice. These spleens were dispersed into a single-cell suspension through 70-µm meshes (BD Biosciences, Germany), and splenocytes were isolated using Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Sweden). Splenocytes from DBA/2 male mice were inactivated with mitomycin C. Pregnant female CBA/J splenocytes were labeled with 5 µM of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, USA) before co-culture with male DBA/2 splenocytes. 2.5×10^5 pregnant female CBA/J splenocytes were co-cultured with an equivalent number of male DBA/2 splenocytes in a final volume of 200 µL in 96-well plates. Control wells contained CBA/J splenocytes without cells from the male partner. Cultures were maintained for 4 days, and proliferation was assessed using flow cytometry.

Statistical analysis

Animals were allocated to different treatment conditions through random assignment. Results were expressed as means ± standard deviation (SD). Statistical significance was assessed using various methods: the Mann–Whitney test for non-parametric data and one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test for comparisons involving three or more groups. A significance threshold of $p < 0.05$ was adopted. All statistical analyses were conducted utilizing Prism 9.2 software.

Results

Isolation of ESCs and induction of *in-vitro* decidualization

The estrus phase was confirmed by a combination of vaginal wet smear examination and histologic evaluation of uterine horn sections. Vaginal cytology revealed the predominance of superficial epithelial cells appearing in some areas as epithelial sheets (Figures 1A, B), and histologic examination showed stromal cell proliferation, stromal congestion, and uterine gland proliferation (Figure 1C). ESCs were isolated from uterine horns and cultured (Figure 1D). Vimentin and cytokeratin immunofluorescence staining was performed to assess the purity of isolated ESCs, and our results indicated that stromal cells express vimentin but failed to express cytokeratin with purity of approximately 95%. As a positive control for cytokeratin staining, hAECs were stained with the same antibody (Figure 1E). Cultured ESCs were *in-vitro* decidualized using a cocktail of cAMP and MPA for 6 days. Decidualization was characterized by the differentiation of ESCs from elongated fibroblast-like endometrial stromal cells into more rounded cells with enlarged cell size, larger nuclei, and abundant cytoplasm (Figure 1F). Prl and Pgr mRNA levels were assessed in the in-vitro decidualized ESCs by qRT-PCR as an indication for decidualization. Compared to undecidualized ESCs, the expression of prl and pr mRNA was significantly increased in decidualized ESCs (Figure 1G).

Decidualization is associated with an altered secretome profile of ESCs

We then proceeded to proteome profiling after a 6-day period of cAMP- and MPA-induced decidualization on CBA/J endometrial

stromal cells. Principal component analysis (PCA) showed a clear separation of proteins differentially regulated between decidualized and undecidualized endometrial cells (Figure 2A). Secretion of various chemokines and cytokines from both the decidua cells and the embryo is crucial for immune cell recruitment and activation of multiple signaling networks. So, we comparatively analyzed the secreted proteome profile of the decidualized and undecidualized ESCs using a membrane protein array system. We identified a totally different pattern of protein expression in decidualized versus undecidualized ESCs leading to two segregated clusters (Figure 2B). Chemokine production by ESCs was considerably altered during decidualization exemplified by significantly higher expression of chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 1 (CXCL1), and CXCL2. Of note, secretion of CCL11 increased several folds in response to decidualization (Figure 2C). Decidualization was associated with a profound increase in IL-6 production (Figure 3A). The expression of various growth factors and their receptors in the uterus is important for the implantation process. So, we examined the secretion of various growth factors and growth factor binding proteins in decidualized endometrial cells. We found that decidualization caused an increased expression of granulocyte colony-stimulating factor (G-CSF), M-CSF, insulin-like growth factor binding protein 2 (IGFBP-2), IGFBP-3, IGFBP-5, and IGFBP-6 (Figure 3B). The expression of other proteins involved in different regulatory pathways including proteins involved in tissue remodeling and wound healing, hemostasis, coagulation, extracellular matrix organization, angiogenesis, and inflammatory response was also altered upon ESC decidualization. Decidualization caused a significant increase in the secretion of serpin E1, serpin F1, proprotein convertase 9 (PCSK9), periostin, pentraxin 3 (PTX3), MMP2, cystatin C, chemerin, and low-density lipoprotein receptor (LDL-R). This process is also associated with a decrease of tissue factor (TF), endostatin, intercellular adhesion molecule 1 (ICAM1), retinol-binding protein 4 (RBP4), MMP9, B-cell activating factor (BAFF), endoglin, and osteopontin (OPN) (Figures 3C, D).

Decidualized ESCs display an inflammatory and senescence-associated protein signature

GSVA analysis of differentially expressed proteins pointed to several pathways related to decidualization. We identified higher expression of several proteins involved in inflammatory responses including IL-6, G-CSF, M-CSF, LDL-R, PTX3, SERPIN E1, CCL2, CCL11, and CXCL2 (Figures 2C, 3) (36, 37) upon ESC decidualization. Notably, decidualization was associated with the downregulation of the IL-1 pathway (Figure 4A) suggesting a mixture of inflammatory and anti-inflammatory responses upon ESC decidualization. The cyclooxygenase 2 (COX-2)-induced

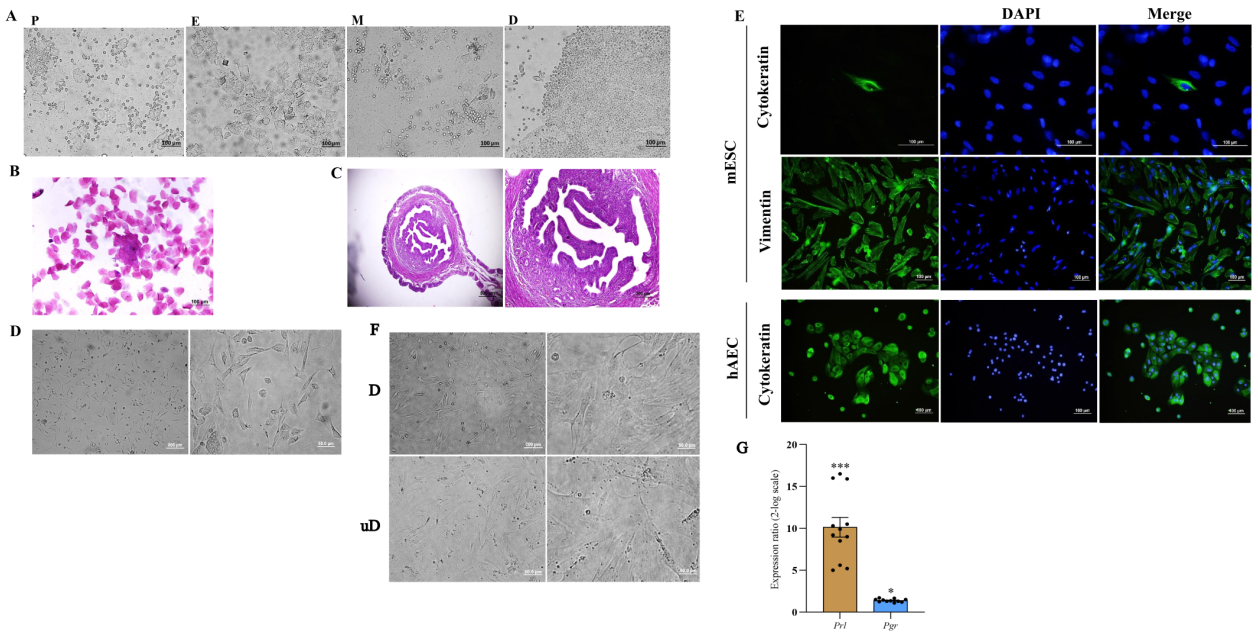


FIGURE 1
Isolation of mouse endometrial stromal cells, characterization, and *in-vitro* decidualization. The phases of the estrous cycle were determined by inspection of vaginal wet smear **(A)**, Papanicolaou staining of vaginal cells **(B)**, and histologic evaluation of uterine tissue **(C)**. Endometrial stromal cells were isolated from the uterine horn of non-pregnant CBA/J female mice and cultured **(D)**. Expression of vimentin and cytokeratin was assessed by immunofluorescence staining of isolated cells. Human amniotic epithelial cells were used as a positive control for cytokeratin staining **(E)**. *In-vitro* decidualized endometrial stromal cells transformed to a distinct morphology of rounded cells with enlarged cell size, larger nuclei, and abundant cytoplasm **(F)** and expressed Prl and Pgr mRNA **(G)**. In **(A)** P, proestrus; E, estrus; M, metestrus; D, diestrus. In **(F)** D, decidualized; unD, undecidualized. In **(E)**: mESC, mouse endometrial stromal cell; hAEC, human amniotic epithelial cell. *p < 0.05, ***p < 0.001.

10.3389/fimmu.2024.1440388

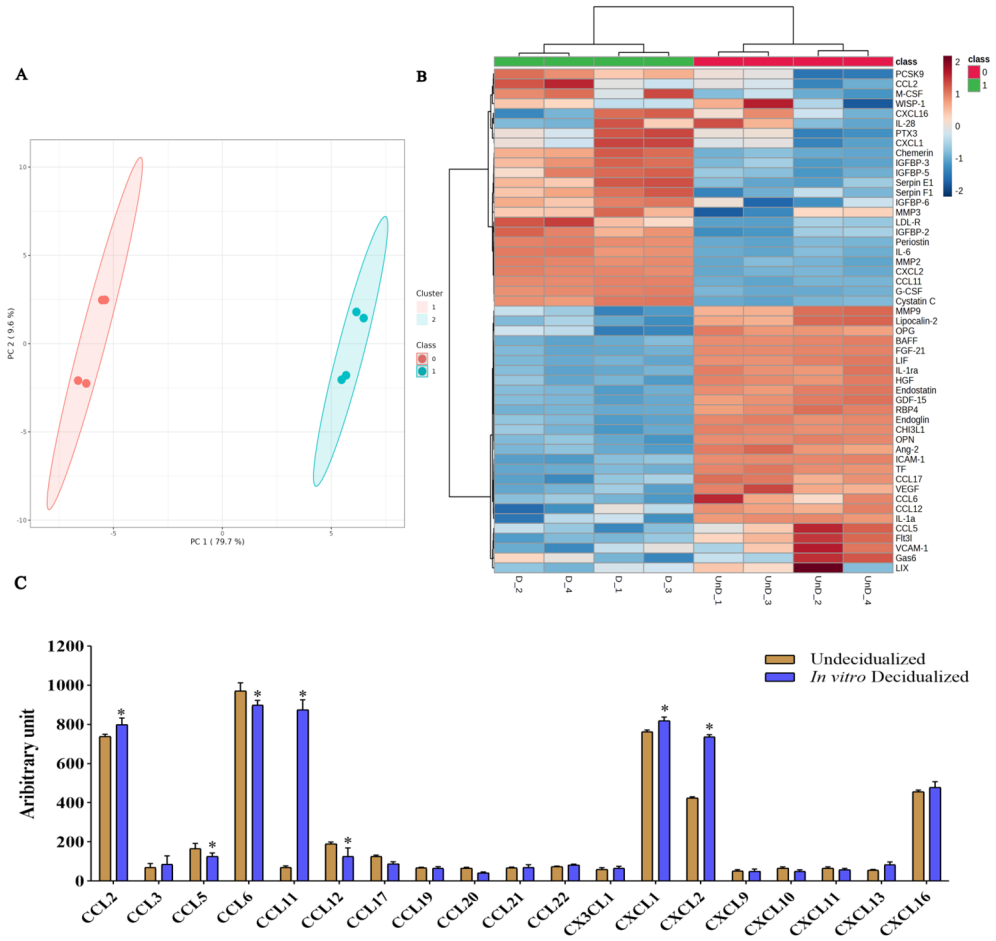


FIGURE 2
Secretome protein profiling of *in-vitro* decidualized endometrial stromal cells. Endometrial stromal cells of female CBA/J mice were *in-vitro* decidualized for 6 days, and the proteome profile of the secretome in decidualized and undecidualized cells was assessed by membrane array (n = 4). PCA showed a clear separation of proteins differentially regulated between decidualized and undecidualized endometrial cells **(A)**. A totally different pattern of protein expression in decidualized versus undecidualized ESCs was identified leading to two segregated clusters **(B)**. Production of different chemokines **(C)** was analyzed in the secretome of decidualized and undecidualized endometrial cells. *p < 0.05.

prostaglandin E2 (PGE2) is actively involved in the induction of decidualization through the cAMP signaling pathway (38). Prostaglandins are active lipid molecules that are shown to have a great impact on cellular senescence (39). By focusing on senescence-associated secretory proteins including IL-6, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, and serpin E1, we confirmed that 6-day decidualization induces a secretome of active senescence in ESCs (Figure 3). Heat map analysis of wound healing, prostaglandin signaling, and extracellular matrix (ECM) remodeling proteins clearly showed differential expression of related proteins in decidualized and undecidualized ESCs (Figure 4B).

Adoptive *In-utero* transfer of ESCs did not affect the number of embryos or implantation sites

Examination of the number of implantation sites showed that mice in C×D, C×D/P, C×D/ND, C×D/D, and C×B groups had an average of 10, 12.8, 10.2, 10.8, and 9.25 embryos per mouse, respectively, with no statistical difference (Figure 5A). To assess whether the transfer of cells to the right uterine horn could differentially affect a number of embryos in this horn, the number of embryos in the right and left uterine horns was counted separately. The results showed that the number of embryos in the right horn of the five groups (mean: 4.6, 5.6, 5.6, 6.4, and 4.75) and in the left uterine horn (mean: 5.4, 3.8, 4.75, and 4.75) did not show a statistically intergroup or intragroup significant difference (Figure 5B). Nonetheless, the mean fetal weight in groups C×D/P, C×D/ND, and C×D/D (0.183 g, 0.213 g, and 0.194 g) who underwent surgical procedure was found significantly lower compared to the other two control groups: C×D and C×B (mean: 0.287 g and 0.258 g, respectively) (Figure 5C). To give insight into the morphology of the implantation sites, PAS and H&E stainings were performed on the cross sections prepared from implantation units (decidua and placenta). PAS-reactive uterine NK cells were present in the decidua and metrial glands of all groups (Supplementary Figure S2).

10.3389/fimmu.2024.1440388

Adoptive *In-utero* transfer of decidualized ESCs normalized the abortion rate in CBA/J-DBA2 mating

To assess the potential effect of *in-utero* transfer of decidualized ESCs on the correction of the abortion rate, the percentage of resorption was assessed in the five groups. The mean abortion rate in the C×D, C×D/P, C×D/ND, C×D/D, and C×B groups was 20%, 16.72%, 21.44%, 8.3%, and 2.8%, respectively. The transfer of undecidualized ESCs (C×D/P) or PBS (C×D/ND) did not affect the resorption rate in DBA2-mated female CBA/J mice compared to the C×D group, while the C×D/D group showed a statistically comparable level of resorption rate with that of the C×B group (Figures 5D, E).

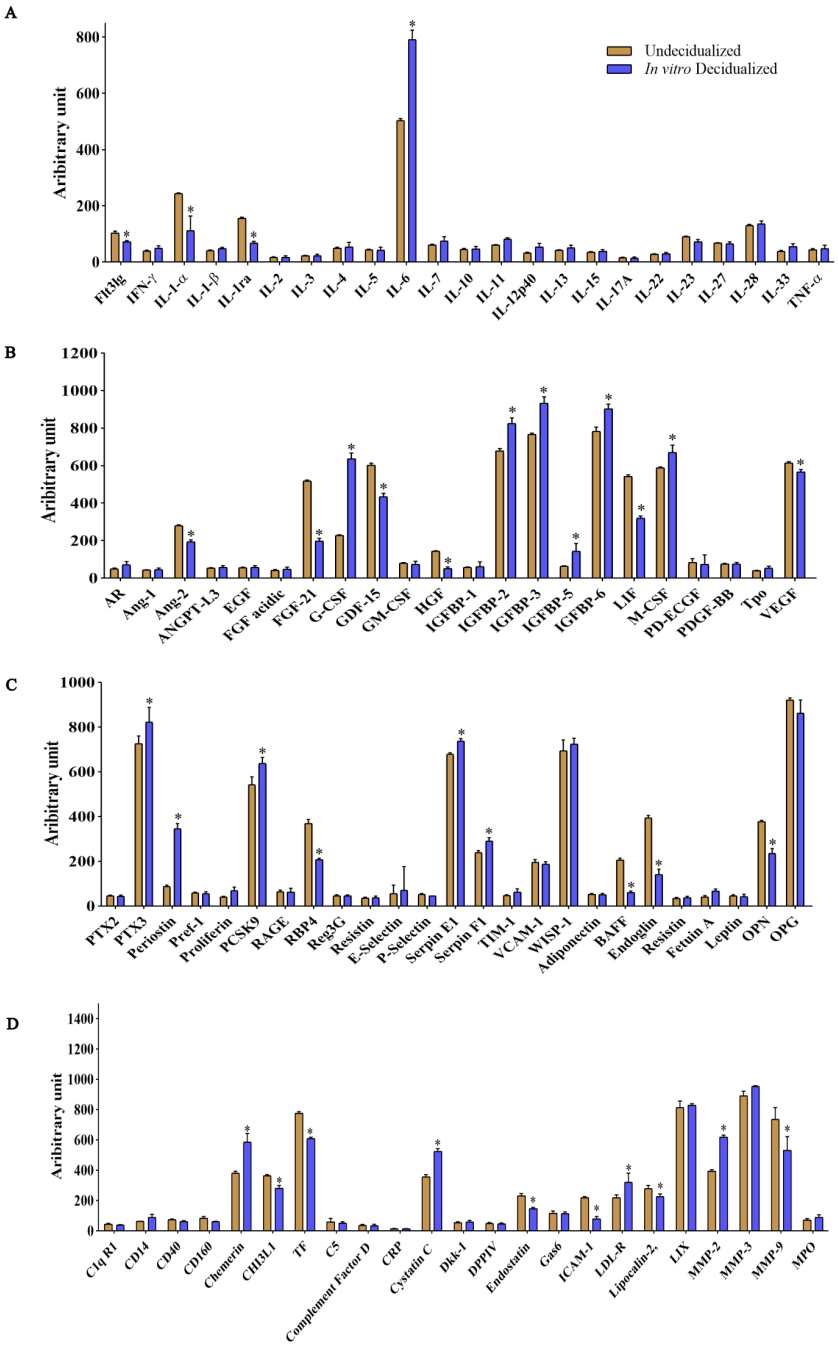


FIGURE 3
Altered expression of inflammatory cytokines, growth factors, and proteins involved in tissue remodeling and senescence in *in-vitro* decidualized endometrial stromal cells. Endometrial stromal cells of female CBA/J mice were *in-vitro* decidualized for 6 days, and the production of cytokines **(A)**, growth factors and growth factor binding proteins **(B)**, and proteins involved in different regulatory pathways including proteins involved in tissue remodeling and wound healing, hemostasis, coagulation, extracellular matrix organization, angiogenesis, inflammatory response, and senescence **(C, D)** was assessed (n = 4). *p < 0.05.

10.3389/fimmu.2024.1440388

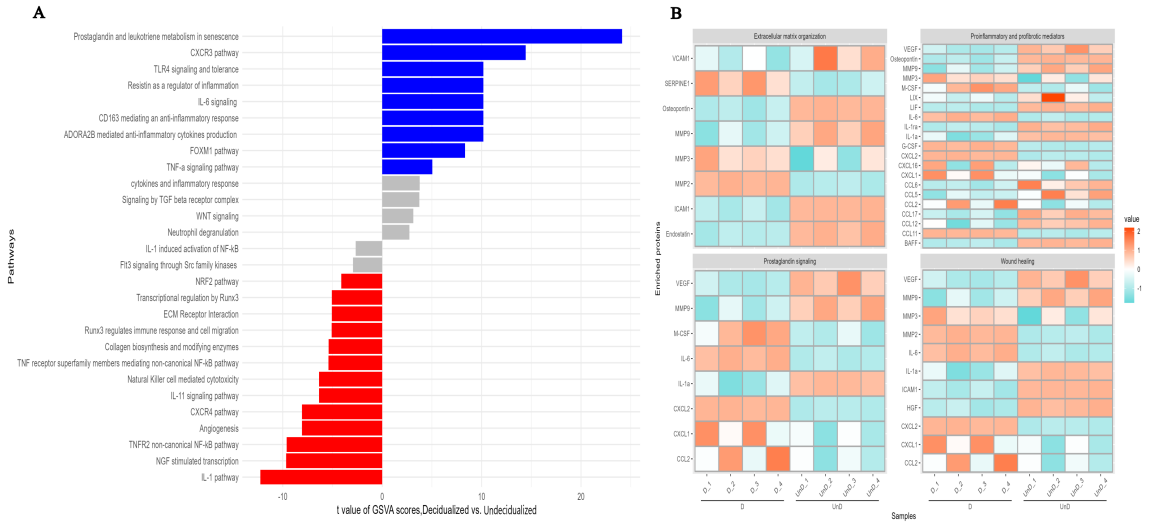


FIGURE 4
GSVA analysis of differentially expressed proteins in *in-vitro* decidualized endometrial stromal cells. Decidualization of mouse endometrial stromal cells is associated with profound changes in different signaling pathways **(A)**, extracellular organization, prostaglandin signaling, wound healing, and proinflammatory and profibrotic mediators **(B)**.

Intrauterine perfusion of ESCs resulted in their repopulation in the uterine stroma

EdU-labeled ESCs were perfused to the lumen of the right horn and tracked by Alexa-594 staining on days 2, 5, and 12 post-perfusion. The results showed that perfused ESCs were able to survive for at least 12 days (Figure 6). We did not observe a decline in the fluorescent signal of the labeled cells over the 12 days of study. Interestingly, besides the right horn of the uterus, where EDU-labeled ESCs were perfused, these cells could also be tracked in the left horn of the uterus with a lower frequency indicating dynamic traffic of the perfused cells in the uterus. Contrary to expectation, the fluorescent signal of Alexa-594 was not always confined to the cell nucleus, and cytoplasmic labeling was also observed indicating background staining. This issue had been reported earlier with the Click-iT technique without any known reason (40).

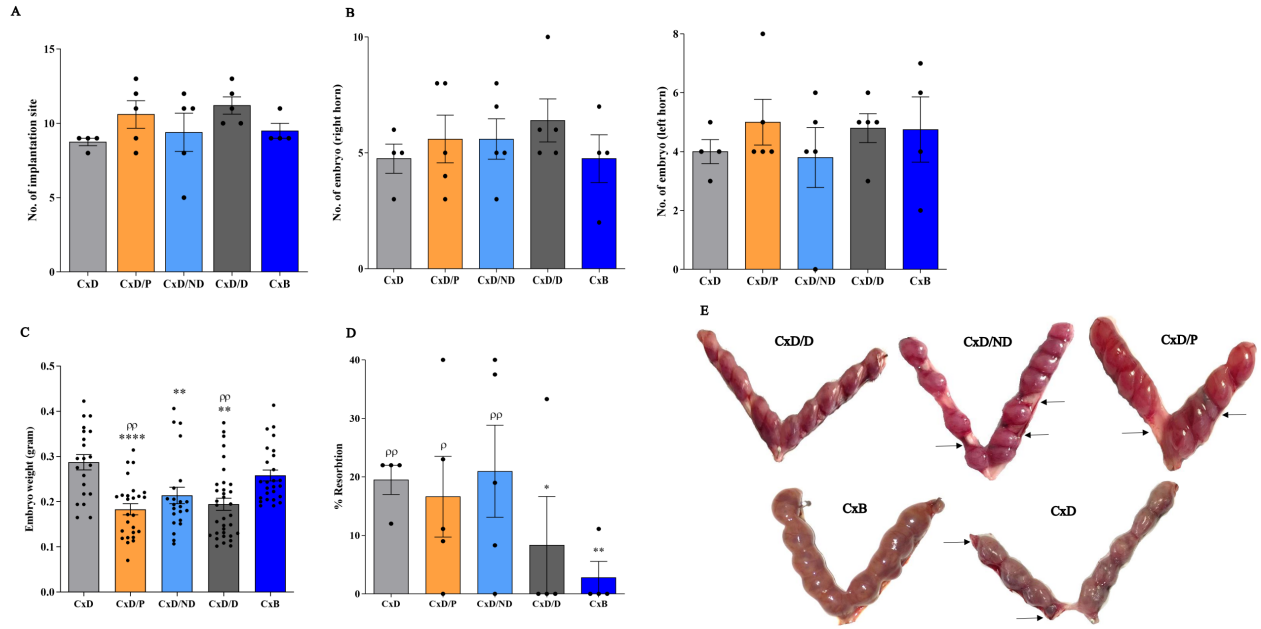


FIGURE 5
Reproduction parameters in different experimental and control groups. Five experimental groups, each consisting of four to five mice, were employed. In the CxD/D group, female CBA/J mice underwent surgical *in-utero* perfusion of decidualized ESCs, while mice in the CxD/ND and CxD/P groups received undecidualized ESCs or PBS, respectively, before mating with DBA/2 males. Two groups of female CBA/J mice were not manipulated and were mated with either male DBA/2 (CxD) or Balb/c mice (CxB). Reproduction parameters were then assessed on day 13.5 of pregnancy including the number of implantation sites **(A)**, number of embryos in the left and right horns **(B)**, embryo weight **(C)**, and resorption rate **(D, E)**. *compared to CxD, $^{\rho}$compared to CxB, *p < 0.05, **p < 0.01, ****p < 0.0001, $^{\rho}$p < 0.05, $^{\rho\rho}$p < 0.01.

10.3389/fimmu.2024.1440388

In-utero transfer of decidualized but not undecidualized ESCs increased the frequency of Tregs in paraaortic and renal lymph nodes

The gating strategy of Tregs is provided in Figure 7A. The percentage of $CD4^+CD25^+FOXP3^-$, $CD4^+FOXP3^+CD25^-$, and $CD4^+CD25^+FOXP3^+$ cells was analyzed separately in five groups by flow cytometry in inguinal and paraaortic/renal lymph nodes. In inguinal lymph nodes, the percentage of $CD4^+CD25^+FOXP3^-$ cells in C×D/ND and C×D/D mice was significantly lower than in the C×D group. The percentage of $CD4^+FOXP3^+CD25^-$ cells in the C×D/D and C×B groups was higher than in the other groups except group C×D. No significant differences were observed between the

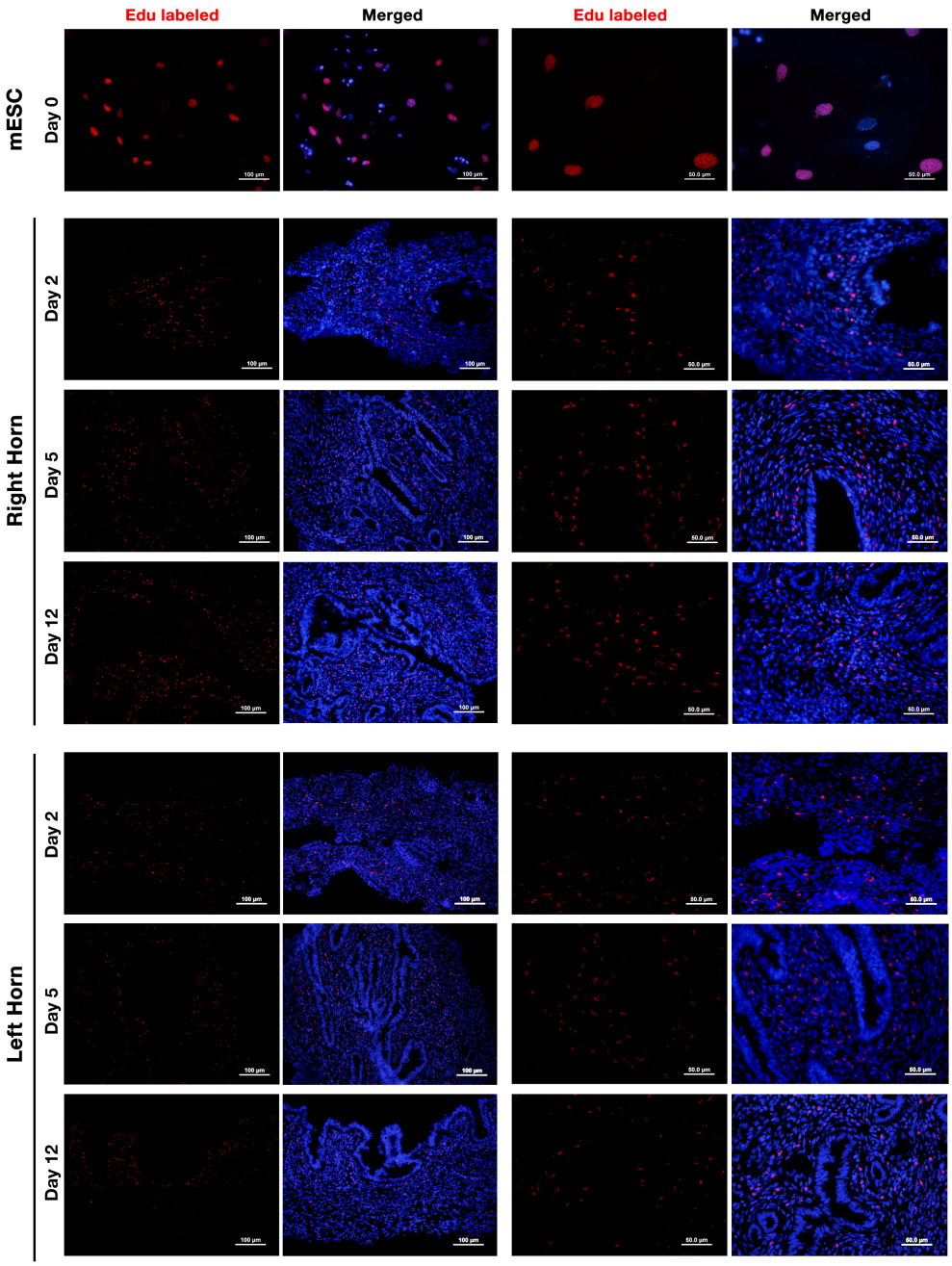


FIGURE 6
Tracking of intrauterine perfused ESCs. Endometrial stromal cells were isolated from non-pregnant CBA/J females and labeled with 10 µm of EdU for 24 h (upper panel). EdU-labeled cells were collected, and 5×10^5 labeled cells were perfused in the right horn of the uterus of three CBA/J female mice. EDU-labeled ESCs were then tracked in the upper one-third of the right (middle panel) and left (lower panel) uterine horns after 2, 5, and 12 days post-perfusion by Click-iT reaction fluorescent staining followed by staining with Hoechst 33342 for nuclear staining.

10.3389/fimmu.2024.1440388

five groups in terms of the percentage of $CD4^+CD25^+FOXP3^+$ regulatory T cells in the inguinal lymph node (Figure 7B). In paraaortic/renal lymph nodes, the percentage of $CD4^+FOXP3^+CD25^-$ cells in the C×D/D and C×B groups was significantly higher compared to the C×B/ND group, but no statistical difference was observed between the C×D/D and C×B groups. Interestingly, the percentage of $CD4^+CD25^+FOXP3^+$ regulatory T cells in C×D/D was increased to the level observed in the C×B group and showed a statistically higher level compared to the other three groups (Figure 7C).

In-utero transfer of ESCs did not affect the proliferative response of CBA/j splenocytes to male DBA2 splenocytes

To assess whether the immunoregulatory effects of decidualized ESCs also exerted at the systemic level, female CBA/J splenocytes on day 13.5 of pregnancy were isolated and stimulated with equal numbers of mitomycin-treated male DBA/2 splenocytes, and the proliferation rate of female CBA/J mice splenocytes was measured by CFSE flow cytometry. Data analysis did not show a significant difference in splenocyte proliferation of CBA/J mice in the C×D/ND and C×D/D groups compared to the C×D group (Figure 7D).

Discussion

Despite many reports underscoring the role of immune dysregulation in inducing abortion in the CBA/J × DBA/2 model, there is strong evidence that immune dysregulation alone cannot explain abortion in this mouse model. First, mating of CBA/J females with Balb/c males, which have similar MHC haplotypes with DBA/2 mice, is considered normal. Second, reverse mating of DBA/2 females with CBA/J males is not associated with abortion (26). Third, the abortion protective effects of pre-pregnancy immunotherapy of CBA/J female mice with Balb/c splenocytes before mating with DBA/2 mice are temporary and last up to 6 weeks (41). Fourth, an organism cannot have a collection of immunological disorders but experience no adverse consequences except in pregnancy. Finally, looking at various immune dysfunctions in this mating model is like the story of Rumi's elephant; many of the components of the immune system reported to be defective in this model are closely related to each other, and dysfunction of one component will eventually lead to the dysfunction of downstream components. Therefore, the main question that arises is what the main cause of abortion in this model is.

The endometrium plays a key role in embryo implantation and development by directing the initial phase of embryo attachment,

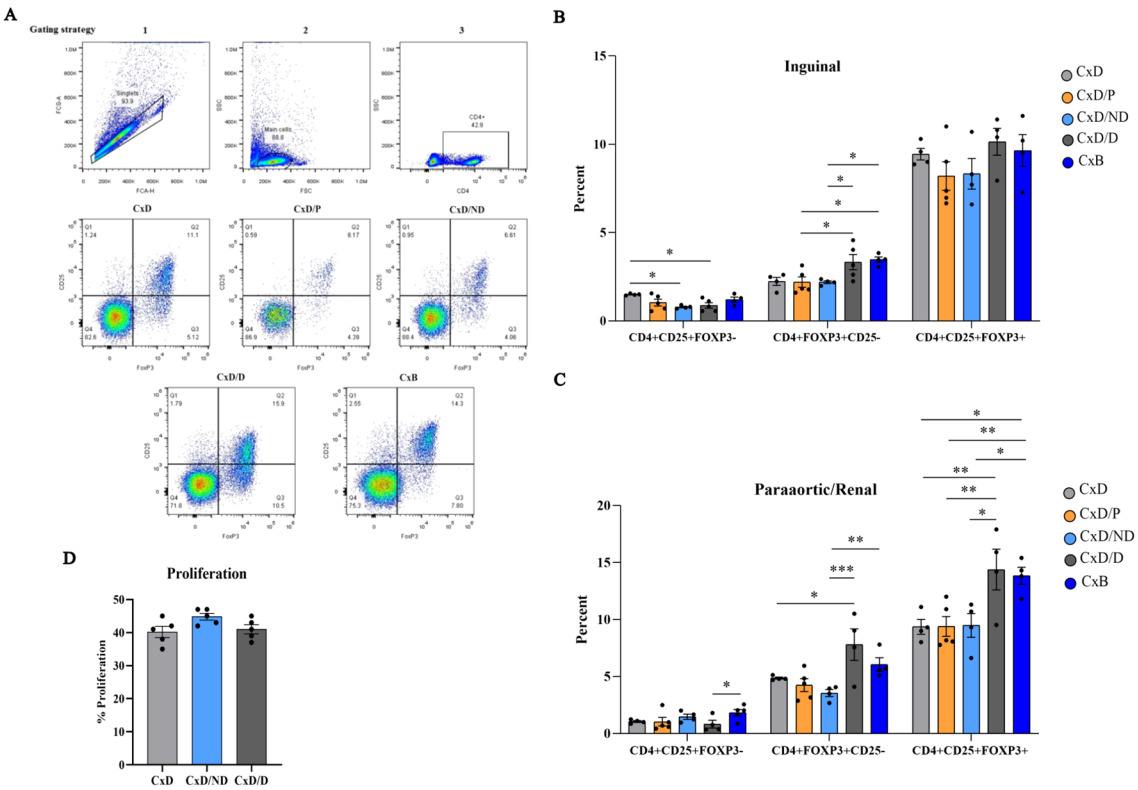


FIGURE 7
Assessment of frequency of regulatory T cells and proliferation of splenocytes. Inguinal and paraaortic/renal lymph nodes of the experimental and control groups (n = 4–5) were excised, and the frequency of CD4+CD25+FOXP3–, CD4+FOXP3+CD25–, and CD4+CD25+FOXP3+ regulatory T cells was measured by flow cytometry **(A)**. **(B, C)** The frequency of these cells in inguinal and paraaortic/renal lymph nodes, respectively. The proliferation capacity of pregnant CBA/J spleen cells in response to the stimulation with DBA/2 splenocytes in the CxD/ND and CxD/D groups was compared to the CxD group **(D)**. *p < 0.05, **p < 0.01 and ***p < 0.001

aiding placental development and invasion, and establishing a safe microenvironment for fetal growth (30, 42). Transformation of endometrial stromal cells to the decidualized state is a fundamental step before the implantation of the human embryo. Decidualization is a multifaceted morphological transformation of endometrial stromal cells, which is associated with profound changes in the epigenetic, metabolomic, transcriptome, and proteome profiles of ESCs (4). Impaired decidualization has been associated with pregnancy loss (1, 10, 11). Decidualized human ESCs not only act as biosensors of embryo quality (43–46) but also, through extensive cross-talk, modulate the function of uterine immune cells (30, 47). Our results showed that adoptive *in-utero* transfer of decidualized, but not undecidualized, ESCs normalized the abortion rate in CBA/J-DBA2 mating comparable to the level observed in CBA/J × Balb/c mating. Interestingly, this intervention was associated with increased frequency of Tregs in the uterine draining (paraaortic and renal) but not in inguinal lymph nodes, while undecidualized ESCs failed to exert such an effect. Of note, in the C×D/D group, the frequency of unconventional Tregs ($CD4^+CD25^-FOXP3^+$) was increased both in renal/paraaortic and inguinal lymph nodes. It has been shown that unconventional Tregs inhibited polyclonal T-cell proliferation and IFN-γ production and similarly exerted regulatory functions akin to $CD25^+$ conventional Tregs (48). These results suggest that decidualization is associated with increased frequency of Tregs and that reduced frequency of these regulatory cells might not be considered as the primary cause of abortion in this model. Several lines of evidence propose that the adoptive transfer of Tregs (20, 21, 49) prevents abortion in the CBA/J × DBA/2 model. Although the robustness of these studies is beyond reasonable doubt, it can be assumed that reduced Treg frequency or impaired function is secondary to impaired decidualization. In line with this assumption, a recent study demonstrated that decidualization increased the frequency of IL-10-producing tolerogenic dendritic cells to induce Tregs (50). In humans, decidualization of ESCs inhibits the expression by these cells of chemokines that cause recruitment of TH1 and cytotoxic T cells (51). Decidualization is also associated with the huge recruitment of uterine NK cells and their transformation to regulatory phenotype with high expression of CD56 (52). More recently, Yang et al. reported a dysfunctional and spatially disordered subpopulation of decidual stromal cells with features like immune cells that cause abnormal accumulation of immune cells in the vascular hub, have aberrant increased inflammatory responses, disrupt decidual hub specification, and eventually lead to pregnancy complications in DBA/2-mated CBA/J mice (53). Collectively, these data highlight decidualized ESCs as the master regulators of the uterine immune system.

Our results showed that perfusion of EDU-labeled ESCs into the uterine lumen resulted in the appearance of EDU-positive cells in uterine stroma after 2 days, which remained there for at least 12 days. Perfusion of mesenchymal stem cells (MSCs) in the mare uterus resulted in the transient early appearance of these cells in the lumen but not in endometrial tissue (54). On the other hand, there are many reports in rodentia including mice and rats indicating that mesenchymal stem cells are easily repopulated in the endometrium for a relatively long time following intrauterine perfusion and exert functional effects (55, 56). We believe that both reports in mares and mice, although seem contradictory, are correct. The different placentation type in the mares and rodentia explains the differential localization of perfused mesenchymal stem cells, as mares have epitheliochorial placentation, while placentation in mice is of hemochorial type (57). Evidence suggests that in species with epitheliochorial placentation, the intact uterine epithelium functions as a barrier to prevent the invasion of blastocyst to the endometrial lumen. From the molecular aspect, gap junctions such as connexins 26, 32, and 43 are not detectable in the uterine epithelium of mares and pigs, whereas these molecules are readily expressed in rodents and modulated under the control of hormones (58).

We did not observe that the adoptive transfer of either decidualized or undecidualized ESCs affects the proliferative response of CBA/j splenocytes to male DBA/2 splenocytes reinforcing the fact that immunoregulation during pregnancy is mainly confined to the uterus and its draining lymph nodes.

The decidualization process in human endometrial stromal cells leads to changes in the expression of over 3,300 genes. These alterations primarily affect genes involved in regulating the cell cycle, restructuring the cytoskeleton, remodeling tissue, promoting angiogenesis, modulating the immune system, combating oxidative stress, facilitating ion and water transfer, responding to steroid hormones, and signaling chemokines, cytokines, and growth factors (53). We observed that decidualization was associated with an altered secretome profile of ESCs. Notably, we observed that decidualization caused a significant increase in IL-6 and a decrease in IL-1α production by ESCs. It is interesting to note that IL-6 knockout mice are infertile (59). IL-6 is produced cyclically by the endometrium, and its production reaches the highest level during the window of implantation (60). Of note, secretion of CCL11 increased several folds in response to decidualization. The effects of eotaxin CCL11 on extravillous trophoblast invasion and endometrial vessel remodeling have already been documented (61). Our data indicated that chemerin is upregulated during mouse ESC decidualization, which contributes to NK cell accumulation and vascular remodeling during early pregnancy (62). Analysis of ECM remodeling and senescence proteins clearly showed differential expression of related proteins in decidualized and undecidualized ESCs. During decidualization, the ECM undergoes remodeling, which involves changes in the synthesis, degradation, and organization of ECM components such as collagen, fibronectin, and proteoglycans (63). By focusing on senescence-associated secretory proteins including IL-6, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, and serpin E1, we confirmed that 6-day decidualization induces a secretome of active senescence in ESCs. Cellular senescence plays a crucial role in the normal process of ESC decidualization. During decidualization, a subset of cells expressing p16, a marker associated with cellular senescence, along with the activation of genes linked to senescence emerged. The severity of the initial proinflammatory response during decidualization is influenced by the quantity of pre-senescent ESCs (5, 13, 64–68).

Although our results showed that intrauterine perfusion of decidualized ESCs could significantly decrease the resorption rate

in the CBA/J × DBA/2 model, correction of decidualization following cell transplantation was not directly investigated in this study. Indeed, early decidualization could be investigated in this model after mating with DBA/2 males and before when pregnancy loss occurs to define whether this is compromised in this model.

If decidualization in CBA/J mice is compromised, the immediate question that should be addressed is why mating between CBA/J females with Balb/c males is normal. Our preliminary results show that the endometrium of CBA/J mice experiences excessive inflammation upon exposure to DBA/2, but not Balb/c, mice seminal plasma, which is the main focus of our future research.

Overall, our findings showed that intrauterine perfusion of decidualized but not undecidualized ESCs in CBA/J females before mating with DBA/2 males could control abortion in this mouse model. This was associated with increased levels of regulatory T cells in the uterine drainage lymph nodes and profound alterations in proteins involved in extracellular matrix remodeling, immune cell trafficking, senescence, and inflammatory responses. These findings reinforce the pivotal role of endometrium in regulating local immune system (69) and indicate that abortion in this mouse model could not simply be attributed to the primary immune dysfunction; rather, impaired decidualization might be the cause of abortion in these mice. These findings offer a fresh outlook on reproductive immunology and fundamentally alter the approach to treating individuals experiencing recurrent miscarriages.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

All experimental procedures done in this study were approved by the Ethics Committee of Animal Experiments of Avicenna Research Institute (ARI). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KaZ: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. KiZ: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. NM-L: Methodology, Project administration, Validation, Writing – review & editing. BZ: Project administration, Validation, Writing – review & editing. SV: Methodology, Writing – review & editing. M-RS: Formal analysis, Methodology, Writing – review & editing. NV: Formal analysis, Methodology, Writing – review & editing. HS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. EM: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. HE: Methodology, Writing – review & editing. M-MN: Methodology, Writing – review & editing. AS: Methodology, Writing – review & editing. FA: Project administration, Supervision, Validation, Writing – review & editing. MJ-T: Project administration, Supervision, Validation, Writing – review & editing. A-HZ: Conceptualization, Formal analysis, Project administration, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The authors declare that financial support was received from the Avicenna Research Institute (grant no. IR.ACECR.AVICENNA.REC.1397.020).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declare that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1440388/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Perfusion of Trypan blue in to the mouse uterus. For evaluation of leakage and tracing the medium flow in the uterus, 10 µL trypan blue was perfused under a 10X operating microscope into the top of the right uterine horn at the utero-tubal junction (UTJ) **(A)** and medium flow was tracked **(B)**.

SUPPLEMENTARY FIGURE 2
H&E and PAS staining of mouse implantation unite: DBA/2-mated female CBA/J mice in different study groups were sacrificed at day 13.5 of pregnancy and implantation unites containing placenta and decidua were removed. Cross sections were prepared and stained with H&E and PAS. PAS-reactive uterine NK cells were present in the decidua (D) and metrial glands (MG) of all groups. JZ: Junctional zone, L: Labyrinth, C: Chorionic plate.

References

1. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med*. (2012) 18:1754–67. doi: 10.1038/nm.3012

2. Evans J, Salamonsen LA, Winship A, Menkhorst E, Nie G, Gargett CE, et al. Fertile ground: human endometrial programming and lessons in health and disease. *Nat Rev Endocrinol*. (2016) 12:654–67. doi: 10.1038/nrendo.2016.116

3. Okada H, Tsuzuki T, Murata H. Decidualization of the human endometrium. *Reprod Med Biol*. (2018) 17:220–7. doi: 10.1002/rmb2.12088

4. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev*. (2014) 35:851–905. doi: 10.1210/er.2014-1045

5. Lucas ES, Vrljicak P, Muter J, Diniz-da-Costa MM, Brighton PJ, Kong C-S, et al. Recurrent pregnancy loss is associated with a pro-senescent decidual response during the peri-implantation window. *Commun Biol*. (2020) 3:37. doi: 10.1038/s42003-020-0763-1

6. Grewal S, Carver JG, Ridley AJ, Mardon HJ. Implantation of the human embryo requires rac1-dependent endometrial stromal cell migration. *Proc Natl Acad Sci U.S.A*. (2008) 105:16189–94. doi: 10.1073/pnas.0806219105

7. Weimar CH, Kavelaars A, Brosens JJ, Gellersen B, de Vreeden-Elbertse JM, Heijnen CJ, et al. Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos. *PloS One*. (2012) 7:e41424. doi: 10.1371/journal.pone.0041424

8. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med*. (2007) 25:445–53. doi: 10.1055/s-2007-991042

9. Ramathal CY, Bagchi IC, Taylor RN, Bagchi MK. Endometrial decidualization: of mice and men. *Semin Reprod Med*. (2010) 28:17–26. doi: 10.1055/s-0029-1242989

10. Salker MS, Christian M, Steel JH, Nautiyal J, Lavery S, Trew G, et al. Deregulation of the serum- and glucocorticoid-inducible kinase sgk1 in the endometrium causes reproductive failure. *Nat Med*. (2011) 17:1509–13. doi: 10.1038/nm.2498

11. Salker MS, Nautiyal J, Steel JH, Webster Z, Sućurović S, Nicou M, et al. Disordered il-33/st2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PloS One*. (2012) 7:e52252. doi: 10.1371/journal.pone.0052252

12. Lucas ES, Dyer NP, Murakami K, Lee YH, Chan YW, Grimaldi G, et al. Loss of endometrial plasticity in recurrent pregnancy loss. *Stem Cells*. (2016) 34:346–56. doi: 10.1002/stem.2222

13. Brighton PJ, Maruyama Y, Fishwick K, Vrljicak P, Tewary S, Fujihara R, et al. Clearance of senescent decidual cells by uterine natural killer cells in cycling human endometrium. *eLife*. (2017) 6:e31274. doi: 10.7554/eLife.31274

14. Williams PJ, Bulmer JN, Searle RF, Innes BA, Robson SC. Altered decidual leucocyte populations in the placental bed in pre-eclampsia and foetal growth restriction: A comparison with late normal pregnancy. *Reproduction*. (2009) 138:177–84. doi: 10.1530/rep-09-0007

15. Ticconi C, Pietropolli A, Di Simone N, Piccione E, Fazleabas A. Endometrial immune dysfunction in recurrent pregnancy loss. *Int J Mol Sci*. (2019) 20:1–29. doi: 10.3390/ijms20215332

16. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. *Int J Dev Biol*. (2010) 54:281–94. doi: 10.1387/ijdb.082763jb

17. Bonney EA, Brown SA. To drive or be driven: the path of a mouse model of recurrent pregnancy loss. *REPRODUCTION*. (2014) 147:R153–R67. doi: 10.1530/REP-13-0583

18. Clark DA, Petitbarat M, Chaouat G. Original article: how should data on murine spontaneous abortion rates be expressed and analyzed? *Am J Reprod Immunol*. (2008) 60:192–6. doi: 10.1111/j.1600-0897.2008.00612.x

19. Fatemi R, Mirzadegan E, Vahedian Z, Zarnani AH, Jeddi-Tehrani M, Idali F. Low 17β-estradiol levels are better inducers of regulatory conditioned T cells in-vitro. *Iran J Immunol*. (2017) 14:159–71.

20. Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, et al. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced cd4+Cd25+ T regulatory cells prevents fetal rejection in a murine abortion model. *Am J Pathol*. (2005) 166:811–22. doi: 10.1016/S0002-9440(10)62302-4

21. Wafula PO, Teles A, Schumacher A, Pohl K, Yagita H, Volk HD, et al. Pd-1 but not ctla-4 blockage abrogates the protective effect of regulatory T cells in a pregnancy murine model. *Am J Reprod Immunol*. (2009) 62:283–92. doi: 10.1111/j.1600-0897.2009.00737.x

22. Idali F, Rezaii-Nia S, Golshahi H, Fatemi R, Naderi MM, Goli LB, et al. Adoptive cell therapy with induced regulatory T cells normalises the abortion rate in abortion-prone mice. *Reprod Fertil Dev*. (2021) 33:220–8. doi: 10.1071/rd20063

23. Chen T, Darrasse-Jèze G, Bergot A-S, Courau T, Churlaud G, Valdivia K, et al. Self-specific memory regulatory T cells protect embryos at implantation in mice. *J Immunol*. (2013) 191:2273–81. doi: 10.4049/jimmunol.1202413

24. Muzikova E, Clark DA. Is spontaneous resorption in the dba/2-mated cba/J mouse due to a defect in "Seed" or in "Soil"? *Am J Reprod Immunol*. (1995) 33:81–5. doi: 10.1111/j.1600-0897.1995.tb01142.x

25. Brown LY, Bonney EA, Raj RS, Nielsen B, Brown S. Generalized disturbance of DNA methylation in the uterine decidua in the cba/J X dba/2 mouse model of pregnancy failure. *Biol Reprod*. (2013) 89:120. doi: 10.1095/biolreprod.113.113142

26. Dixon ME, Chien EK, Osol G, Callas PW, Bonney EA. Failure of decidual arteriolar remodeling in the cba/J X dba/2 murine model of recurrent pregnancy loss is linked to increased expression of tissue inhibitor of metalloproteinase 2 (Timp-2). *Am J Obstet Gynecol*. (2006) 194:113–9. doi: 10.1016/j.ajog.2005.06.063

27. Shokri M-R, Bozorgmehr M, Ghanavatinejad A, Falak R, Aleahmad M, Kazemnejad S, et al. Human menstrual blood-derived stromal/stem cells modulate functional features of natural killer cells. *Sci Rep*. (2019) 9:10007. doi: 10.1038/s41598-019-46316-3

28. Aleahmad M, Bozorgmehr M, Nikoo S, Ghanavatinejad A, Shokri MR, Montazeri S, et al. Endometrial mesenchymal stem/stromal cells: the enigma to code messages for generation of functionally active regulatory T cells. *Stem Cell Res Ther*. (2021) 12:536. doi: 10.1186/s13287-021-02603-3

29. Ghanavatinejad A, Bozorgmehr M, Shokri MR, Aleahmad M, Tavakoli M, Shokri F, et al. Menscs exert a supportive role in establishing a pregnancy-friendly microenvironment by inhibiting th17 polarization. *J Reprod Immunol*. (2021) 144:103252. doi: 10.1016/j.jri.2020.103252

30. Khorami-Sarvestani S, Vanaki N, Shojaeian S, Zarnani K, Stensballe A, Jeddi-Tehrani M, et al. Placenta: an old organ with new functions. *Front Immunol*. (2024) 15:1385762. doi: 10.3389/fimmu.2024.1385762

31. De Clercq K, Hennes A, Vriens J. Isolation of mouse endometrial epithelial and stromal cells for in vitro decidualization. *J Vis Exp*. (2017) 12:1–10. doi: 10.3791/55168

32. Zarnani AH, Moazzeni SM, Shokri F, Salehnia M, Jeddi Tehrani M. Analysis of endometrial myeloid and lymphoid dendritic cells during mouse estrous cycle. *J Reprod Immunol*. (2006) 71:28–40. doi: 10.1016/j.jri.2006.01.003

33. Tabatabaei M, Mosaffa N, Ghods R, Nikoo S, Kazemnejad S, Khanmohammadi M, et al. Vaccination with human amniotic epithelial cells confer effective protection in a murine model of colon adenocarcinoma. *Int J Cancer*. (2018) 142:1453–66. doi: 10.1002/ijc.31159

34. Sarvari A, Naderi Mm, Sadeghi MR, Akhondi MM. A technique for facile and precise transfer of mouse embryos. *AVICENNA J OF Med Biotechnol (AJMB)*. (2013) 5:62–5.

35. Lin G, Huang Y-C, Shindel AW, Banie L, Wang G, Lue TF, et al. Labeling and tracking of mesenchymal stromal cells with edu. *Cytotherapy*. (2009) 11:864–73. doi: 10.3109/14653240903180084

36. Raats S, Jadhav A, Martens M, Sener DD, Weitz E. Overview of proinflammatory and profibrotic mediators (WP5095). Available online at: https://www.wikipathways.org/instance/WP5095 (Accessed March 15 2024).

37. Zhou Q, Amar S. Identification of signaling pathways in macrophage exposed to porphyromonas gingivalis or to its purified cell wall components. *J Immunol*. (2007) 179:7777–90. doi: 10.4049/jimmunol.179.11.7777

38. Stadtmauer DJ, Wagner GP. Single-cell analysis of prostaglandin E2-induced human decidual cell in vitro differentiation: A minimal ancestral deciduogenic signal†. *Biol Reprod*. (2022) 106:155–72. doi: 10.1093/biolre/ioab183

39. Wiley CD, Sharma R, Davis SS, Lopez-Dominguez JA, Mitchell KP, Wiley S, et al. Oxylipin biosynthesis reinforces cellular senescence and allows detection of senolysis. *Cell Metab*. (2021) 33:1124–36.e5. doi: 10.1016/j.cmet.2021.03.008

40. Lin G, Ning H, Banie L, Qiu X, Zhang H, Lue TF, et al. Bone marrow cells stained by azide-conjugated alexa fluors in the absence of an alkyne label. *Stem Cells Dev*. (2012) 21:2552–9. doi: 10.1089/scd.2012.0092

41. Clark DA, Rahmati M, Gohner C, Bensussan A, Markert UR, Chaouat G. Seminal plasma peptides may determine maternal immune response that alters success or failure of pregnancy in the abortion-prone cbaxdba/2 model. *J Reprod Immunol*. (2013) 99:46–53. doi: 10.1016/j.jri.2013.03.006

42. Critchley HOD, Maybin JA, Armstrong GM, Williams ARW. Physiology of the endometrium and regulation of menstruation. *Physiol Rev*. (2020) 100:1149–79. doi: 10.1152/physrev.00031.2019

43. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. *Biol Reprod*. (2014) 91:98. doi: 10.1095/biolreprod.114.122846

44. Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, et al. Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PloS One*. (2010) 5:e10258. doi: 10.1371/journal.pone.0010258

45. Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, et al. Uterine selection of human embryos at implantation. *Sci Rep*. (2014) 4:3894. doi: 10.1038/srep03894

46. Ticconi C, Pietropolli A, D'Ippolito S, Chiaramonte C, Piccione E, Scambia G, et al. Time-to-pregnancy in women with unexplained recurrent pregnancy loss: A controlled study. *Reprod Sci*. (2020) 27:1121–8. doi: 10.1007/s43032-019-00122-4

47. Dimitriadis E, Menkhorst E, Saito S, Kutteh WH, Brosens JJ. Recurrent pregnancy loss. *Nat Rev Dis Primers*. (2020) 6:98. doi: 10.1038/s41572-020-00228-z

48. Angerami MT, Suarez GV, Vecchione MB, Laufer N, Ameri D, Ben G, et al. Expansion of cd25-negative forkhead box P3-positive T cells during hiv and mycobacterium tuberculosis infection. *Front Immunol*. (2017) 8. doi: 10.3389/fimmu.2017.00528

49. Yin Y, Han X, Shi Q, Zhao Y, He Y. Adoptive transfer of cd4+Cd25+ Regulatory T cells for prevention and treatment of spontaneous abortion. *Eur J Obstet Gynecol Reprod Biol*. (2012) 161:177–81. doi: 10.1016/j.ejogrb.2011.12.023

50. Gori S, Soczewski E, Fernández L, Grasso E, Gallino L, Merech F, et al. Decidualization process induces maternal monocytes to tolerogenic il-10-producing dendritic cells (Dc-10). *Front Immunol*. (2020) 11:1571. doi: 10.3389/fimmu.2020.01571

51. Llorca T, Ruiz-Magaña MJ, Martinez-Aguilar R, García-Valdeavero OM, Rodríguez-Doña L, Abadia-Molina AC, et al. Decidualized human decidual stromal cells inhibit chemotaxis of activated T cells: A potential mechanism of maternal-fetal immune tolerance. *Front Immunol*. (2023) 14. doi: 10.3389/fimmu.2023.1223539

52. Zhu H, Hou C-C, Luo L-F, Hu Y-J, Yang W-X. Endometrial stromal cells and decidualized stromal cells: origins, transformation and functions. *Gene*. (2014) 551:1–14. doi: 10.1016/j.gene.2014.08.047

53. Yang M, Ong J, Meng F, Zhang F, Shen H, Kitt K, et al. Spatiotemporal insight into early pregnancy governed by immune-featured stromal cells. *Cell*. (2023) 186:4271–88.e24. doi: 10.1016/j.cell.2023.08.020

54. Rink BE, Beyer T, French HM, Watson E, Aurich C, Donadeu FX. The fate of autologous endometrial mesenchymal stromal cells after application in the healthy equine uterus. *Stem Cells Dev*. (2018) 27:1046–52. doi: 10.1089/scd.2018.0056

55. Zhao J, Zhang Q, Wang Y, Li Y. Uterine infusion with bone marrow mesenchymal stem cells improves endometrium thickness in a rat model of thin endometrium. *Reprod Sci*. (2015) 22:181–8. doi: 10.1177/1933719114537715

56. Margiana R, Markov A, Zekiy AO, Hamza MU, Al-Dabbagh KA, Al-Zubaidi SH, et al. Clinical application of mesenchymal stem cell in regenerative medicine: A narrative review. *Stem Cell Res Ther*. (2022) 13:366. doi: 10.1186/s13287-022-03054-0

57. D'Souza AW, Wagner GP. Malignant cancer and invasive placentation: A case for positive pleiotropy between endometrial and Malignancy phenotypes. *Evolution Medicine Public Health*. (2014) 2014:136–45. doi: 10.1093/emph/eou022

58. Malassiné A, Cronier L. Involvement of gap junctions in placental functions and development. *Biochim Biophys Acta*. (2005) 1719:117–24. doi: 10.1016/j.bbamem.22005.09.019

59. van Mourik MSM, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *J Leukocyte Biol*. (2009) 85:4–19. doi: 10.1189/jlb.0708395

60. Perrier d'Hauterive S, Charlet-Renard C, Berndt S, Dubois M, Munaut C, Goffin F, et al. Human chorionic gonadotropin and growth factors at the embryonic-endometrial interface control leukemia inhibitory factor (Lif) and interleukin 6 (Il-6) secretion by human endometrial epithelium. *Hum Reprod*. (2004) 19:2633–43. doi: 10.1093/humrep/deh450

61. Chau SE, Murthi P, Wong MH, Whitley GS, Brennecke SP, Keogh RJ. Control of extravillous trophoblast function by the eotaxins ccl11, ccl24 and ccl26. *Hum Reprod*. (2013) 28:1497–507. doi: 10.1093/humrep/det060

62. Carlino C, Trotta E, Stabile H, Morrone S, Bulla R, Soriani A, et al. Chemerin regulates nk cell accumulation and endothelial cell morphogenesis in the decidua during early pregnancy. *J Clin Endocrinol Metab*. (2012) 97:3603–12. doi: 10.1210/jc.2012-1102

63. White CA, Robb L, Salamonsen LA. Uterine extracellular matrix components are altered during defective decidualization in interleukin-11 receptor alpha deficient mice. *Reprod Biol Endocrinol*. (2004) 2:76. doi: 10.1186/1477-7827-2-76

64. Liao Y, Jiang Y, He H, Ni H, Tu Z, Zhang S, et al. Nedd8-mediated neddylation is required for human endometrial stromal proliferation and decidualization. *Hum Reprod*. (2015) 30:1665–76. doi: 10.1093/humrep/dev117

65. Kusama K, Yoshie M, Tamura K, Nakayama T, Nishi H, Isaka K, et al. The role of exchange protein directly activated by cyclic amp 2-mediated calreticulin expression in the decidualization of human endometrial stromal cells. *Endocrinology*. (2014) 155:240–8. doi: 10.1210/en.2013-1478

66. Ochiai A, Kuroda K, Ozaki R, Ikemoto Y, Murakami K, Muter J, et al. Resveratrol inhibits decidualization by accelerating downregulation of the crabp2-rar pathway in differentiating human endometrial stromal cells. *Cell Death Dis*. (2019) 10:276. doi: 10.1038/s41419-019-1511-7

67. Ochiai A, Kuroda K, Ikemoto Y, Ozaki R, Nakagawa K, Nojiri S, et al. Influence of resveratrol supplementation on ivf-embryo transfer cycle outcomes. *Reprod BioMed Online*. (2019) 39:205–10. doi: 10.1016/j.rbmo.2019.03.205

68. Deryabin P, Griukova A, Nikolsky N, Borodkina A. The link between endometrial stromal cell senescence and decidualization in female fertility: the art of balanc. *Cell Mol Life Sci*. (2020) 77:1357–70. doi: 10.1007/s00018-019-03374-0

69. Bozorgmehr M, Gurung S, Darzi S, Nikoo Sh, Kazemnejad S, Zarnani AH, et al. Endometrial and menstrual blood mesenchymal stem/stromal cells: biological properties and clinical application. *Front Cell Dev Biol*. (2020) 8:494. doi: 10.3389/fcell.2020.00497

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 07 October 2024
DOI 10.3389/fimmu.2024.1456171

Check for updates

OPEN ACCESS

EDITED BY
Amir-Hassan Zarnani,
Tehran University of Medical Sciences, Iran

REVIEWED BY
Anne Schumacher,
Helmholtz Association of German Research Centres (HZ), Germany
Chiara Agostinis,
Institute for Maternal and Child Health Burlo Garofolo (IRCCS), Italy

*CORRESPONDENCE
Qingqing Zhang
zhangqq1@hku.hk
Philip C. N. Chiu
pchiucn@hku.hk

RECEIVED 28 June 2024
ACCEPTED 23 September 2024
PUBLISHED 07 October 2024

CITATION
Liu J-C, Zeng Q, Duan Y-G, Yeung WSB, Li RHW, Ng EHY, Cheung KW, Zhang Q and Chiu PCN (2024) B cells: roles in physiology and pathology of pregnancy.
Front. Immunol. 15:1456171.
doi: 10.3389/fimmu.2024.1456171

COPYRIGHT
© 2024 Liu, Zeng, Duan, Yeung, Li, Ng, Cheung, Zhang and Chiu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

B cells: roles in physiology and pathology of pregnancy

Jin-Chuan Liu[1,2], Qunxiong Zeng[1,2], Yong-Gang Duan[2], William S. B. Yeung[1,2], Raymond H. W. Li[1], Ernest H. Y. Ng[1], Ka-Wang Cheung[1], Qingqing Zhang[1,2]* and Philip C. N. Chiu[1,2]*

[1]Department of Obstetrics and Gynaecology, School of Clinical Medicine, Li Ka Shing (LKS) Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong SAR, China, [2]Shenzhen Key Laboratory of Fertility Regulation, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China

B cells constitute a diverse and adaptable immune cell population with functions that can vary according to the environment and circumstances. The involvement of B cells in pregnancy, as well as the associated molecular pathways, has yet to be investigated. This review consolidates current knowledge on B cell activities and regulation during pregnancy, with a particular focus on the roles of various B cell subsets and the effects of B cell-derived factors on pregnancy outcomes. Moreover, the review examines the significance of B cell-associated autoantibodies, cytokines, and signaling pathways in relation to pregnancy complications such as pregnancy loss, preeclampsia, and preterm birth.

KEYWORDS

B cells, pregnancy, maternal-fetal interface, immune tolerance, pregnancy complications

1 Introduction

Pregnancy poses a considerable immunological challenge as a mother must support her baby with half of genetic makeup different from hers. A successful pregnancy depends on the intricate interaction between the fetal placenta, and the maternal decidua at the maternal-fetal interface. Throughout pregnancy, the immune cells at the maternal-fetal interface create an environment of immune tolerance. This not only protects the fetus from the maternal immune system but also offers defense for the mother against pathogens. The decidua contains various immune cell populations. Among the immune cells, natural killer (NK) cells, macrophages, T cells and dendritic cells are the prevalent leukocytes (1, 2). They not only prevent immune rejection of the fetus but also support trophoblast invasion, tissue remodeling, and angiogenesis to establish a healthy placenta (2–4). Disruption of the immune microenvironment at the maternal-fetal interface as often caused by inflammation can result in adverse pregnancy outcomes such as preterm labor, preeclampsia (PE), fetal growth restriction, or pregnancy loss. Inflammatory responses can be triggered by infections, autoimmune diseases, stress, or other unknown factors.

B cells in humans and mice originate from hematopoietic stem cells (HSCs) and mature in the fetal liver and bone marrow (5, 6). They play a significant role in both humoral and cellular

immunity. In humoral immunity, they produce specific antibodies in response to infections or vaccinations. In cellular immunity, they activate T cells by presenting antigens, offering co-stimulation signals, and generating cytokines (7, 8). Since their discovery over a century ago, scientists have identified several subsets of B cells, including B1 and B2 lymphocytes. The latter are further divided into marginal zone (MZ) and follicular (FO) B cells (8).

Although B cells account for only 1–2% of the decidual lymphocytes, their importance in promoting a healthy pregnancy is gaining attention (9) due to their ability to produce antibodies as well as other cell regulatory factors. The proportion of decidual B cells modestly increased between 27 and 33 weeks of gestation followed by a slight decline at term (10). In mice, a lack of mature B cells correlates with reduced litter size, smaller embryos, and heightened susceptibility to prenatal infections (11). In women with common variable immunodeficiency, a condition characterized by reduced levels of switched memory B cells (12), there is an increased risk for preterm birth (PTB) and other pregnancy complications, including PE, stillbirth, and vaginal bleeding (13). Both recurrent pregnancy loss (RPL) and PE have been linked to alterations in the development of B cell memory (14–16).

Current research suggests that during pregnancy, B cells create a tolerant environment by generating protective antibodies against foreign paternal antigens. In addition, a subset of interleukin 10 (IL-10) secreting regulatory B cells (Bregs) is important for establishing an anti-inflammatory microenvironment to sustain pregnancy (17). However, B cells can also negatively affect pregnancy by producing autoantibodies that target the mother's tissues and complicate pregnancy. For example, these autoantibodies may attack the placenta, hindering its functionality and causing PE, RPL, or intrauterine fetal death. Consistently, certain autoimmune diseases exacerbated by these autoantibodies, such as systemic lupus erythematosus (SLE), can increase the risk of RPL, PTB, and other adverse pregnancy outcomes (18–23). Despite their significance, research on B cells in normal and complicated pregnancies has been largely neglected, making this area underexplored in the field.

2 B cell development and differentiation

2.1 B cell development and function

B cells originate from HSCs. They undergo a tightly regulated developmental process to ensure their functionality and avoid self-reactivity. The B1 cells primarily develop in the fetal liver, while the B2 cells emerge from the bone marrow (Figure 1A) (8, 24). B cell differentiation in the bone marrow consists of several key stages: early stage, pro-B cells, pre-B cells, and immature B cells (Figure 1A). The process begins with HSCs differentiation into common lymphoid progenitors. Those committed to the B cell lineage become pro-B cells characterized by expression of specific surface markers and immunoglobulin (Ig) gene rearrangement (8). In humans, pro-B cells express CD19, a crucial B cell marker, along with other markers such as CD34 and CD45R (B220). Successful IgH gene rearrangement enables the pro-B cells to advance to the pre-B cell stage, during which light chain gene rearrangement occurs.

The pre-B cells express the pre-B cell receptor (pre-BCR), which consists of the antibody's heavy chain (μH), surrogate light chains, and signaling molecules Igα and Igβ (25). Upon successful light chain rearrangement, the cells display the B cell receptor (BCR) on their surface and become immature B cells. These cells have a strong affinity for self-antigens and undergo negative selection, eliminating self-reactive B cells through central tolerance.

The immature B cells migrate to peripheral lymphoid organs, such as the spleen, where they develop into transitional B cells

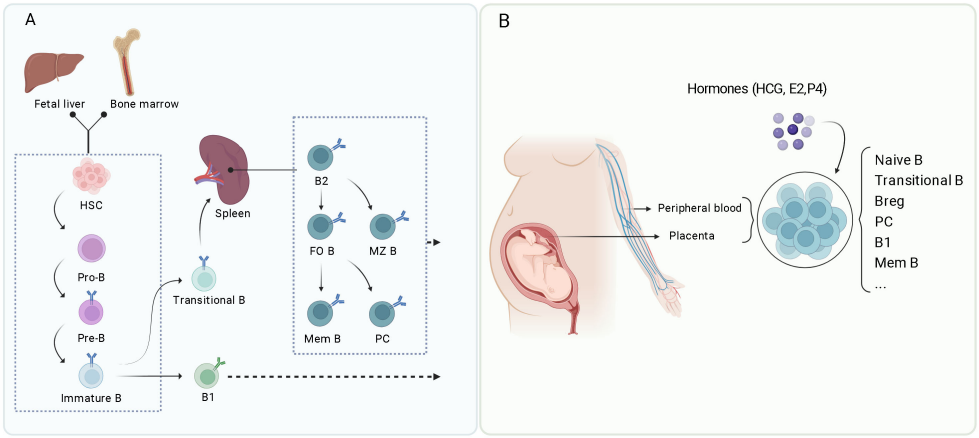


FIGURE 1
(Created with BioRender.com): Overview of B cell development and differentiation. **(A)** B cells develop from hematopoietic stem cells (HSC) in the fetal liver and bone marrow, leading to the formation of various B cell subsets including B1 cells, B2 cells (including follicular B cells or FO B, marginal zone B cells or MZ B), memory B cells (Mem B), and plasma cells (PC). **(B)** During pregnancy, various B cell subsets play important roles in maintaining a health environment, including naïve B cells, transitional B cells, Bregs, plasma cells, B1 B cells, and memory B cells. These subsets are regulated by hormones such as human chorionic gonadotropin (hCG), estradiol (E2), and progesterone (P4).

before reaching full maturity. In the spleen, the transitional B cells receive signals from their BCR and other surface molecules within the surrounding microenvironment, including interactions with follicular dendritic cells and T cells. The combination of these interactions and survival signals, such as B-cell activating factor (BAFF), facilitate the development of transitional B cells into two distinct types: FO B cells and MZ B cells. FO B cells are predominantly located in the follicles of secondary lymphoid organs and play a crucial role in producing high-affinity antibody responses. MZ B cells are situated in the spleen's marginal zone, specializing in rapid reactions to pathogens in the bloodstream. Both FO and MZ B cells are fully mature B cells that can respond to antigens effectively (Figure 1A).

Upon encountering antigens, mature FO B cells further differentiate into memory B cells or antibody-secreting plasma cells. This differentiation involves germinal center (GC) reactions and is influenced by T follicular helper cells (Tfh) (8). Tfh cells are a subset of T cells that play a significant role in the formation of GCs within lymphoid tissues (26). These GCs facilitate B cell proliferation, differentiation, and somatic hypermutation (SHM). Following antigen detection, FO B cells increase the expression of chemokine receptors, such as C-C chemokine receptor 7 (CCR7) and Epstein-Barr virus-induced receptor 2. This upregulation enables FO B cells to move to the T-B border, a region adjacent to the T-cell zone within the follicle. At the T-B border, FO B cells interact with antigen-specific Tfh cells, which provide crucial costimulatory signals, such as CD40L, and cytokines like interleukin 4 (IL-4), interleukin 21 (IL-21), and interferon gamma (IFN-γ). These factors contribute to B cell proliferation and differentiation. A portion of activated FO B cells increases B-cell lymphoma 6 (Bcl6) expression and returns to the follicles to establish GCs alongside Tfh cells. Within GCs, B cells experience extensive proliferation, SHM, and class switch recombination (CSR). The SHM process introduces mutations into immunoglobulin genes, thereby improving the affinity of the resulting antibodies. Meanwhile, CSR alters the antibody isotype, enabling it to perform various effector functions. High-affinity B cells that successfully compete for antigens in the GC and receive sufficient Tfh signals differentiate into memory B cells, which can endure long durations and quickly react to future exposures of the same antigen. Additionally, some B cells differentiate into long-lived plasma cells that migrate to the bone marrow and secrete substantial amounts of high-affinity antibodies (8, 26).

2.2 B cell receptor signaling and activation

BCR signaling is essential for B cell activation, differentiation, and immune response. It begins when the BCR on the surface of B cells binds to a specific antigen (27). The BCR complex consists of a membrane-bound immunoglobulin molecule (mIg) that recognizes antigens and a signaling module composed of Igα and Igβ chains (28, 29). These components collaborate to transmit signals into the cell, leading to B cell activation, differentiation, and antibody production. Upon binding with the antigens, the BCRs cluster together and initiate a series of intracellular signaling events, involving the recruitment and activation of various signaling molecules, such as kinases like Lyn and Syk, adaptors such as B cell linker protein (BLNK), and enzymes like phosphoinositide 3-kinase (PI3K) (29). This results in the activation of downstream signaling molecules and transcription factors. The signaling cascade increases intracellular calcium levels and activates nuclear factors such as nuclear factor kappa B (NFκB) and nuclear factor of activated T cells (NFAT) (29). These factors move to the nucleus and drive the transcription of genes vital for B cell activation and function. To achieve full activation and differentiation into antibody-producing plasma cells or memory B cells, B cells require a second signal, provided by helper T cells through CD40L-CD40 interactions or by pattern-recognition receptors recognizing pathogen-associated molecular patterns (PAMPs) (29).

Proper BCR signaling is crucial for protective immunity but can contribute to pathological conditions if dysregulated. For instance, BCR signaling dysregulation can cause autoimmune diseases or B cell malignancies (30, 31), while defective BCR signaling can lead to immunodeficiencies (32, 33). During pregnancy, BCR develops a diverse repertoire through processes such as somatic recombination, class switch recombination, and somatic hypermutation. This diversity enables the fetus to establish a stable immune response (34, 35). The repertoire is achieved by selecting and rearranging variable (V), diversity (D), and joining (J) gene segments, as well as random nucleotide insertions and deletions facilitated by terminal deoxynucleotidyl transferase (TdT) (35). BCR activity is also modulated to enhance the functions of Bregs, which help to prevent maternal immune responses against the fetus by producing anti-inflammatory cytokines (36). Moreover, maternal B lymphocytes with BCRs specific for paternal antigens or trophoblast giant cells trigger the deletion of these B cells in the spleen and bone marrow, beginning in mid-pregnancy and reversing after childbirth. This mechanism aids in preventing maternal immune rejection of the semi-allogeneic fetus by reducing the population of B cells that could recognize and attack fetal antigens (37, 38).

3 Localization of B cells in the maternal-fetal interface

B cells are found in the decidua basalis, decidua parietalis, and placental tissue within the interface. They are present in the human term decidua (39, 40); the number of B cells is higher in the term decidua basalis than in the decidua parietalis tissues, and the decidua parietalis has a greater proportion of mature/naive B cells while the decidua basalis has more transitional B cells (40). During preterm labor and placental inflammation, the total B cell count increases and the proportion of various B cell subsets changes in human choriodecidua (39). The functional role of B cells in the decidua parietalis of pregnant women remains to be investigated.

Decidual B cells express high levels of interleukin 12 (IL-12), interleukin 6 (IL-6), and interleukin 35 (IL-35). The number of B1 cells and plasmablasts (a short-lived, antibody-secreting cells that are an intermediate stage between activated B cells and fully differentiated plasma cells) also increases in women experiencing labor and chronic chorioamnionitis. The observed increase in

immune memory and the consistent presence of B cells at the fetal-maternal interface suggest that B cells may be a normal decidual component of a healthy pregnancy rather than indicators of uterine abnormalities. In this context, the decidual B cells produce IL-10 and are found in clusters alongside the Foxp3$^+$ T cells (41). Further research is necessary to clarify the role of B cells in pregnancy.

B cells have been found in the chorionic villi and the decidua (42–44). The immune landscape in the villous placenta is distinct from the choriodecidua, characterized by a significant population of monocytes, including fetal-origin Hofbauer cells, and a diverse immune cell population such as T cells, monocytes, dendritic cells, NK cells, neutrophils, and B cells (44). The B cells are also found within the trophoblast boundary and beyond fetal blood vessels in the second-trimester placenta (43). Advanced single-cell techniques have identified B cells in healthy placentas from women who delivered at term, with or without spontaneous labor (42, 45). In full-term healthy pregnancies, the mature/naïve B cells are retained in placental intervillous blood due to specific placenta-produced chemokines (46), indicating the potential role of these cells in fetal immune responses. The presence of B cells in both maternal and fetal compartments is consistent with their involvement in immune interactions at the maternal-fetal interface.

There is currently no evidence of the presence of B cells in the fetal membranes. However, extracts from fetal membranes during labor induce greater leukocyte chemotaxis than those from non-labor membranes, implying that B cells and other leukocytes may be attracted to the fetal membranes during labor (47). Furthermore, the pre-B-cell colony-enhancing factor (PBEF), known to play a role in the maturation of B cell precursors, is consistently expressed in the fetal membranes and placenta, with increased expression during labor (48, 49). These findings suggest that the role of B cells in the immune environment of fetal membranes and their impact on pregnancy outcomes is a potential research area.

4 Role of B cells at the maternal-fetal interface

4.1 B cells in antigen presentation

B cells process and present antigens in association with either MHC class I or MHC class II molecules to CD8 and CD4 cells, respectively, ultimately leading to the activation of the adaptive immune response (50). Antigen presentation is crucial for enabling a targeted immunogenic response from the immune system. The fetal-placental antigens that interact with the maternal immune system include blood group antigens, MHC antigens, and minor histocompatibility antigens. Despite being significantly different from the mother, these antigens do not elicit the robust cellular or humoral immune reactions typically seen following organ transplantation (51). Previous research suggests that B cells specific to the fetal antigens are eliminated during pregnancy to maintain immune tolerance at the maternal-fetal interface. When the MHC class I molecule, specifically H-2Kb, is expressed in mouse trophoblast giant cells, it is recognized by the maternal immune system and triggers a response in the maternal bone marrow, leading to the deletion of a B cell subpopulation, including immature and transitional B cells (Figure 2A). This deletion is essential for developing maternal tolerance towards the fetus (38). Recent research has also shown that B cells play a vital role in mediating the MHC-class-II-restricted presentation of antigens to CD4$^+$ T cells, ultimately resulting in T cell suppression after mid-gestation during pregnancy (52) (Figure 2A). The study offers evidence that B cells specific to a model trophoblast antigen are significantly suppressed via CD22–LYN inhibitory signaling (52). A complete understanding of the role of B cells as antigen-presenting cells during pregnancy is still elusive. Much of the current insights into this conundrum come from T-cell research in mouse models. Additional research in this area, including the effects of microchimerism on B cell function, is required to fully elucidate the complex interplay between maternal B cells and fetal antigens during pregnancy.

4.2 Antibody production

B cells serve a dual role within the immune system during pregnancy (Figure 2A); they produce protective antibodies contributing to a stable immune microenvironment at the maternal-fetal interface but may also generate autoantibodies that could disrupt pregnancy (Figure 2). The protective antibodies, also known as asymmetric antibodies (AAb), cannot form antigen-antibody complexes due to a structural irregularity involving an oligosaccharide residue (53, 54). These antibodies cannot trigger immune responses, potentially offering protection to the paternal antigens. Low maternal serum AAb levels in the first trimester are associated with spontaneous abortion later in pregnancy (55). The production of AAb can be influenced by IL-6, and progesterone-induced blocking factor (PIBF) (56–58). Estrogen and progesterone regulate the synthesis of PIBF and IL-6, suggesting that these steroids also play crucial roles in AAb production (59, 60).

Over the past several decades, the relationship between autoantibodies and pregnancy has been a subject of ongoing debate. Studies have shown that a significant number of non-organ-specific autoantibodies are linked to adverse pregnancy outcomes, such as RPL, intrauterine growth restriction, PE, and PTB. However, besides antiphospholipid (aPL) antibodies and anti-thyroid antibodies, many findings remain inconsistent. In patients with antiphospholipid syndrome (APS), an autoimmune condition characterized by the production of aPL, there is an increase in peripheral CD27$^-$IgM$^+$ naïve B cells, transitional B cells, CD19$^+$CD5$^+$ B cells, and plasmablasts, which are potential sources of autoreactive antibodies (61). Additionally, a decrease in IL-10-producing Bregs has been observed in the peripheral blood of patients with primary antiphospholipid syndrome (PAPS) and SLE with antiphospholipid syndrome (SLE/APS) (61). These B cell dysregulations may contribute to the pathogenesis of APS by disrupting the regulatory mechanisms that typically suppress the autoreactive B cell responses (61, 62). While most studies have focused on peripheral blood, the B cells in reproductive tissue during pregnancy have seldom been explored.

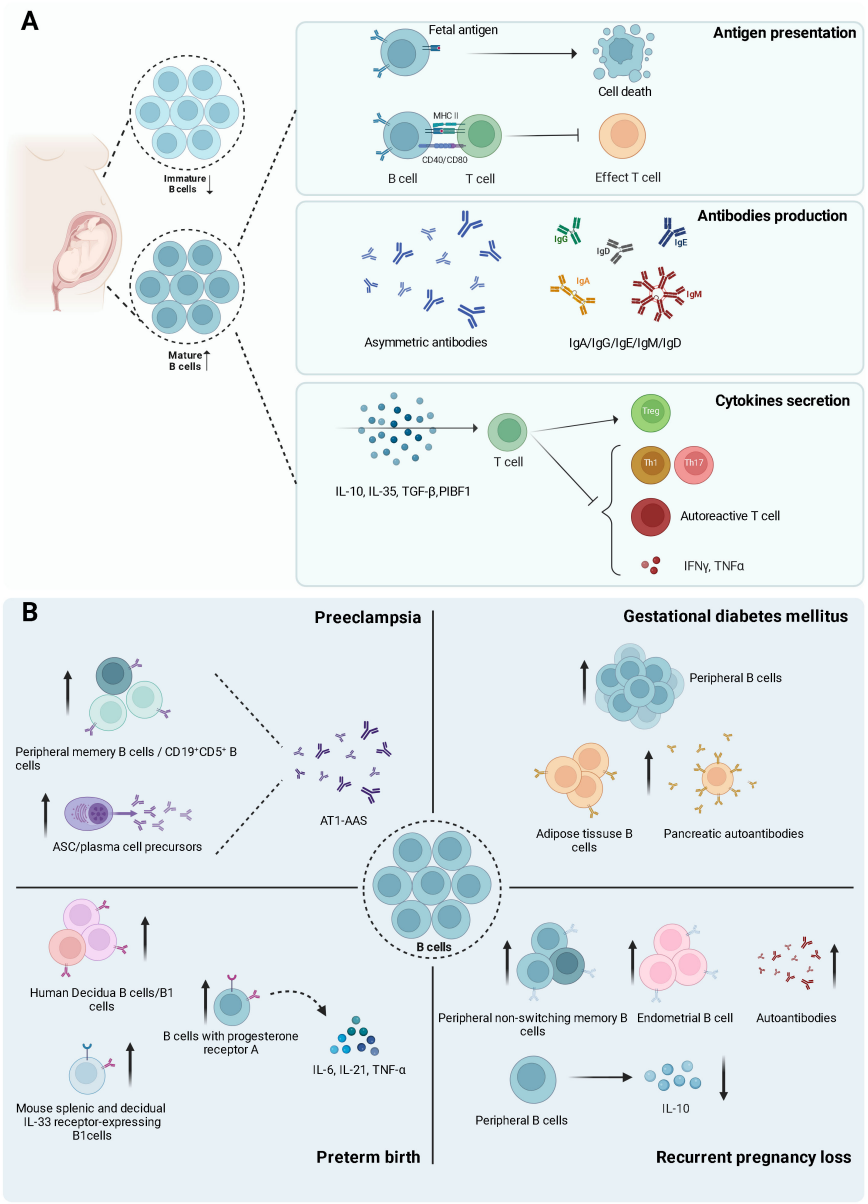


FIGURE 2
(Created with BioRender.com): Diverse functions of B cells during normal pregnancy and pregnancy-related disorders. **(A)** The Multifaceted Roles of B Cells During Pregnancy. The left panel illustrates the altered B cell populations during pregnancy, characterized by decreased immature B cells and increased mature B cells. The right panel delineates three primary functions of B cells: 1) Antigen Presentation: B cells encountering fetal antigens may undergo deletion, potentially eliminating autoreactive B cells. Additionally, B cells present antigens to T cells via MHC II, leading to the inhibition of effector T cell differentiation. 2). Antibody Production: B cells generate a diverse repertoire of antibodies during pregnancy, including asymmetric antibodies and various immunoglobulin isotypes (IgA, IgG, IgE, IgM, and IgD). 3) Cytokine Secretion: B cell-derived cytokines (IL-10, IL-35, TGF-β, and PIBF1) exert immunomodulatory effects on T cell subsets. These cytokines promote the differentiation of regulatory T cells (Treg) while suppressing Th1, Th17, and autoreactive T cells that produce pro-inflammatory cytokines such as IFN-γ and TNF-α. **(B)** The diverse roles and alterations of B cells in four pregnancy-related complications are as follows: 1) Preeclampsia: Increased peripheral memory B cells, CD19+CD5+ B cells, and antibody-secreting cell (ASC)/plasma cell precursors. These cells contribute to the production of AT1-AAS (Angiotensin II Type 1 Receptor Agonistic Autoantibodies), implicated in preeclampsia pathogenesis. 2) Gestational Diabetes Mellitus: Elevated peripheral and adipose tissue B cells, accompanied by increased pancreatic autoantibodies, suggesting a potential autoimmune component in its development. 3) Preterm Birth: Increased human decidual B cells/B1 cells, B cells expressing progesterone receptor A, and murine splenic and decidual IL-33 receptor-expressing B1 cells. B cells expressing progesterone receptor A are associated with enhanced production of pro-inflammatory cytokines (IL-6, IL-21, TNF-α), potentially contributing to preterm birth. 4) Recurrent Pregnancy Loss: Increased peripheral non-switching memory B cells, endometrial B cells, and autoantibodies. Conversely, there is a decrease in peripheral B cells producing the anti-inflammatory cytokine IL-10.

4.3 B cell secretion

At the maternal-fetal interface, B cells play a role in immune regulation through the secretion of cytokines. Key cytokines such as IL-10, IL-35, PIBF1, and transforming growth factor beta (TGF-β) are crucial in modulating immune responses and ensuring a successful pregnancy (Figure 2A).

IL-10 has anti-inflammatory properties. The IL-10-producing B cells can suppress Th1 and Th17 differentiation and prevent autoimmune disease development or alleviate established disease (63). These B cells can also inhibit T cells' ability to generate tumor necrosis factor alpha (TNF-α) and IFN-γ. Additionally, the IL-10-producing B cells have longer contact times with the $CD4^+CD25^-$ T cells compared to IL-10-negative B cells, promoting the differentiation of these T cells into regulatory T cells (Tregs) (64).

B cells that produce and respond to IL-35 have been found in the uterine draining lymph nodes and spleens of pregnant mice, as well as in the peripheral blood of pregnant women. The function of these B cells is similar to that of the IL-10-producing B cells and contributes to maintaining immune tolerance during gestation (65–67). Serum IL-35 levels increase during a healthy pregnancy but decrease in cases of recurrent spontaneous abortion (68). One potential mechanism by which IL-35 operates during pregnancy is by regulating T-cell responses. B cell-produced IL-35 assists in controlling T cell-mediated autoimmunity by restricting the activation and proliferation of pathogenic T cells. In contrast, the absence of IL-35 enhances T cells responses against infections (69).

Besides IL-10 and IL-35, B cells can also secrete PIBF1 to help prevent pre-term labor in the later stages of pregnancy, an effect that is amplified by the therapeutic administration of interleukin 33 (IL-33) (39). PIBF1 protects normal pregnancy by reducing inflammation through the inhibition of pro-inflammatory cytokines and neutrophil activation, and by modulating immune responses, including the suppression of NK cell activity and support of B cell function (39). Furthermore, TGF-β produced by B cells plays a crucial role in fostering the development of Tregs. Specifically, TGF-β can cause dendritic cells to adopt a tolerogenic phenotype, supporting the expansion and maintenance of Tregs and contributing to an immune-tolerant environment (70). TGF-β is also part of a broader set of mechanisms through which B cells exercise regulatory functions, such as producing other cytokines like IL-10 and IL-35 (71).

4.4 Functional roles of B cell subsets

At the maternal-fetal interface, a unique immunological environment, various B cell subsets play vital roles in maintaining tolerance and immune protection. These subsets include B1 cells, and B2 cells (which encompass Bregs, transitional B cells, naïve B cells, memory B cells, MZ B cells, and plasma cells) (Figure 1B). Each subset exhibits distinct functions and mechanisms, contributing to the delicate balance between immune tolerance and protection in pregnancy (41, 46, 72–81). It is crucial to recognize that significant differences exist between B cells found in peripheral blood and those at the maternal-fetal interface. Due to the challenges associated with obtaining human tissue, most research focuses on peripheral blood B cells. Circulating B cell numbers are lower during pregnancy compared to non-pregnant women, possibly because they are concentrated at the maternal-fetal interface (36, 82).

4.4.1 B1 cells

B1 cells are a subset of B lymphocytes crucial to humoral immune response. These cells predominantly reside in the peritoneal cavity of mice and peripheral blood in humans. Mouse B1 cells specifically express CD19, and B220, low levels of CD23 and IgD, and high levels of CD43 and IgM. Based on CD5 expression, the B1 cells can be categorized into the B1a ($CD5^+$ cells) and B1b ($CD5^-$ cells) subtypes (83). In humans, B1 cells are identified by the presence of $CD20^+CD27^+CD43^+CD70^-$ and may also express $CD5^+$. There is ongoing debate regarding the identification and functional roles of human B1 cells compared to their mouse counterparts.

B1 cells contribute to the production of natural antibodies and autoantibodies in the serum, which are essential for regulating immune responses (84). Additionally, B1 cells participate in mucosal immunity by generating immunoglobulin A (IgA) plasma cells in the lamina propria, with $CD5^-$ B1b cells potentially playing a crucial role in the mucosal IgA system (85). In older animals, B1 and B2 cells are implicated in B cell expansion and plasma cell accumulation. A homeostatic mechanism helps control the deregulated growth of B cells in aged mice. B1a cells, in particular, have been linked to dysregulated growth and tumor development. Studies suggest that peritoneal B1a cells consistently express the Stat3 oncogene, which may predispose them to oncogenesis due to their unique proliferation (86).

B1 cells have a complex role in pregnancy involving a delicate balance between their protective roles and potential for complications. During pregnancy, B1a B cells with high expressions of plasma cell alloantigen 1 (PC1) are present in the mouse peritoneal cavity. These cells generate regulatory substances such as IL-10, which contribute to immune tolerance and safeguard the fetus from maternal immune rejection. This process is achieved by influencing the balance between different T cell populations. Conversely, the transfer of B1a B cells with low PC1 levels has been shown to increase the likelihood of fetal rejection in pregnant mice (87). Damián Oscar Muzzio et al. demonstrated that B1a B cells, found in the mouse peritoneal cavity, have lower CD86 expression during normal pregnancy and suppress the differentiation of pro-inflammatory Th1 and Th17 cells. In contrast, during instances of pregnancy complications, these B1a B cells retain higher CD86 expression levels and promote Th1 and Th17 cell differentiation (74).

Disruptions in B1 cell function or numbers have been linked to pregnancy complications. B1 cells can also produce autoantibodies that target maternal or fetal tissue, causing PE or recurrent spontaneous abortion. These autoantibodies may disturb the critical immune balance needed for a healthy pregnancy (72). It is important to recognize that the precise mechanisms and effects of B1 cells on pregnancy are still under investigation.

4.4.2 Regulatory B cells

In both mice and humans, the Bregs exhibit a variety of markers, including $CD21^{hi}CD23^{+}IgM^{hi}$ (T2-MZP) B cells, $TIM\text{-}1^{+}$ B cells, $CD9^{+}$ B cells, $CD5^{+}$ B1a cells, GIFT15 B cells, and $CD44^{hi}CD138^{+}$ plasmocytes (88). Human Bregs are present in peripheral blood, lymphoid tissues, and the uterus, whereas in mice, they reside in the spleen, lymph nodes, and bone marrow (79, 89).

The primary function of Bregs is to regulate immune responses. They produce anti-inflammatory cytokines, such as IL-10, which suppresses excessive immune reactions and maintains immune balance. This action is vital in preventing autoimmune diseases, where the immune system mistakenly targets the body's cells, and in managing chronic inflammation (90). Furthermore, Bregs control inflammation during infections, ensuring an effective immune response without causing extensive damage to host tissues (91). They also influence other immune cells, including T cells, dendritic cells, and macrophages, through direct cell-to-cell interactions or cytokine secretion.

During pregnancy, Bregs in fetal-maternal interface play a vital role in promoting maternal-fetal tolerance. Along with their functions in the periphery, they also release immunosuppressive cytokines like IL-10, IL-35, and TGF-β1 to reduce inflammatory responses and create a tolerogenic environment (89, 92). They also contribute to tissue remodeling essential for placental development (93). Moreover, Bregs in the uterus or circulation can interact with immune cells, such as T cells, to encourage the development of Tregs and suppress the functions of effector T cells, preventing pregnancy complications (79). Bregs promote the expansion and function of Tregs primarily through the secretion of IL-10 and TGF-β, which create an immunosuppressive environment that enhances the proliferation and stability of Tregs. Additionally, Bregs interact directly with Tregs via cell surface molecules such as CD40 and CD86, further supporting their expansion and suppressive functions. Human peripheral Bregs suppress effector T cell functions through a combination of cytokine production, expression of inhibitory surface markers, induction of Tregs, modulation of antigen-presenting cells, production of proapoptotic molecules, and TLR interactions (94, 95).

4.4.3 Transitional B cells

Transitional B cells in mice and humans are characterized by the expression of specific surface markers CD24 and CD38 (96). They are located in peripheral lymphoid organs, such as the spleen and lymph nodes, where B cells undergo maturation. As the transitional B cells exit the bone marrow, they are tested for autoreactivity (97). If they exhibit strong binding to self-antigens, they are typically eliminated or in specificity through receptor editing. Conversely, if transitional B cells recognize foreign antigens with adequate affinity, they are allowed to continue maturing. This mechanism ensures an effective immune response against pathogens while protecting the body's tissues (98). As a result, the transitional B cells help establish central tolerance by eliminating self-reactive B cells and preventing autoimmunity (99). Moreover, the transitional B cells suppress autoreactive $CD4^{+}$ T cell proliferation, inhibit pro-inflammatory T cell differentiation, promote the conversion of effector T cells into Tregs, and suppress $CD8^{+}$ T cell responses (99).

The role of transitional B cells in pregnancy is yet to be completely understood. As pregnancy progresses, the number of decidual transitional B cells increases, suggesting a protective role in fetal tolerance and immune regulation (78). In contrast, the percentage of transitional B cells in peripheral blood reduces during pregnancy (100). Furthermore, human circulating transitional B cells in the third trimester of pregnancy are capable of producing anti-inflammatory cytokines, such as IL-10, which are crucial for maintaining pregnancy (82).

4.4.4 Naïve B cells

In mice, naive B cells are mainly identified by their surface expression of IgM and IgD. They are primarily located in the bone marrow and secondary lymphoid organs, including the spleen and lymph nodes. Human naive B cells, on the other hand, are marked by IgD and CD27 and are produced in bone marrow and subsequently migrate to the peripheral blood and secondary lymphoid organs (96).

Naive B cells initiate the primary immune response and act as the first line of defense for the adaptive immune system upon an initial pathogen encounter (101). Each naive B cell possesses a distinct BCR, enabling it to recognize a broad range of pathogens. Some naive B cells exhibit SHM to enhance their BCRs' affinity for antigens. When an antigen is encountered, naive B cells activate and differentiate into either plasma cells, which produce antigen-specific antibodies, or memory B cells for long-term immunity (102). Although naive B cells initially produce IgM antibodies, they may transition to other antibody classes, such as immunoglobulin G (IgG), IgA, or immunoglobulin E (IgE), through CSR (103).

During pregnancy, naive B cells can be localized in the placental intervillous blood, a region within the placenta containing maternal blood. The interaction between CCL20, which is produced by trophoblasts and decidual stromal cells, and CCR6, which is present on mature naive B cells, enables the movement of these B cells toward the intervillous blood areas within the placenta (46). These mature naive B cells serve as guardians against pathogens, protecting both the mother and fetus from infections by producing natural antibodies (IgM) in a T-cell independent manner (46). These natural antibodies, which are generated without previous exposure to antigens, can identify and attach themselves to PAMPs and modified self-antigens. This ability enables them to provide an immediate defense against infections, promote the removal of dying cells, manage inflammation, and exhibit anti-cancer properties (104).

4.4.5 Memory B cells

Memory B cells are situated in various locations, including the spleen, lymph nodes, bone marrow, and peripheral blood of both mice and humans. Their primary role is to remember past infections, enabling the body to launch a faster and more effective immune response when encountering the same pathogen again. After an initial infection or vaccination, the memory B cells can remain in the body for years or even decades. If the same germ invades again, these cells can rapidly transform into plasma cells,

producing high-affinity antibodies to neutralize the germ. Thus, memory B cells are vital in immunological memory, offering long-term protection and enabling secondary immune responses (101, 105). In humans, CD27 is a surface marker commonly used for the memory B cells. However, not all memory B cells express CD27 (96, 106). The discovery of other markers, such as PD-L2, CD80, and CD73, has unveiled considerable phenotypic diversity of memory B cells, leading to the identification of at least five unique subsets of these cells (107). Interestingly, Marilen Benner et al. discovered that a subset of memory B cells increases in the decidua during pregnancy (41).

During pregnancy, the primary function memory B cells is to assist the mother's immune system in tolerating the fetus by avoiding the initiation of a strong immune response against fetal antigens. In addition, human circulating memory B cells are prepared to combat infections that may endanger the mother or fetus (108). B cells can identify paternal antigens in the fetus and placenta that are foreign to the mother's immune system, such as specific Human Leukocyte Antigen (HLA) molecules. The memory B cells generated during a woman's first pregnancy can influence her immune response in later pregnancies. If these cells encounter the same paternal antigens in future pregnancies, they can promote faster immune recognition and tolerance. It is noteworthy that uterine-resident B cells may not be involved in the production of alloantibodies. Instead, B cells located outside the uterus predominantly differentiate into plasma cells to produce antibodies (109, 110). Acquiring an in-depth understanding of memory B cells' functions and regulation during pregnancy is essential for addressing complications like spontaneous abortion, PE, and other immune-related pregnancy issues. This knowledge also bears significance for organ transplantation, as the memory B cells specific to HLA antigens can affect transplant acceptance and rejection.

4.4.6 Marginal zone B cells

MZ B cells form a unique subset of B cells located primarily in the spleen's marginal zone. The strategic position allows them to play a vital role in immune responses, particularly in rapidly responding to blood-borne pathogens. MZ B cells can initiate an immune response more quickly than other B cell subsets due to their direct antigen recognition capability, bypassing the need for antigen processing and presentation by other cells. Once an antigen is captured, MZ B cells initiate an immune response by transforming into plasma cells that generate antibodies. They can also present antigens to T cells, triggering a customized adaptive immune response. Additionally, MZ B cells help maintain immune memory, as they can swiftly respond to previously encountered antigens for a potent secondary immune response.

Human and mouse MZ B cells exhibit a distinct set of surface markers. In mice, MZ B cells are marked by a high expression of CD21/CD35 and typically low or negative for CD23, differentiating them from the FO B cells, which exhibit high CD23 expression. Compared to the FO B cells, MZ B cells express higher levels of CD21, CD1d, CD38, CD9, and CD25. Human MZ B cells are generally $CD27^{+}$, a marker shared with memory B cells. Similar to mice, human MZ B cells express high levels of IgM and CD21, and low levels of IgD. Additionally, CD1c is present in human MZ B cells (96, 111).

During pregnancy, the immune system of mice is suppressed to promote fetal tolerance by inhibition of differentiation of T cell-dependent FO B cells, which generate highly specific IgG antibodies. To counterbalance this suppression, the T cell-independent MZ B cells from mouse spleen or lymph nodes form short-lived plasma cells producing non-specific antibodies (IgM/IgA) that protect the mother from infections during pregnancy and minimize the likelihood of producing autoreactive B cells that target fetal tissues (112, 113).

4.4.7 Plasma cells

In both mice and humans, plasma cells are generally recognized by CD138, CD27, and CD38 (114). They are predominantly located in the bone marrow, where they generate and release antibodies. During an immune response, plasma cells may be found in secondary lymphoid organs, including the spleen and lymph nodes (115). These cells are the main producers of antibodies that defend against pathogens by attaching to them, obstructing their entrance into host cells, and triggering the complement cascade. Moreover, the antibodies can coat pathogens, improving their recognition by other immune cells, such as macrophages and neutrophils, which then engulf and eliminate the pathogens (114). Certain plasma cells also secrete cytokines like IL-35 and interleukin 17 (IL-17), which modulate the activity of other immune cells (116).

Plasma cells in the placenta are vital in maintaining immune tolerance during pregnancy by producing specific antibodies that interact with the Fc receptors on various immune cells. This interaction results in suppression of inflammatory responses and promotion of tolerance (117). IgG antibodies can also cross the placenta, providing the fetus with passive immunity against infections and supporting healthy fetal development (118, 119). Additionally, these antibodies can be found in breast milk, offering passive immunity to the infant after birth (120).

For pregnant women with SLE, modulation of circulating plasma cell function can lead to reduced disease severity due to shifting of the immune system towards a more regulatory and less inflammatory state. However, in SLE patients with pregnancy complications, certain plasma cell-related transcriptomic modules may not be downregulated to the same degree as in healthy or uncomplicated SLE pregnancies. This inadequate downregulation may contribute to the persistence or worsening of autoimmune activity (36, 121).

5 Hormonal regulation of B cell development and functions

Hormonal regulation of B cell development and function is a complex process that involves a multitude of hormones and signaling pathways. A notable difference is observed between the genders, with females typically exhibiting higher levels of immunoglobulins IgM and IgG than males, which is thought to

be due to the influence of sex hormones (122). Hormonal regulation not only maintains standard immune responses but also enables the immune system to adapt to the evolving physiological conditions throughout pregnancy. Pregnancy-related hormones, such as human chorionic gonadotropin (hCG), progesterone, and estrogen, are essential for a successful pregnancy (Table 1) (123, 124). Each hormone has a specific role: hCG supports the corpus luteum in the ovary and prompts it to produce progesterone, which prepares the uterus for pregnancy and suppresses the immune response to avoid embryo rejection. Concurrently, estrogen assists in modulating the mother's immune system and promotes fetal growth (125).

In humans, progesterone levels increase following ovulation and stay elevated throughout pregnancy. By interacting with specific receptors, progesterone efficiently suppresses inflammatory immune responses and initiates tolerance pathways. Simultaneously, estrogen concentrations consistently rise until term, modulating immune cell populations and functions to support fetal tolerance through estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). During early pregnancy, hCG levels rapidly escalate, reaching their peak between 9-12 weeks of gestation (125, 125). This hormone serves a vital function in stimulating progesterone production, which in turn assists in maintaining the pregnancy. In combination, these hormones work together to regulate the immune system during pregnancy, fostering a favorable environment for fetal growth.

Estrogen has been demonstrated to have suppressive effects on B cell lymphopoiesis in bone marrow, specifically hindering the differentiation from pro-B cells to pre-B cells and consequently leading to a reduction in B cell precursors (126). This suppressive action is mediated through both direct effects on B cells and indirect effects on stromal cells (127). It is partially attributed to decreased production of the homeostatic cytokine interleukin-7 (IL-7) and increased expression of soluble frizzled-related protein 1 (sFRP1) by bone-lining stromal cells (128, 129). The observed decline in B cell production during pregnancy is suggested to serve as a protective mechanism against the development of autoimmune diseases.

On the other hand, estrogen enhances B cell survival by elevating the expression of anti-apoptotic proteins, such as bcl-2, and fostering the differentiation of B cells into antibody-generating plasma cells. This process leads to an increase in antibody production, particularly IgG and IgA. Estrogen also enhances CSR and SHM in mature B cells, mechanisms that expand antibody diversity and affinity. This is achieved by directly stimulating the transcription and function of activation-induced deaminase (AID), a vital enzyme involved in these processes that are crucial for adaptive immunity (130). Moreover, estrogen affects

TABLE 1 The roles of key hormones in B cell development and function during pregnancy.

Hormones	Secreted organ	Process	Effects	References
Estrogens	Ovaries, placenta	Development	Pro-B, Pre-B, immature B ↓ mature B cells ↑ memory B cells ↑ Plasma B cells ↑ Plasmablasts ↑ Bregs ↑	(142–145)
		Activation	activation-induced deaminase (AID) ↑ CD80 ↓	(146, 147)
		Survival	Apoptosis ↓	(146)
		Antibodies	IgA, IgG1,IgG2 IgG3 and IgE, anti-DNA antibodies↑	(144, 148)
Progesterone	Ovaries, placenta	Development	Pro-B, Pre-B, immature B ↓ Plasma B cells ↓/↑ Plasmablasts ↓	(135, 145, 149)
		Activation	BAFF ↓ CD80 and CD86 ↓ activation-induced deaminase (AID) ↓	(135, 147, 150)
		Survival	Apoptosis ↑	(134)
		Antibodies	IgA, IgG ↑	(135)
Human Chorionic Gonadotropin (hCG)	Placenta	Development	B1 cells ↑ Bregs ↑ plasma cells ↓	(73, 76, 137, 141)
		Activation	/	
		Survival	/	
		Antibodies	IgG ↑	(137)

symbols ↓: Decrease in expression.
symbols ↑: Increase in expression.

the formation of Bregs, contributing to the establishment of an immunosuppressive environment necessary for a successful pregnancy (131).

Progesterone affects B cells through both genomic and non-genomic mechanisms by interacting with progesterone receptors (PR-A and PR-B) present on B cells. It modulates B cell development in the bone marrow by decreasing the amount of BAFF, a key cytokine for B cell maturation and survival. Progesterone can also hinder CSR and SHM of B cells by reducing AID mRNA levels. This is accomplished through binding to the promoter region of the AID gene, which leads to the assembly of an inhibitory transcription complex (132). This inhibition changes the Ig glycosylation patterns and affects the immune response, contributing to the alleviation of autoimmune diseases during pregnancy. The modified glycosylation patterns can reduce the inflammatory potential of antibodies, leading to a more regulated immune response (132, 133). Progesterone also promotes the production of Bregs that generate immunosuppressive cytokines like IL-10 and TGF-β. Moreover, progesterone has been observed to increase the apoptosis of certain B cell lines and suppress the activation of B cells by reducing the expression of co-stimulatory molecules such as CD80 and CD86 on B cells, as seen in an *in vitro* study (134, 135).

The combined effect of progesterone and estrogen enhances humoral immunity during pregnancy by controlling the differentiation and growth of specific B cell subsets, such as memory B cells and plasma cells, as well as modulating Ig production, particularly IgG, through influencing Tfh cells and B cell interactions (136). The interplay between Tfh cells and B cells is vital for generating high-affinity antibodies and establishing immunological memory (26). These hormones boost the activity and functionality of Tfh cells, which in turn offer essential support to B cells, such as providing signals for B cell activation, survival, and differentiation into antibody-producing plasma cells or memory B cells (124).

HCG can bind to the luteinizing hormone/choriogonadotropin receptor (LHCGR) found on B cells, subsequently initiating signaling pathways that influence B cell proliferation. Higher hCG levels have been detected in the serum of PE patients compared to normal pregnancies. The persistently elevated hCG levels appear to stimulate the expansion of $CD19^+$ $CD5^+$ cells in peripheral blood through its receptor, which is highly expressed in these cells. *In vitro* exposure to hCG leads to significant proliferation of $CD19^+$ $CD5^+$ cells. The increased presence of these B cells, capable of producing autoantibodies such as angiotensin II type 1 receptor autoantibodies (AT1-AA), may contribute to the autoimmune-like symptoms observed in PE (73). In contrast, hCG can raise the levels of asymmetrically glycosylated IgG antibodies, an unusual structure of IgG molecules that protect pregnancy by reducing alloreactive immune responses (137–139). In addition, hCG triggers the conversion of $CD19^+$ cells into Bregs in peripheral blood, similar to the effect of estrogen, contributing to pregnancy tolerance (76). It can also promote pregnancy success by enhancing the function of human Bregs for fetal survival (76). Interestingly, hCG has been found to inhibit the differentiation of murine splenic B cells into plasma cells, suggesting that hCG may regulate the function of different B cell subsets variably during pregnancy (140, 141).

In summary, hormonal regulation during pregnancy greatly affects B cell development and functionality. Key hormones such as estrogen, progesterone, and hCG modulate B cell proliferation, survival, antibody production, and the balance of various B cell subsets. This hormonal regulation ensures the immune system appropriately adapts to the distinct demands of pregnancy, maintaining a fine balance between immune tolerance and protection against infections.

6 B cells and pregnancy complications

B cells have been associated with the development of various pregnancy complications, such as PE, gestational diabetes mellitus (GDM), PTB, and RPL (Figure 2B).

6.1 Preeclampsia

PE is a common gestational disease affecting 2-5% of all pregnancies and is the leading cause of prenatal mortality and morbidity. PE is also associated with a high incidence of fetal growth restriction, and short- and long-term maternal and perinatal complications, making it a significant burden on the healthcare system. The etiology of PE is linked to defective trophoblast differentiation and functions, leading to abnormal placental development, high blood pressure, insufficient placental perfusion, and maternal-fetal exchange defects. Impaired fetus-maternal tolerance and altered immune homeostasis are widely believed to be significant factors in the development of PE (151). The systematic immune system is activated while the immunoregulatory system is suppressed in women with PE (152, 153). When activated by AT1-AAs in PE, the anti-angiotensin II type 1 (AT1) receptor, which is expressed by endothelial cells, vascular smooth muscle cells, cardiac myocytes, and renal cells, enhances immune responses by increasing the production of pro-inflammatory cytokines, promoting oxidative stress, and contributing to endothelial dysfunction, thereby disrupting immune homeostasis (151). B cells, with the help of T cells, are thought to produce antibodies that activate the immune system and target the AT1 receptor in women with PE (151). These antibodies can also form immune complexes (ICs) when they bind to target antigens, including those from fetal trophoblasts, which serve as a natural antigenic source during normal pregnancy. Although ICs are present in normal pregnancies, their elevated levels and altered composition in PE can trigger inflammatory responses through circulation or tissue deposition. This may contribute to the inflammatory response and endothelial dysfunction characteristic of the condition (154, 155).

Ai-Hua Liao et al. investigated the functional changes in human peripheral B cells in women with PE compared to healthy ones. The study found that women with PE had a higher percentage of memory B cells ($CD27^+CD38^-$) and plasma cell precursors ($CD27^+CD38^+$). Additionally, irrespective of stimulation, the mean percentages of

generated plasma cells were significantly higher in the PE group. There were also more antibody-producing cells in women with PE following activation. These findings suggest that the functional changes in circulating B cells may contribute to the etiology of PE (156). Federico Jensen et al. found that the frequency of $CD19^+CD5^+$ B1a B cells was significantly higher in the peripheral blood of preeclamptic patients compared to normal pregnant women. These $CD19^+CD5^+$ B cells were identified as the source of AT1-AAs and found in the placenta of preeclamptic patients but are almost absent in normal pregnancies. Additionally, the elevated levels of hCG in the serum and placenta supernatant of these patients drive the increase in $CD19^+CD5^+$ B cells. Approximately 95% of these cells express the hCG receptor and expand upon hCG stimulation, which further promotes the production of pathogenic autoantibodies (73). In animal models of PE, depletion of maternal B cells with rituximab reduces blood pressure and increases fetal weight (157). Reducing the B cell numbers decreases the AT1-AA levels and alleviates PE symptoms (158). Kristin Malinowsky demonstrated that the *in vivo* transfer of B1a B cells, which were strongly activated by progesterone, led to the accumulation of deposits in the mothers' kidneys, resulting in kidney damage (159). This finding highlights the significance of the B cells in PE pathogenesis. Since PE is a complex condition with poorly understood mechanisms, further research is crucial to fully comprehend B cells' contribution to PE.

6.2 Gestational diabetes mellitus

GDM is a condition in which women with no prior history of diabetes exhibit high blood glucose levels during pregnancy, which usually resolve after delivery (160). It is the most prevalent medical complication in pregnancy. The risk factors of GDM include maternal obesity, advanced maternal age, a history of GDM, a family history of type 2 diabetes, and certain ethnic backgrounds. GDM can lead to complications such as hypertensive disorders in the mother, excessive fetal growth and adiposity, and increased long-term risks of diabetes, obesity, and cardiovascular disease for both mother and child (160). GDM is associated with a relative insufficiency in insulin response or β-cell defects that cannot compensate for the increased insulin resistance during pregnancy (161). The significance of B cells in GDM is shown by a positive correlation between the percentage of B cells in peripheral blood and insulin resistance (162), and an increased proportion of adipose tissue B cells in pregnant women with GDM, particularly in those who produce pancreatic autoantibodies (163, 164). These finding suggests that the B cells may serve as a predictor for insulin resistance in women with GDM. Despite these observations, the exact role of B cells in GDM remains uncertain. Further research is needed to clarify the contribution of B cells to the pathophysiology and development of GDM.

6.3 Preterm birth

PTB refers to the delivery of a baby before 37 weeks of gestation. PTB affects approximately 11% of births worldwide, with significant variations due to differences in gestational age measurement and definitions (165–167). Major risk factors for PTB include socio-demographic, nutritional, medical, obstetric, and environmental factors, although most PTB cases occur without clear risk factors. Complications of PTB often involve increased risks of neurodevelopmental impairments, respiratory issues, and gastrointestinal complications, contributing significantly to perinatal morbidity and mortality (168). There is a significant increase in B cells, particularly the B1 cells, in the decidua of women experiencing preterm labor compared to term labor (78). In a mouse model of LPS-induced PTB, significant increases in IL-33 receptor-expressing B1cells in the spleen and decidual B cells are observed during the acute phase, indicating their critical role in the immune mechanisms underlying PTB (169). There is also increased PR-A expression in B cells among women with PTB, along with elevated levels of pro-inflammatory cytokines such as IL-6, IL-21, and TNF-α, particularly in cases of spontaneous PTB and PE/HELLP syndrome. This correlation suggests a pronounced inflammatory response preceding PTB, emphasizing the potential of $PR\text{-}A^+$ B cells as biomarkers for predicting PTB risk (170).

6.4 Recurrent pregnancy loss

RPL is characterized by the failure of two or more clinically recognized pregnancies before reaching 20-24 weeks of gestation, encompassing both embryonic and fetal losses (171). RPL affects approximately 2-5% of couples attempting to conceive (172). RPL can have significant psychological impacts on the affected couples, including grief, anxiety, and depression, and may increase risks of future pregnancy complications (171). RPL has been associated with parental chromosomal abnormalities, maternal thrombotic diseases, endocrine disorders, and immune dysfunction (171, 172). Autoantibodies produced by B cells are associated with RPL (173). The absolute count of non-switching memory B cells from peripheral blood is significantly elevated in women with RPL (14). Moreover, the endometrial B cell levels are higher in women with RPL compared to their healthy counterparts (174). The production of IL-10 by B cells from human peripheral blood, as well as overall IL-10 levels, are lower in women with RPL. And a negative correlation exists between the proportion of these IL-10-producing B cells and serum autoantibody levels in the RPL patients (175). Although there is no relevant human data, the abortion rate in mouse models with a tendency for miscarriage (CBA/J×DBA/2J) decreased after exogenous administration of regulatory B10 cells (138). These findings suggest that RPL may be linked to abnormal maturation and activation of B lymphocytes.

7 B cells and autoimmune: implications for pregnancy and target therapies

The function of B-cells during pregnancy is complex and multifaceted. The development of B cell-targeting therapies offers a promising opportunity for preventing and treating pregnancy

complications. Nonetheless, this approach necessitates a more profound comprehension of the delicate equilibrium between immune tolerance and response throughout pregnancy.

Autoimmune diseases are characterized by dysregulated immune tolerance, which is similar to pregnancy complications. It is worth investigating whether drugs approved for autoimmune diseases could be repurposed for treating pregnancy complications. B-cell dysregulation has been identified as a critical factor in pregnancy-related autoimmune diseases, particularly in conditions such as APS, SLE, and autoimmune thyroid disease (AITD) (176–180). In these disorders, autoreactive B cells produce pathogenic autoantibodies that directly affect pregnancy outcomes. For instance, in APS, B cells generate aPL antibodies that target phospholipid-binding proteins on placental tissues, potentially leading to thrombosis, placental insufficiency, and fetal loss (170). In SLE, autoantibodies such as anti-Ro and anti-La can cross the placenta, possibly causing neonatal lupus and congenital heart block (181). In AITD, thyroid peroxidase (TPO) and thyroglobulin (Tg) antibodies may also cross the placenta, potentially interfering with maternal and fetal thyroid function (182). The discovery of TPO expression in the endometrium and placenta suggests that these antibodies may directly impact reproductive tissues. These antibodies have the potential to cause local damage, activate inflammatory responses, and disrupt local thyroid hormone production, thereby creating an unfavorable environment for embryo implantation and fetal development (177). Furthermore, Lee et al. showed that TPO antibodies might bind directly to embryos (183). B cells further contribute to pregnancy complications through cytokine production, with increased levels of pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-6, which promote placental inflammation and dysfunction (179, 184). B cells can also present autoantigens to T cells, activating them and further intensifying the autoimmune response at the maternal-fetal interface (185). The formation of immune complexes by autoantibodies may trigger complement activation, leading to placental damage and hindered fetal development. Various B cell subsets, including naïve, memory, plasma, and MZ B cells, participate in these processes, while Bregs function to suppress inflammation through IL-10 and TGF-β production (89, 93). This intricate interplay of B cell-mediated mechanisms may result in various pregnancy complications, such as RPL, PE, intrauterine growth restriction, and PTB in women with autoimmune diseases (180).

B cell-targeted therapies have become a significant approach in treating various autoimmune diseases. These encompass several strategies such as: 1) direct depletion using monoclonal antibodies like rituximab, ocrelizumab, and obinutuzumab; 2) indirect depletion by blocking cytokines essential for B cell survival, such as belimumab targeting BAFF; 3) blockade of the co-stimulatory signal with CD40/CD40L antibody; 4) B cell modulation using agents like Bruton Tyrosine Kinase (BTK) inhibitors, Ibrutinib, and acalabrutinib; 5) targeting plasma cells with anti-CD38 antibody daratumumab (179, 186, 187). While first-generation therapies like rituximab have been widely used, second-generation agents and newer approaches, including targeting CD19, proteasome inhibition, and chimeric antigen receptor (CAR) T-cell therapy, have shown improved efficacy in certain conditions (186, 187). These treatments have had varied success across diseases like rheumatoid arthritis, SLE, multiple sclerosis, and ANCA-associated vasculitis, with their effectiveness often depending on the specific disease mechanism and the balance between pathogenic and protective B cell functions (186). In pregnancy, current therapies for B cell-mediated autoimmune disorders focus on managing symptoms and reducing risks, including the cautious use of B cell depletion therapy, intravenous immunoglobulin, hydroxychloroquine, and anticoagulants for conditions like APS (180, 188–191). Future research efforts to develop more targeted and safer treatments, including new B cell-specific therapies, enhancement of regulatory B cell function, development of antigen-specific immunotherapies, and investigation of cytokine-targeted approaches, are needed. There's also growing interest in microbiome modulation, nanoparticle-based drug delivery systems, and gene editing technologies (192–194). Researchers are working to identify biomarkers for personalized treatment and conduct long-term safety studies, with the ultimate goal of developing more effective, tailored treatments that can modulate the immune system with minimal side effects, thus maintaining maternal health while ensuring optimal fetal development.

8 Conclusions and perspectives

In conclusion, the current lack of research on the role B cells play during pregnancy indicates a valuable area for future investigation. Future studies should concentrate on the roles of different B cell subsets, their regulation by hormones and cytokines, and potential therapeutic targets for pregnancy-related disorders. This includes understanding the dynamics of B cells at the maternal-fetal interface and their interactions with other immune cells, such as T cells, NK cells, and DCs. Moreover, the impact of B cell-derived factors, including cytokines, chemokines, and antibodies, on pregnancy outcomes should be explored. Enhancing our understanding of B cells in pregnancy could lead to better strategies for improving maternal and neonatal health.

The role of B cells in pregnancy is critical but underexplored. These cells balance immune tolerance for successful gestation and defense against pathogens. Despite their significance, the specific contributions of B cells to pregnancy outcomes, including complications like PTB and PE, remain unclear. This article overviews the current understanding of B cells' multifaceted functions at the maternal-fetal interface, emphasizing their dual role in creating a favorable environment for fetal development while also being implicated in various pregnancy-related disorders. Bregs and their secretion of IL-10 are crucial in maintaining tolerance towards the fetus, showcasing the body's sophisticated mechanism to prevent immune responses against the semi-allogeneic fetus. In contrast, the production of autoantibodies by certain B cell subsets highlights the complexity of immune regulation, where abnormalities can result in adverse pregnancy outcomes. The paper also emphasizes the significant hormonal regulation of B cell function during pregnancy. These findings suggest that the immune system's adaptability during pregnancy is not only a response to the fetus's presence but also to the hormonal environment, which modulates B cell activity and consequently influences pregnancy outcomes.

This review paper also explores the specific types of B cells at the maternal-fetal interface and clarifies their unique roles. The presence of naive B cells, memory B cells, and plasma cells emphasizes the immune system's readiness to respond to pathogens, ensuring the fetus's protection while maintaining tolerance. Although significant progress has been made in understanding B cell subsets and their activities during pregnancy, many questions still remain. It is important to recognize that identifying B cell subpopulations and their characteristics and roles during pregnancy, particularly within the maternal-fetal interface, is an area of interest because the current identification of these subpopulations is primarily based on studies of human peripheral blood and mouse models. Filling this knowledge gap could have substantial implications for both maternal and fetal health.

A new subset of B lymphocytes, termed age-associated B cells (ABCs), characterized by the expression of $CD11c^{+}CD19^{+}B220^{+}$, has been identified and is associated with infection, aging, and autoimmune disease. Additionally, the long-term effects of B cell dysregulation during pregnancy on both maternal and offspring health should be investigated. This research will help determine if abnormal B cell function during pregnancy has lasting consequences for the health of both the mother and her child and may identify potential targets for interventions to improve long-term health outcomes. By addressing these gaps in knowledge, future research can offer valuable insights into the complex immune regulation that occurs during pregnancy and may lead to the development of novel therapeutic approaches to enhance maternal and fetal health.

The discussion on pregnancy complications and the involvement of B cells provides a new perspective on the pathogenesis of these conditions. The relationship between increased B cell activity, especially in the context of autoantibody production, and the development of conditions such as PE and RPL, highlights the need for further research. Unraveling the molecular mechanisms by which B cells contribute to these complications could lead to the development of innovative therapeutic interventions aimed at modulating B cell activity to prevent or alleviate these conditions. Consequently, potential therapeutic strategies might focus on B cells, including B cell depletion, modulation of B cell function (inhibiting or activating BCR signaling, targeting the co-stimulatory signal required for B cell activation), targeting specific antibodies, and enhancing the function of Bregs. However, due to the unique nature of pregnancy, the safety, timing, and dosage of intervention require careful and comprehensive evaluation.

Author contributions

J-CL: Writing – review & editing, Investigation, Writing – original draft, Data curation, Visualization. QXZ: Writing – original draft, Writing – review & editing, Visualization. Y-GD: Writing – review & editing, Conceptualization, Supervision. WY: Writing – review & editing, Supervision. RL: Supervision, Writing – review & editing. EN: Supervision, Writing – review & editing. CK: Supervision, Writing – review & editing. QQZ: Supervision, Writing – original draft, Writing – review & editing, Conceptualization, Validation. PC: Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Validation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This project was supported by Hong Kong Research Grant Council Grant (17112822), Health and Medical Research Fund (11222326), Guangdong Basic and Applied Basic Research Foundation (2022A1515220017), the High Level-Hospital Program, Health Commission of Guangdong Province, China (HKUSZH201902015), and HKU-SZH Fund for Shenzhen Key Medical Discipline (SZXK2020089).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Fu B, Wei H. Decidual natural killer cells and the immune microenvironment at the maternal-fetal interface. *Sci China Life Sci*. (2016) 59:1224–31. doi: 10.1007/s11427-016-0337-1

2. Ander SE, Diamond MS, Coyne CB. Immune responses at the maternal-fetal interface. *Sci Immunol*. (2019) 4(31):eaat6114. doi: 10.1126/sciimmunol.aat6114

3. Robertson SA, Care AS, Moldenhauer LM. Regulatory T cells in embryo implantation and the immune response to pregnancy. *J Clin Invest*. (2018) 128:4224–35. doi: 10.1172/jci122182

4. Robertson SA, Moldenhauer LM, Green ES, Care AS, Hull ML. Immune determinants of endometrial receptivity: A biological perspective. *Fertil Steril*. (2022) 117:1107–20. doi: 10.1016/j.fertnstert.2022.04.023

5. Popescu D-M, Botting RA, Stephenson E, Green K, Webb S, Jardine L, et al. Decoding human fetal liver haematopoiesis. *Nature*. (2019) 574:365–71. doi: 10.1038/s41586-019-1652-y

6. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. *J Allergy Clin Immunol*. (2013) 131:959–71. doi: 10.1016/j.jaci.2013.01.046

7. Cyster JG, Allen CDC. B cell responses: cell interaction dynamics and decisions. *Cell*. (2019) 177:524–40. doi: 10.1016/j.cell.2019.03.016

8. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies, and more. *Clin J Am Soc Nephrol*. (2016) 11:137–54. doi: 10.2215/cjn.09430915

9. Wang L, Jiang P, Zhao S, Liu H, Liu L, Mor G, et al. The dynamic profile and potential function of B-cell subsets during pregnancy. *Cell Mol Immunol*. (2021) 18:1082–4. doi: 10.1038/s41423-020-00535-1

10. Bartmann C, Segerer SE, Rieger L, Kapp M, Sütterlin M, Kämmerer U. Quantification of the predominant immune cell populations in decidua throughout human pregnancy. *Am J Reprod Immunol*. (2014) 71:109–19. doi: 10.1111/aji.12185

11. Busse M, Campe K-NJ, Nowak D, Schumacher A, Plenagl S, Langwisch S, et al. Il-10 producing B cells rescue mouse fetuses from inflammation-driven fetal death and are able to modulate T cell immune responses. *Sci Rep*. (2019) 9:9335. doi: 10.1038/s41598-019-45860-2

12. Bright P, Grigoriadou S, Kamperidis P, Buckland M, Hickey A, Longhurst HJ. Changes in B cell immunophenotype in common variable immunodeficiency: cause or effect – is bronchiectasis indicative of undiagnosed immunodeficiency? *Clin Exp Immunol*. (2013) 171:195–200. doi: 10.1111/cei.12010

13. Kralickova P, Kurecova B, Andrys C, Krcmova I, Jilek D, Vlkova M, et al. Pregnancy outcome in patients with common variable immunodeficiency. *J Clin Immunol*. (2015) 35:531–7. doi: 10.1007/s10875-015-0188-7

14. Carbone J, Sarmiento E, Gallego A, Lanio N, Navarro J, García S, et al. Peripheral blood T- and B-cell immunophenotypic abnormalities in selected women with unexplained recurrent miscarriage. *J Reprod Immunol*. (2016) 113:50–3. doi: 10.1016/j.jri.2015.11.003

15. Ota K, Dambaeva S, Lee J, Gilman-Sachs A, Beaman K, Kwak-Kim J. Persistent high levels of igm antiphospholipid antibodies in a patient with recurrent pregnancy losses and rheumatoid arthritis. *Am J Reprod Immunol*. (2014) 71:286–92. doi: 10.1111/aji.12196

16. Zeng B, Kwak-Kim J, Liu Y, Liao AH. Treg cells are negatively correlated with increased memory B cells in pre-eclampsia while maintaining suppressive function on autologous B-cell proliferation. *Am J Reprod Immunol*. (2013) 70:454–63. doi: 10.1111/aji.12154

17. Dutta S, Sengupta P, Haque N. Reproductive immunomodulatory functions of B cells in pregnancy. *Int Rev Immunol*. (2020) 39:53–66. doi: 10.1080/08830185.2019.1674299

18. Kutteh WH. Antiphospholipid antibody syndrome and reproduction. *Curr Opin Obstet Gynecol*. (2014) 26:260–5. doi: 10.1097/gco.0000000000000086

19. Knight CL, Nelson-Piercy C. Management of systemic lupus erythematosus during pregnancy: challenges and solutions. *Open Access Rheumatol*. (2017) 9:37–53. doi: 10.2147/oarrr.S87828

20. D'Ippolito S, Barbaro G, Paciullo C, Tersigni C, Scambia G, Di Simone N. Antiphospholipid syndrome in pregnancy: new and old pathogenetic mechanisms. *Int J Mol Sci*. (2023) 24(4):3195. doi: 10.3390/ijms24043195

21. Knight JS, Branch DW, Ortel TL. Antiphospholipid syndrome: advances in diagnosis, pathogenesis, and management. *Bmj*. (2023) 380:e069717. doi: 10.1136/bmj-2021-069717

22. Magatti M, Masserdotti A, Cargnoni A, Papait A, Stefani FR, Silini AR, et al. The role of B cells in pe pathophysiology: A potential target for perinatal cell-based therapy? *Int J Mol Sci*. (2021) 22(7):3405. doi: 10.3390/ijms22073405

23. Yeung HY, Dendrou CA. Pregnancy immunogenetics and genomics: implications for pregnancy-related complications and autoimmune disease. *Annu Rev Genomics Hum Genet*. (2019) 20:73–97. doi: 10.1146/annurev-genom-083118-014943

24. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity*. (2012) 36:13–21. doi: 10.1016/j.immuni.2011.11.017

25. Winkler TH, Mårtensson IL. The role of the pre-B cell receptor in B cell development, repertoire selection, and tolerance. *Front Immunol*. (2018) 9:2423. doi: 10.3389/fimmu.2018.02423

26. Crotty S. T follicular helper cell biology: A decade of discovery and diseases. *Immunity*. (2019) 50:1132–48. doi: 10.1016/j.immuni.2019.04.011

27. Kwak K, Akkaya M, Pierce SK. B cell signaling in context. *Nat Immunol*. (2019) 20:963–9. doi: 10.1038/s41590-019-0427-9

28. Tkachenko A, Kupcova K, Havranek O. B-cell receptor signaling and beyond: the role of igα (Cd79a)/igβ (Cd79b) in normal and Malignant B cells. *Int J Mol Sci*. (2024) 25:10. doi: 10.3390/ijms25010010

29. Wen Y, Jing Y, Yang L, Kang D, Jiang P, Li N, et al. The regulators of bcr signaling during B cell activation. *Blood Sci*. (2019) 1:119–29. doi: 10.1097/bs9.0000000000000026

30. Rickert RC. New insights into pre-bcr and bcr signalling with relevance to B cell Malignancies. *Nat Rev Immunol*. (2013) 13:578–91. doi: 10.1038/nri3487

31. Wang Q, Feng D, Jia S, Lu Q, Zhao M. B-cell receptor repertoire: recent advances in autoimmune diseases. *Clin Rev Allergy Immunol*. (2024) 66(1):76–98. doi: 10.1007/s12016-024-08984-6

32. Ghraichy M, Galson JD, Kelly DF, Trück J. B-cell receptor repertoire sequencing in patients with primary immunodeficiency: A review. *Immunology*. (2018) 153:145–60. doi: 10.1111/imm.12865

33. van de Ven AA, Compeer EB, van Montfrans JM, Boes M. B-cell defects in common variable immunodeficiency: bcr signaling, protein clustering and hardwired gene mutations. *Crit Rev Immunol*. (2011) 31:85–98. doi: 10.1615/critrevimmunol.v31.i2.10

34. Huang J, Zhang W, Zhao Y, Li J, Xie M, Lu Y, et al. Deciphering the intercellular communication network of peripartum decidua that orchestrates delivery. *Front Cell Dev Biol*. (2021) 9:770621. doi: 10.3389/fcell.2021.770621

35. Rechavi E, Somech R. Survival of the fetus: fetal B and T cell receptor repertoire development. *Semin Immunopathol*. (2017) 39:577–83. doi: 10.1007/s00281-017-0626-0

36. Abu-Raya B, Michalski C, Sadarangani M, Lavoie PM. Maternal immunological adaptation during normal pregnancy. *Front Immunol*. (2020) 11:575197. doi: 10.3389/fimmu.2020.575197

37. Aït-Azzouzene D, Gendron MC, Houdayer M, Langkopf A, Bürki K, Nemazee D, et al. Maternal B lymphocytes specific for paternal histocompatibility antigens are partially deleted during pregnancy. *J Immunol*. (1998) 161:2677–83. doi: 10.4049/jimmunol.161.6.2677

38. Aït-Azzouzene D, Caucheteux S, Tchang F, Wantyghem J, Moutier R, Langkopf A, et al. Transgenic major histocompatibility complex class I antigen expressed in mouse trophoblast affects maternal immature B cells1. *Biol Reprod*. (2001) 65:337–44. doi: 10.1095/biolreprod65.2.337

39. Huang B, Faucette AN, Pawlitz MD, Pei B, Goyert JW, Zhou JZ, et al. Interleukin-33-induced expression of pibf1 by decidual B cells protects against preterm labor. *Nat Med*. (2017) 23:128–35. doi: 10.1038/nm.4244

40. Solders M, Gorchs L, Gidlöf S, Tiblad E, Lundell AC, Kaipe H. Maternal adaptive immune cells in decidua parietalis display a more activated and coinhibitory phenotype compared to decidua basalis. *Stem Cells Int*. (2017) 2017:8010961. doi: 10.1155/2017/8010961

41. Benner M, Feyaerts D, García CC, Inci N, López SC, Fasse E, et al. Clusters of tolerogenic B cells feature in the dynamic immunological landscape of the pregnant uterus. *Cell Rep*. (2020) 32:108204. doi: 10.1016/j.celrep.2020.108204

42. Pique-Regi R, Romero R, Tarca AL, Sendler ED, Xu Y, Garcia-Flores V, et al. Single cell transcriptional signatures of the human placenta in term and preterm parturition. *Elife*. (2019) 8:e52004. doi: 10.7554/eLife.52004

43. Toothaker JM, Olaloye O, McCourt BT, McCourt CC, Silva TN, Case RM, et al. Immune landscape of human placental villi using single-cell analysis. *Development*. (2022) 149(8):dev200013. doi: 10.1242/dev.200013

44. Toothaker JM, Presicce P, Cappelletti M, Stras SF, McCourt CC, Chougnet CA, et al. Immune cells in the placental villi contribute to intra-amniotic inflammation. *Front Immunol*. (2020) 11:866. doi: 10.3389/fimmu.2020.00866

45. Garcia-Flores V, Romero R, Tarca AL, Peyvandipour A, Xu Y, Galaz J, et al. Deciphering maternal-fetal cross-talk in the human placenta during parturition using single-cell rna sequencing. *Sci Transl Med*. (2024) 16:eadh8335. doi: 10.1126/scitranslmed.adh8335

46. Solders M, Lundell AC, Gorchs L, Gidlöf S, Tiblad E, Kaipe H. Mature naïve B cells are retained in the placental intervillous blood and positively associate with specific chemokines in full-term healthy pregnancy. *Am J Reprod Immunol*. (2019) 82:e13154. doi: 10.1111/aji.13154

47. Gomez-Lopez N, Estrada-Gutierrez G, Jimenez-Zamudio L, Vega-Sanchez R, Vadillo-Ortega F. Fetal membranes exhibit selective leukocyte chemotaxic activity during human labor. *J Reprod Immunol*. (2009) 80:122–31. doi: 10.1016/j.jri.2009.01.002

48. Astern JM, Collier AC, Kendal-Wright CE. Pre-B cell colony enhancing factor (Pbef/nampt/visfatin) and vascular endothelial growth factor (Vegf) cooperate to increase the permeability of the human placental amnion. *Placenta*. (2013) 34:42–9. doi: 10.1016/j.placenta.2012.10.008

49. Pavlová T, Novák J, Bienertová-Vašků J. The role of visfatin (Pbef/nampt) in pregnancy complications. *J Reprod Immunol*. (2015) 112:102–10. doi: 10.1016/j.jri.2015.09.004

50. Rastogi I, Jeon D, Moseman JE, Muralidhar A, Potluri HK, McNeel DG. Role of B cells as antigen presenting cells. *Front Immunol*. (2022) 13:954936. doi: 10.3389/fimmu.2022.954936

51. Petroff MG, Nguyen SL, Ahn SH. Fetal-placental antigens and the maternal immune system: reproductive immunology comes of age. *Immunol Rev*. (2022) 308:25–39. doi: 10.1111/imr.13090

52. Rizzuto G, Brooks JF, Tuomivaara ST, McIntyre TI, Ma S, Rideaux D, et al. Establishment of Fetomaternal Tolerance through Glycan-Mediated B cell Suppression. *Nature*. (2022) 603:497–502. doi: 10.1038/s41586-022-04471-0

53. Zenclussen AC, Gentile T, Kortebani G, Mazzolli A, Margni R. Asymmetric antibodies and pregnancy. *Am J Reprod Immunol*. (2001) 45:289–94. doi: 10.1111/j.8755-8920.2001.450504.x

54. Yang X, Zhang C, Chen G, Sun C, Li J. Antibodies: the major participants in maternal-fetal interaction. *J Obstet Gynaecol Res*. (2019) 45:39–46. doi: 10.1111/jog.13839

55. Barrientos G, Fuchs D, Schröcksnadel K, Ruecke M, Garcia MG, Klapp BF, et al. Low levels of serum asymmetric antibodies as a marker of threatened pregnancy. *J Reprod Immunol*. (2009) 79:201–10. doi: 10.1016/j.jri.2008.11.002

56. Dubinsky V, Junovich G, Gentile T, Gutiérrez G. Il-6 as a regulatory factor of the humoral response during pregnancy. *Am J Reprod Immunol*. (2008) 60:197–203. doi: 10.1111/j.1600-0897.2008.00614.x

57. Szekeres-Bartho J, Polgar B. Pibf: the double edged sword. Pregnancy and tumor. *Am J Reprod Immunol*. (2010) 64:77–86. doi: 10.1111/j.1600-0897.2010.00833.x

58. Margni RA, Zenclussen AC. During pregnancy, in the context of a th2-type cytokine profile, serum il-6 levels might condition the quality of the synthesized antibodies. *Am J Reprod Immunol*. (2001) 46:181–7. doi: 10.1034/j.1600-0897.2001.d01-1.x

59. Canellada A, Alvarez I, Berod L, Gentile T. Estrogen and progesterone regulate the il-6 signal transduction pathway in antibody secreting cells. *J Steroid Biochem Mol Biol*. (2008) 111:255–61. doi: 10.1016/j.jsbmb.2008.06.009

60. AbdulHussain G, Azizieh F, Makhseed M, Raghupathy R. Effects of progesterone, dydrogesterone and estrogen on the production of th1/th2/th17 cytokines by lymphocytes from women with recurrent spontaneous miscarriage. *J Reprod Immunol*. (2020) 140:103132. doi: 10.1016/j.jri.2020.103132

61. Dieudonné Y, Guffroy A, Poindron V, Sprauel PS, Martin T, Korganow AS, et al. B cells in primary antiphospholipid syndrome: review and remaining challenges. *Autoimmun Rev*. (2021) 20:102798. doi: 10.1016/j.autrev.2021.102798

62. van den Hoogen LL, Bisoendial RJ. B-cells and baff in primary antiphospholipid syndrome, targets for therapy? *J Clin Med*. (2022) 12(1):18. doi: 10.3390/jcm12010018

63. Wu H, Su Z, Barnie PA. The role of B regulatory (B10) cells in inflammatory disorders and their potential as therapeutic targets. *Int Immunopharmacol*. (2020) 78:106111. doi: 10.1016/j.intimp.2019.106111

64. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Muñoz-Suano A, Kamanaka M, et al. Mice lacking endogenous il-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of th1/th17 but a decrease in regulatory T cells. *J Immunol*. (2011) 186:5569–79. doi: 10.4049/jimmunol.1100284

65. Liu J, Chen X, Hao S, Zhao H, Pang L, Wang L, et al. Human chorionic gonadotropin and il-35 contribute to the maintenance of peripheral immune tolerance during pregnancy through mediating the generation of il-10+ or il-35+ Breg cells. *Exp Cell Res*. (2019) 383:111513. doi: 10.1016/j.yexcr.2019.111513

66. Slawek A, Lorek D, Kedzierska AE, Chelmonska-Soyta A. Regulatory B cells with il-35 and il-10 expression in a normal and abortion-prone murine pregnancy model. *Am J Reprod Immunol*. (2020) 83:e13217. doi: 10.1111/aji.13217

67. Guzman-Genuino RM, Diener KR. Chapter 4 - B cell pathology and recurrent pregnancy loss. In: Kwak-Kim J, editor. *Immunology of Recurrent Pregnancy Loss and Implantation Failure*. Elsevier_Amsterdam, Netherlands: Academic Press (2022). p. 55–70.

68. Yue CY, Zhang B, Ying CM. Elevated serum level of il-35 associated with the maintenance of maternal-fetal immune tolerance in normal pregnancy. *PloS One*. (2015) 10:e0128219. doi: 10.1371/journal.pone.0128219

69. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, et al. Il-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature*. (2014) 507:366–70. doi: 10.1038/nature12979

70. Seeger P, Musso T, Sozzani S. The tgf-B Superfamily in dendritic cell biology. *Cytokine Growth Factor Rev*. (2015) 26:647–57. doi: 10.1016/j.cytogfr.2015.06.002

71. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity*. (2015) 42:607–12. doi: 10.1016/j.immuni.2015.04.005

72. Muzzio D, Zenclussen AC, Jensen F. The role of B cells in pregnancy: the good and the bad. *Am J Reprod Immunol*. (2013) 69:408–12. doi: 10.1111/aji.12079

73. Jensen F, Wallukat G, Herse F, Budner O, El-Mousleh T, Costa SD, et al. Cd19 +Cd5+ Cells as indicators of preeclampsia. *Hypertension*. (2012) 59:861–8. doi: 10.1161/hypertensionaha.111.188276

74. Muzzio DO, Soldati R, Rolle L, Zygmunt M, Zenclussen AC, Jensen F. B-1a B cells regulate T cell differentiation associated with pregnancy disturbances. *Front Immunol*. (2014) 5:6. doi: 10.3389/fimmu.2014.00006

75. Muzzio D, Zygmunt M, Jensen F. The role of pregnancy-associated hormones in the development and function of regulatory B cells. *Front Endocrinol (Lausanne)*. (2014) 5:39. doi: 10.3389/fendo.2014.00039

76. Rolle L, Memarzadeh Tehran M, Morell-García A, Raeva Y, Schumacher A, Hartig R, et al. Cutting edge: il-10-producing regulatory B cells in early human pregnancy. *Am J Reprod Immunol*. (2013) 70:448–53. doi: 10.1111/aji.12157

77. Fu M, Zhang X, Liu C, Lyu J, Liu X, Zhong S, et al. Phenotypic and functional alteration of cd45+ Immune cells in the decidua of preeclampsia patients analyzed by mass cytometry (Cytof). *Front Immunol*. (2022) 13:1047986. doi: 10.3389/fimmu.2022.1047986

78. Leng Y, Romero R, Xu Y, Galaz J, Slutsky R, Arenas-Hernandez M, et al. Are B cells altered in the decidua of women with preterm or term labor? *Am J Reprod Immunol*. (2019) 81:e13102. doi: 10.1111/aji.13102

79. Guzman-Genuino RM, Hayball JD, Diener KR. Regulatory B cells: dark horse in pregnancy immunotherapy? *J Mol Biol*. (2021) 433:166596. doi: 10.1016/j.jmb.2020.07.008

80. Muzzio DO, Ziegler KB, Ehrhardt J, Zygmunt M, Jensen F. Marginal zone B cells emerge as a critical component of pregnancy well-being. *Reproduction*. (2016) 151:29–37. doi: 10.1530/rep-15-0274

81. Esteve-Solé A, Teixidó I, Deyà-Martínez A, Yagüe J, Plaza-Martín AM, Juan M, et al. Characterization of the highly prevalent regulatory cd24(Hi)Cd38(Hi) B-cell population in human cord blood. *Front Immunol*. (2017) 8:201. doi: 10.3389/fimmu.2017.00201

82. Kövér Á, Lampé R, Szabó K, Tarr T, Papp G. A comprehensive investigation into the distribution of circulating B cell subsets in the third trimester of pregnancy. *J Clin Med*. (2022) 11(11):3006. doi: 10.3390/jcm11113006

83. Srikakulapu P, Pattarabanjird T, Upadhye A, Bontha SV, Osinski V, Marshall MA, et al. B-1b cells have unique functional traits compared to B-1a cells at homeostasis and in aged hyperlipidemic mice with atherosclerosis. *Front Immunol*. (2022) 13:909475. doi: 10.3389/fimmu.2022.909475

84. She Z, Li C, Wu F, Mao J, Xie M, Hun M, et al. The role of B1 cells in systemic lupus erythematosus. *Front Immunol*. (2022) 13:814857. doi: 10.3389/fimmu.2022.814857

85. Meyer-Bahlburg A. B-1 cells as a source of iga. *Ann N Y Acad Sci*. (2015) 1362:122–31. doi: 10.1111/nyas.12801

86. Aziz M, Holodick NE, Rothstein TL, Wang P. The role of B-1 cells in inflammation. *Immunol Res*. (2015) 63:153–66. doi: 10.1007/s12026-015-8708-3

87. Schumacher A, Ehrentraut S, Scharm M, Wang H, Hartig R, Morse HC3rd, et al. Plasma cell alloantigen 1 and il-10 secretion define two distinct peritoneal B1a B cell subsets with opposite functions, pc1(High) cells being protective and pc1(Low) cells harmful for the growing fetus. *Front Immunol*. (2018) 9:1045. doi: 10.3389/fimmu.2018.01045

88. Baba Y, Saito Y, Kotetsu Y. Heterogeneous subsets of B-lineage regulatory cells (Breg cells). *Int Immunol*. (2019) 32:155–62. doi: 10.1093/intimm/dxz068

89. Esteve-Solé A, Luo Y, Vlagea A, Deyà-Martínez Á, Yagüe J, Plaza-Martín AM, et al. B regulatory cells: players in pregnancy and early life. *Int J Mol Sci*. (2018) 19:2099. doi: 10.3390/ijms19072099

90. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol*. (2012) 30:221–41. doi: 10.1146/annurev-immunol-020711-074934

91. Catalán D, Mansilla MA, Ferrier A, Soto L, Oleinika K, Aguillón JC, et al. Immunosuppressive mechanisms of regulatory B cells. *Front Immunol*. (2021) 12:611795. doi: 10.3389/fimmu.2021.611795

92. Ghaebi M, Nouri M, Ghasemzadeh A, Farzadi L, Jadidi-Niaragh F, Ahmadi M, et al. Immune regulatory network in successful pregnancy and reproductive failures. *Biomed Pharmacother*. (2017) 88:61–73. doi: 10.1016/j.biopha.2017.01.016

93. Guzman-Genuino RM, Diener KR. Regulatory B cells in pregnancy: lessons from autoimmunity, graft tolerance, and cancer. *Front Immunol*. (2017) 8:239670. doi: 10.3389/fimmu.2017.00172

94. Berthelot J-M, Jamin C, Amrouche K, Le Goff B, Maugars Y, Youinou P. Regulatory B cells play a key role in immune system balance. *Joint Bone Spine*. (2013) 80:18–22. doi: 10.1016/j.jbspin.2012.04.010

95. Kessel A, Haj T, Peri R, Snir A, Melamed D, Sabo E, et al. Human cd19 +Cd25high B regulatory cells suppress proliferation of cd4+ T cells and enhance foxp3 and ctla-4 expression in T-regulatory cells. *Autoimmun Rev*. (2012) 11:670–7. doi: 10.1016/j.autrev.2011.11.018

96. Velounias RL, Tull TJ. Human B-cell subset identification and changes in inflammatory diseases. *Clin Exp Immunol*. (2023) 210:201–16. doi: 10.1093/cei/uxac104

97. Vossenkämper A, Spencer J. Transitional B cells: how well are the checkpoints for specificity understood? *Archivum Immunol Therapiae Experimentalis*. (2011) 59:379–84. doi: 10.1007/s00005-011-0135-0

98. Martin VG, Wu Y-CB, Townsend CL, Lu GHC, O'Hare JS, Mozeika A, et al. Transitional B cells in early human B cell development – time to revisit the paradigm? *Front Immunol*. (2016) 7:546. doi: 10.3389/fimmu.2016.00546

99. Zhou Y, Zhang Y, Han J, Yang M, Zhu J, Jin T. Transitional B cells involved in autoimmunity and their impact on neuroimmunological diseases. *J Trans Med*. (2020) 18:131. doi: 10.1186/s12967-020-02289-w

100. Ziegler KB, Muzzio DO, Matzner F, Bommer I, Ventimiglia MS, Malinowsky K, et al. Human pregnancy is accompanied by modifications in B cell development and immunoglobulin profile. *J Reprod Immunol*. (2018) 129:40–7. doi: 10.1016/j.jri.2018.07.003

101. Weisel F, Shlomchik M. Memory B cells of mice and humans. *Annu Rev Immunol*. (2017) 35:255–84. doi: 10.1146/annurev-immunol-041015-055531

102. Weisel NM, Joachim SM, Smita S, Callahan D, Elsner RA, Conter LJ, et al. Surface phenotypes of naive and memory B cells in mouse and human tissues. *Nat Immunol*. (2022) 23:135–45. doi: 10.1038/s41590-021-01078-x

103. Stavnezer J, Schrader CE. Igh chain class switch recombination: mechanism and regulation. *J Immunol*. (2014) 193:5370–8. doi: 10.4049/jimmunol.1401849

104. Schwartz-Albiez R, Monteiro RC, Rodriguez M, Binder CJ, Shoenfeld Y. Natural antibodies, intravenous immunoglobulin and their role in autoimmunity, cancer and inflammation. *Clin Exp Immunol*. (2009) 158 Suppl 1:43–50. doi: 10.1111/j.1365-2249.2009.04026.x

105. Seifert M, Küppers R. Human memory B cells. *Leukemia*. (2016) 30:2283–92. doi: 10.1038/leu.2016.226

106. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A new population of cells lacking expression of cd27 represents a notable component of the B

cell memory compartment in systemic lupus erythematosus. *J Immunol.* (2007) 178:6624–33. doi: 10.4049/jimmunol.178.10.6624

107. Tomayko MM, Steinel NC, Anderson SM, Shlomchik MJ. Cutting edge: hierarchy of maturity of murine memory B cell subsets. *J Immunol.* (2010) 185:7146–50. doi: 10.4049/jimmunol.1002163

108. Sung N, Byeon HJ, Garcia MDS, Skariah A, Wu L, Dambaeva S, et al. Deficiency in memory B cell compartment in a patient with infertility and recurrent pregnancy losses. *J Reprod Immunol.* (2016) 118:70–5. doi: 10.1016/j.jri.2016.09.003

109. Nellore A, Killian JT Jr., Porrett PM. Memory B cells in pregnancy sensitization. *Front Immunol.* (2021) 12:688987. doi: 10.3389/fimmu.2021.688987

110. Sanz-Ureña S, Llinás L, Eguía J, Olivé A, Yang S, Karahan G, et al. Fc 112: hla-specific memory B cells and paternal hla molecular mismatch in female transplant candidates sensitized by pregnancy. *Nephrol Dialysis Transplant*. (2022) 37:gfac123. 001. doi: 10.1093/ndt/gfac123.001

111. Pillai S, Cariappa A, Moran ST. Marginal zone B cells. *Annu Rev Immunol.* (2005) 23:161–96. doi: 10.1146/annurev.immunol.23.021704.115728

112. Chappell CP, Draves KE, Giltiay NV, Clark EA. Extrafollicular B cell activation by marginal zone dendritic cells drives T cell-dependent antibody responses. *J Exp Med*. (2012) 209:1825–40. doi: 10.1084/jem.20120774

113. Dutta S, Sengupta P. Functions of follicular and marginal zone B cells in pregnancy. *Asian Pac J Reprod.* (2018) 7:191–2. doi: 10.4103/2305-0500.237058

114. Sanz I, Wei C, Jenks SA, Cashman KS, Tipton C, Woodruff MC, et al. Challenges and opportunities for consistent classification of human B cell and plasma cell populations. *Front Immunol*. (2019) 10:2458. doi: 10.3389/fimmu.2019.02458

115. Cancro MP, Tomayko MM. Memory B cells and plasma cells: the differentiative continuum of humoral immunity. *Immunol Rev*. (2021) 303:72–82. doi: 10.1111/imr.13016

116. Hilgenberg E, Ries S, Shen P, Fillatreau S. From the regulatory functions of B cells to the identification of cytokine-producing plasma cell subsets. *Curr Opin Immunol.* (2014) 28:77–83. doi: 10.1016/j.coi.2014.02.009

117. Kim CJ, Romero R, Chaemsaithong P, Kim JS. Chronic inflammation of the placenta: definition, classification, pathogenesis, and clinical significance. *Am J Obstet Gynecol.* (2015) 213:S53–69. doi: 10.1016/j.ajog.2015.08.041

118. Palmeira P, Quinello C, Silveira-Lessa AL, Zago CA, Carneiro-Sampaio M. Igg placental transfer in healthy and pathological pregnancies. *Clin Dev Immunol.* (2012) 2012:985646. doi: 10.1155/2012/985646

119. Simister NE. Placental transport of immunoglobulin G. *Vaccine*. (2003) 21:3365–9. doi: 10.1016/s0264-410x(03)00334-7

120. Atyeo C, Alter G. The multifaceted roles of breast milk antibodies. *Cell.* (2021) 184:1486–99. doi: 10.1016/j.cell.2021.02.031

121. Hong S, Banchereau R, Maslow B-SL, Guerra MM, Cardenas J, Baisch J, et al. Longitudinal profiling of human blood transcriptome in healthy and lupus pregnancy. *J Exp Med.* (2019) 216:1154–69. doi: 10.1084/jem.20190185

122. Wilkinson NM, Chen HC, Lechner MG, Su MA. Sex differences in immunity. *Annu Rev Immunol.* (2022) 40:75–94. doi: 10.1146/annurev-immunol-101320-125133

123. Nair RR, Verma P, Singh K. Immune-endocrine crosstalk during pregnancy. *Gen Comp Endocrinol.* (2017) 242:18–23. doi: 10.1016/j.ygcen.2016.03.003

124. Santana-Sánchez P, Vaquero-García R, Legorreta-Haquet MV, Chávez-Sánchez L, Chávez-Rueda AK. Hormones and B-cell development in health and autoimmunity. *Front Immunol.* (2024) 15:1385501. doi: 10.3389/fimmu.2024.1385501

125. Schumacher A, Costa SD, Zenclussen AC. Endocrine factors modulating immune responses in pregnancy. *Front Immunol*. (2014) 5:196. doi: 10.3389/fimmu.2014.00196

126. Chakraborty B, Byemerwa J, Krebs T, Lim F, Chang C-Y, McDonnell DP. Estrogen receptor signaling in the immune system. *Endocrine Rev*. (2022) 44:117–41. doi: 10.1210/endrev/bnac017

127. Cunningham M, Gilkeson G. Estrogen receptors in immunity and autoimmunity. *Clin Rev Allergy Immunol.* (2011) 40:66–73. doi: 10.1007/s12016-010-8203-5

128. Yokota T, Oritani K, Garrett KP, Kouro T, Nishida M, Takahashi I, et al. Soluble frizzled-related protein 1 is estrogen inducible in bone marrow stromal cells and suppresses the earliest events in lymphopoiesis. *J Immunol.* (2008) 181:6061–72. doi: 10.4049/jimmunol.181.9.6061

129. Miyaura C, Onoe Y, Inada M, Maki K, Ikuta K, Ito M, et al. Increased B-lymphopoiesis by interleukin 7 induces bone loss in mice with intact ovarian function: similarity to estrogen deficiency. *Proc Natl Acad Sci U.S.A*. (1997) 94:9360–5. doi: 10.1073/pnas.94.17.9360

130. Casali P, Shen T, Xu Y, Qiu Z, Chupp DP, Im J, et al. Estrogen reverses hdac inhibitor-mediated repression of aicda and class-switching in antibody and autoantibody responses by downregulation of mir-26a. *Front Immunol*. (2020) 11:491. doi: 10.3389/fimmu.2020.00491

131. Moulton VR. Sex hormones in acquired immunity and autoimmune disease. *Front Immunol.* (2018) 9:2279. doi: 10.3389/fimmu.2018.02279

132. Pauklin S, Petersen-Mahrt SK. Progesterone inhibits activation-induced deaminase by binding to the promoter. *J Immunol*. (2009) 183:1238–44. doi: 10.4049/jimmunol.0803915

133. Förger F, Villiger PM. Immunological adaptations in pregnancy that modulate rheumatoid arthritis disease activity. *Nat Rev Rheumatol*. (2020) 16:113–22. doi: 10.1038/s41584-019-0351-2

134. McMurray RW, Suwannaroj S, Ndebele K, Jenkins JK. Differential effects of sex steroids on T and B cells: modulation of cell cycle phase distribution, apoptosis and bcl-2 protein levels. *Pathobiology*. (2001) 69:44–58. doi: 10.1159/000048757

135. Zhang L, Chang KK, Li MQ, Li DJ, Yao XY. Mouse endometrial stromal cells and progesterone inhibit the activation and regulate the differentiation and antibody secretion of mouse B cells. *Int J Clin Exp Pathol.* (2014) 7:123–33.

136. Monteiro C, Kasahara TM, Castro JR, Sacramento PM, Hygino J, Centurião N, et al. Pregnancy favors the expansion of circulating functional follicular helper T cells. *J Reprod Immunol.* (2017) 121:1–10. doi: 10.1016/j.jri.2017.04.007

137. Fettke F, Schumacher A, Canellada A, Toledo N, Bekeredjian-Ding I, Bondt A, et al. Maternal and fetal mechanisms of B cell regulation during pregnancy: human chorionic gonadotropin stimulates B cells to produce il-10 While alpha-fetoprotein drives them into apoptosis. *Front Immunol*. (2016) 7:495. doi: 10.3389/fimmu.2016.00495

138. Jensen F, Muzzio D, Soldati R, Fest S, Zenclussen AC. Regulatory B10 cells restore pregnancy tolerance in a mouse model. *Biol Reprod.* (2013) 89:90. doi: 10.1095/biolreprod.113.110791

139. Schumacher A. Human chorionic gonadotropin as a pivotal endocrine immune regulator initiating and preserving fetal tolerance. *Int J Mol Sci.* (2017) 18(10):2166. doi: 10.3390/ijms18102166

140. Hammarström L, Fuchs T, Smith CI. The immunodepressive effect of human glucoproteins and their possible role in the nonrejection process during pregnancy. *Acta Obstet Gynecol Scand.* (1979) 58:417–22. doi: 10.3109/00016347909154059

141. Nikolaevich KN, Ivanovich SJ, Victorovich SS. Major reproduction hormones as regulators of cell-to-cell interactions in humoral immune responses. *Brain Behav Immun*. (1991) 5:149–61. doi: 10.1016/0889-1591(91)90013-Z

142. Medina KL, Garrett KP, Thompson LF, Rossi MI, Payne KJ, Kincade PW. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. *Nat Immunol.* (2001) 2:718–24. doi: 10.1038/90659

143. Thurmond TS, Murante FG, Staples JE, Silverstone AE, Korach KS, Gasiewicz TA. Role of estrogen receptor alpha in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. *Endocrinology*. (2000) 141:2309–18. doi: 10.1210/endo.141.7.7560

144. Erlandsson MC, Jonsson CA, Islander U, Ohlsson C, Carlsten H. Oestrogen receptor specificity in oestradiol-mediated effects on B lymphopoiesis and immunoglobulin production in male mice. *Immunology*. (2003) 108:346–51. doi: 10.1046/j.1365-2567.2003.01599.x

145. Monteiro C, Kasahara T, Sacramento PM, Dias A, Leite S, Silva VG, et al. Human pregnancy levels of estrogen and progesterone contribute to humoral immunity by activating T(Fh)/B cell axis. *Eur J Immunol.* (2021) 51:167–79. doi: 10.1002/eji.202048658

146. Fu Y, Li L, Liu X, Ma C, Zhang J, Jiao Y, et al. Estrogen promotes B cell activation in vitro through down-regulating cd80 molecule expression. *Gynecol Endocrinol.* (2011) 27:593–6. doi: 10.3109/09513590.2010.507281

147. Pauklin S, Sernández IV, Bachmann G, Ramiro AR, Petersen-Mahrt SK. Estrogen directly activates aid transcription and function. *J Exp Med.* (2009) 206:99–111. doi: 10.1084/jem.20080521

148. Aguilar-Pimentel JA, Cho YL, Gerlini R, Calzada-Wack J, Wimmer M, Mayer-Kuckuk P, et al. Increased estrogen to androgen ratio enhances immunoglobulin levels and impairs B cell function in male mice. *Sci Rep*. (2020) 10:18334. doi: 10.1038/s41598-020-75059-9

149. Muzzio DO, Soldati R, Ehrhardt J, Utpatel K, Evert M, Zenclussen AC, et al. B cell development undergoes profound modifications and adaptations during pregnancy in mice. *Biol Reprod.* (2014) 91:115. doi: 10.1095/biolreprod.114.122366

150. Drehmer MN, Suterio DG, Muniz YC, de Souza IR, Löfgren SE. Baff expression is modulated by female hormones in human immune cells. *Biochem Genet*. (2016) 54:722–30. doi: 10.1007/s10528-016-9752-y

151. Deer E, Herrock O, Campbell N, Cornelius D, Fitzgerald S, Amaral LM, et al. The role of immune cells and mediators in preeclampsia. *Nat Rev Nephrol.* (2023) 19:257–70. doi: 10.1038/s41581-022-00670-0

152. Rambaldi MP, Weiner E, Mecacci F, Bar J, Petraglia F. Immunomodulation and preeclampsia. *Best Pract Res Clin Obstet Gynaecol.* (2019) 60:87–96. doi: 10.1016/j.bpobgyn.2019.06.005

153. Saito S, Shiozaki A, Nakashima A, Sakai M, Sasaki Y. The role of the immune system in preeclampsia. *Mol Aspects Med*. (2007) 28:192–209. doi: 10.1016/j.mam.2007.02.006

154. Derzsy Z, Prohászka Z, Rigó J Jr., Füst G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol.* (2010) 47:1500–6. doi: 10.1016/j.molimm.2010.01.021

155. Massobrio M, Benedetto C, Bertini E, Tetta C, Camussi G. Immune complexes in preeclampsia and normal pregnancy. *Am J Obstet Gynecol.* (1985) 152:578–83. doi: 10.1016/0002-9378(85)90631-3

156. Liao AH, Liu LP, Ding WP, Zhang L. Functional changes of human peripheral B-lymphocytes in pre-eclampsia. *Am J Reprod Immunol.* (2009) 61:313–21. doi: 10.1111/j.1600-0897.2009.00697.x

157. Campbell N, Herrock O, Solise D, Fitzgerald S, Deer E, Turner T, et al. Abstract 19: maternal B cell depletion lowers blood pressure and improves fetal weights in offspring in a rat model of preeclampsia. *Hypertension*. (2021) 78:A19–A. doi: 10.1161/hyp.78.suppl_1.19

158. LaMarca B, Wallace K, Herse F, Wallukat G, Martin JN Jr., Weimer A, et al. Hypertension in response to placental ischemia during pregnancy: role of B lymphocytes. *Hypertension*. (2011) 57:865–71. doi: 10.1161/hypertensionaha.110.167569

159. Malinowsky K. The role of gonadotrophins (Hcg) and female sex hormones in B1-a B cell deregulation in a preeclampsia model. *[dissertation]*. Greifswald: Universitätsmedizin Greifswald. (2020).

160. McIntyre HD, Catalano P, Zhang C, Desoye G, Mathiesen ER, Damm P. Gestational diabetes mellitus. *Nat Rev Dis Primers*. (2019) 5:47. doi: 10.1038/s41572-019-0098-8

161. Sweeting A, Wong J, Murphy HR, Ross GP. A clinical update on gestational diabetes mellitus. *Endocr Rev*. (2022) 43:763–93. doi: 10.1210/endrev/bnac003

162. Zhuang Y, Zhang J, Li Y, Gu H, Zhao J, Sun Y, et al. B lymphocytes are predictors of insulin resistance in women with gestational diabetes mellitus. *Endocr Metab Immune Disord Drug Targets*. (2019) 19:358–66. doi: 10.2174/1871530319666190101130300

163. Cinkajzlová A, Anderlová K, Šimják P, Lacinová Z, Kloučková J, Kratochvílová H, et al. Subclinical inflammation and adipose tissue lymphocytes in pregnant females with gestational diabetes mellitus. *J Clin Endocrinol Metab*. (2020) 105(11):dgaa528. doi: 10.1210/clinem/dgaa528

164. McElwain CJ, McCarthy FP, McCarthy CM. Gestational diabetes mellitus and maternal immune dysregulation: what we know so far. *Int J Mol Sci*. (2021) 22:4261. doi: 10.3390/ijms22084261

165. Crump C. Preterm birth and mortality in adulthood: A systematic review. *J Perinatol*. (2020) 40:833–43. doi: 10.1038/s41372-019-0563-y

166. Walani SR. Global burden of preterm birth. *Int J Gynaecol Obstet*. (2020) 150:31–3. doi: 10.1002/ijgo.13195

167. Vogel JP, Chawanpaiboon S, Moller A-B, Watananirun K, Bonet M, Lumbiganon P. The global epidemiology of preterm birth. *Best Pract Res Clin Obstet Gynaecol*. (2018) 52:3–12. doi: 10.1016/j.bpobgyn.2018.04.003

168. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet*. (2008) 371:75–84. doi: 10.1016/s0140-6736(08)60074-4

169. Valeff N, Juriol L, Quadrana F, Muzzio DO, Zygmunt M, Quiroga MF, et al. 9. *Front Immunol*. (2020) 11:446. doi: 10.3389/fimmu.2020.00446

170. Campe KJ, Redlich A, Zenclussen AC, Busse M. An increased proportion of progesterone receptor a in peripheral B cells from women who ultimately underwent spontaneous preterm birth. *J Reprod Immunol*. (2022) 154:103756. doi: 10.1016/j.jri.2022.103756

171. Dimitriadis E, Menkhorst E, Saito S, Kutteh WH, Brosens JJ. Recurrent pregnancy loss. *Nat Rev Dis Primers*. (2020) 6:98. doi: 10.1038/s41572-020-00228-z

172. El Hachem H, Crepaux V, May-Panloup P, Descamps P, Legendre G, Bouet P-E. Recurrent pregnancy loss: current perspectives. *Int J Women's Health*. (2017) 9:331–45. doi: 10.2147/IJWH.S100817

173. D'Ippolito S, Ticconi C, Tersigni C, Garofalo S, Martino C, Lanzone A, et al. The pathogenic role of autoantibodies in recurrent pregnancy loss. *Am J Reprod Immunol*. (2020) 83:e13200. doi: 10.1111/aji.13200

174. Ângelo-Dias M, Martins C, Dias SS, Borrego LM, Lima J. Association of B cells with idiopathic recurrent pregnancy loss: A systematic review and meta-analysis. *Int J Mol Sci*. (2022) 23(23):15200. doi: 10.3390/ijms232315200

175. Danaii S, Ghorbani F, Ahmadi M, Abbaszadeh H, Koushaeian L, Soltani-Zangbar MS, et al. Il-10-producing B cells play important role in the pathogenesis of recurrent pregnancy loss. *Int Immunopharmacol*. (2020) 87:106806. doi: 10.1016/j.intimp.2020.106806

176. van den Hoogen LL, Bisoendial RJ. B-cells and baff in primary antiphospholipid syndrome, targets for therapy? *J Clin Med*. (2023) 12:18. doi: 10.3390/jcm12010018

177. Rahnama R, Mahmoudi A-R, Kazemnejad S, Salehi M, Ghahiri A, Soltanghoraee H, et al. Thyroid peroxidase in human endometrium and placenta: A potential target for anti-tpo antibodies. *Clin Exp Med*. (2021) 21:79–88. doi: 10.1007/s10238-020-00663-y

178. Canny SP, Jackson SW. B cells in systemic lupus erythematosus: from disease mechanisms to targeted therapies. *Rheum Dis Clin North Am*. (2021) 47:395–413. doi: 10.1016/j.rdc.2021.04.006

179. Lin X, Lu L. B cell-mediated autoimmune diseases. In: Wang J-Y, editor. *B Cells in Immunity and Tolerance*. Springer Singapore, Singapore (2020). p. 145–60.

180. Somers EC. Pregnancy and Autoimmune diseases. *Best Pract Res Clin Obstet Gynaecol*. (2020) 64:3–10. doi: 10.1016/j.bpobgyn.2019.11.004

181. Tan Y, Yang S, Liu Q, Li Z, Mu R, Qiao J, et al. Pregnancy-related complications in systemic lupus erythematosus. *J Autoimmun*. (2022) 132:102864. doi: 10.1016/j.jaut.2022.102864

182. Vissenberg R, Manders VD, Mastenbroek S, Fliers E, Afink GB, Ris-Stalpers C, et al. Pathophysiological aspects of thyroid hormone disorders/thyroid peroxidase autoantibodies and reproduction. *Hum Reprod Update*. (2015) 21:378–87. doi: 10.1093/humupd/dmv004

183. Lee YL, Ng HP, Lau KS, Liu WM, WS O, Yeung WS, et al. Increased fetal abortion rate in autoimmune thyroid disease is related to circulating tpo autoantibodies in an autoimmune thyroiditis animal model. *Fertil Steril*. (2009) 91:2104–9. doi: 10.1016/j.fertnstert.2008.07.1704

184. Yockey LJ, Iwasaki A. Interferons and proinflammatory cytokines in pregnancy and fetal development. *Immunity*. (2018) 49:397–412. doi: 10.1016/j.immuni.2018.07.017

185. Piccinni M-P, Lombardelli L, Logiodice F, Kullolli O, Parronchi P, Romagnani S. How pregnancy can affect autoimmune diseases progression? *Clin Mol Allergy*. (2016) 14:11. doi: 10.1186/s12948-016-0048-x

186. Barnas JL, Looney RJ, Anolik JH. B cell targeted therapies in autoimmune disease. *Curr Opin Immunol*. (2019) 61:92–9. doi: 10.1016/j.coi.2019.09.004

187. Jung SM, Kim WU. Targeted immunotherapy for autoimmune disease. *Immune Netw*. (2022) 22:e9. doi: 10.4110/in.2022.22.e9

188. D'Mello RJ, Hsu CD, Chaiworapongsa P, Chaiworapongsa T. Update on the use of intravenous immunoglobulin in pregnancy. *Neoreviews*. (2021) 22:e7–e24. doi: 10.1542/neo.22-1-e7

189. Velikova T, Sekulovski M, Bogdanova S, Vasilev G, Peshevska-Sekulovska M, Miteva D, et al. Intravenous immunoglobulins as immunomodulators in autoimmune diseases and reproductive medicine. *Antibodies*. (2023) 12:20. doi: 10.3390/antib12010020

190. Liu Y, Wei Y, Zhang Y, Yang H. Hydroxychloroquine significantly decreases the risk of preeclampsia in pregnant women with autoimmune disorders: A systematic review and meta-analysis. *Clin Rheumatol*. (2023) 42:1223–35. doi: 10.1007/s10067-022-06496-2

191. Capecchi M, Abbattista M, Ciavarella A, Uhr M, Novembrino C, Martinelli I. Anticoagulant therapy in patients with antiphospholipid syndrome. *J Clin Med*. (2022) 11:6984. doi: 10.3390/jcm11236984

192. Pabst O, Nowosad CR. B cells and the intestinal microbiome in time, space and place. *Semin Immunol*. (2023) 69:101806. doi: 10.1016/j.smim.2023.101806

193. Li H, Yang YG, Sun T. Nanoparticle-based drug delivery systems for induction of tolerance and treatment of autoimmune diseases. *Front Bioeng Biotechnol*. (2022) 10:889291. doi: 10.3389/fbioe.2022.889291

194. Serra P, Santamaria P. Nanoparticle-based approaches to immune tolerance for the treatment of autoimmune diseases. *Eur J Immunol*. (2018) 48:751–6. doi: 10.1002/eji.201747059

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 24 October 2024
DOI 10.3389/fimmu.2024.1451552

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Buenos Aires, Argentina

REVIEWED BY
Howie Seay,
Cellares Cell Therapy Solutions, South San Francisco, United States
Mahmood Jeddi-Tehrani,
Avicenna Research Institute, Academic Center for Education, Culture and Research, Tehran, Iran

*CORRESPONDENCE
Silvia Sánchez-Ramón
ssramon@salud.madrid.org

RECEIVED 20 June 2024
ACCEPTED 03 October 2024
PUBLISHED 24 October 2024

CITATION
de la Fuente-Munoz E, Fernández-Arquero M, Subbhi-Issa N, Guevara-Hoyer K, Suárez LP, Laborda RG, Sánchez M, Ochoa-Grullón J, Guzmán-Fulgencio M, Villegas Á, Mansilla MD, Pérez N, Cornudella RS, Gastañaga-Holguera T, Urrutia MC, García IC and Sánchez-Ramón S (2024) Recurrent reproductive failure and celiac genetic susceptibility, a leading role of gluten. *Front. Immunol.* 15:1451552. doi: 10.3389/fimmu.2024.1451552

COPYRIGHT
© 2024 de la Fuente-Munoz, Fernández-Arquero, Subbhi-Issa, Guevara-Hoyer, Suárez, Laborda, Sánchez, Ochoa-Grullón, Guzmán-Fulgencio, Villegas, Mansilla, Pérez, Cornudella, Gastañaga-Holguera, Urrutia, García and Sánchez-Ramón. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Recurrent reproductive failure and celiac genetic susceptibility, a leading role of gluten

Eduardo de la Fuente-Munoz[1,2], Miguel Fernández-Arquero[1,2], Nabil Subbhi-Issa[1,2], Kissy Guevara-Hoyer[1,2,3], Lydia Pilar Suárez[4], Raquel Gil Laborda[1], Marina Sánchez[2], Juliana Ochoa-Grullón[1,2], María Guzmán-Fulgencio[1,2], Ángela Villegas[1,2], María Dolores Mansilla[1,2], Noelia Pérez[4], Ricardo Savirón Cornudella[4], Teresa Gastañaga-Holguera[4], Marta Calvo Urrutia[4], Ignacio Cristóbal García[4] and Silvia Sánchez-Ramón[1,2]*

[1]Department of Clinical Immunology, Instituto de Medicina de Laboratorio (IML) and Fundación para la Investigación Biomédica del Hospital Clínico San Carlos (IDISCC), Hospital Universitario Clínico San Carlos, Madrid, Spain, [2]Department of Immunology, Ophthalmology and Otorhinolaryngology (ENT), School of Medicine, Universidad Complutense, Madrid, Spain, [3]Cancer Immunomonitoring and Immune-mediated Diseases Research Unit, Instituto de Investigación Sanitaria San Carlos (Fundación para la Investigación Biomédica del Hospital Clínico San Carlos (IDISCC)), Department of Clinical Immunology, Hospital Universitario Clínico San Carlos, Madrid, Spain, [4]Department of Obstetrics and Gynecology, Hospital Universitario Clínico San Carlos, Madrid, Spain

Introduction: The prevalence of gluten-related disorders, mainly celiac disease (CD) and non-celiac gluten sensitivity (NCGS), varies between 0.6% and 13% in the general population. There is controversial evidence regarding the association of both CD and NCGS with extra-digestive manifestations, including recurrent reproductive failure (RRF), which may have clinical implications.

Objective: To study the prevalence of HLA susceptibility alleles for CD/NCGS in a cohort of female patients with RRF from a single reference center and to evaluate the effect of a gluten-free diet on reproductive success.

Material and methods: A retrospective study was conducted on 173 patients with RRF, consecutively attended at the Reproductive Immunology Unit of San Carlos University Clinical Hospital in Madrid. We collected and analyzed the clinical, analytical, and immunological profiles of RRF patients who presented HLA alleles associated with CD and NCGS (HLA DQ2.2, DQ2.5, DQ8, and DQ7.5).

Results: We observed a significantly higher prevalence of HLA alleles associated with CD and NCGS in our RRF cohort compared to the prevalence in the general population (69% vs. 35%–40%, p<0.0001). Only 2.3% of patients met the criteria for a CD diagnosis. In our RRF cohort, HLA-genetic susceptibility for CD/NCGS (HLA-risk group) was associated with a significantly higher rate of hypothyroidism compared to patients without these alleles (HLA-negative group) (48.7% vs. 26.92%, p=0.03). Patients with HLA-genetic susceptibility for CD/NCGS and thyroid disease had a significantly higher success rate in the subsequent pregnancy after management (55% vs. 30%, p=0.002). Two factors were found to be significant in this group: a gluten-free diet (p=0.019) and the use of levothyroxine (p=0.042).

Conclusions: In our cohort of RRF patients, we observed a significantly higher prevalence of HLA susceptibility genes for CD/NCGS compared to the general population, also associated with a higher incidence of thyroid alterations. A gluten-free diet and the use of levothyroxine in cases of thyroid pathology had significant beneficial effects on pregnancy outcomes. We suggest that HLA typing for CD/NCGS and a gluten-free diet, in the presence of risk alleles, can improve pregnancy outcomes in RRF patients.

KEYWORDS

recurrent reproductive failure (RRF), non-celiac-gluten-sensitivity (NCGS), gluten, celiac disease (CD), HLA

Introduction

Celiac disease (CD) is an immune-mediated disorder associated with gluten intake, primarily manifesting with digestive symptoms, that affects 0.6% –1% of the general population. Currently, there are several methods for diagnosing and monitoring CD, including duodenal biopsy (histopathology and study of epithelial lymphogram) or peripheral blood tests (anti-transglutaminase antibodies, anti-gliadin antibodies, anti-endomysial antibodies, or HLA class II typing for identifying CD-related genetic susceptibility) (1, 2) However, despite these advancements and the growing knowledge of the disease, CD is still considered underdiagnosed, which may lead to long-term complications (3). Cumulative evidence from the last few decades suggests that the spectrum of CD represents a multisystemic disease, with multiple organs and tissues affected (such as the skin, kidneys, nervous system, or endocrine system) (1).

The cornerstone of treatment to date is to follow a strict gluten-free diet, which usually controls the different symptoms and minimizes the risk of severe complications, such as intestinal lymphoma (1, 2).

More recently, the spectrum of conditions related to CD has expanded to include other pathologies associated with wheat and/or gluten intake, such as gluten allergy and non-celiac gluten sensitivity (NCGS). Gluten allergy is an IgE-mediated type 1 hypersensitivity disease to gluten antigens. It has a well-established pathophysiological basis and specific biomarkers. However, these parameters are not as clearly defined in NCGS (1, 4, 5). NCGS is thus considered an emerging systemic (both intestinal and extraintestinal) disease in the context of wheat intake, affecting patients who do not meet the diagnosis criteria for CD or gluten allergy, often due to the absence of autoantibodies. As with CD, symptoms improve or subside when gluten is removed from the diet (4, 6). The correlation between NCGS and HLA is not well understood, although some studies show a correlation of up to 100% in these patients (4). NCGS prevalence varies between 0.6% and 13% in the general population (5).

There is compelling evidence that CD can cause reproductive alterations in men (7) and especially in women, such as sterility, intrauterine growth restriction, and miscarriages (8–12). However, very few studies have assessed the effects of NCGS and HLA-genetic susceptibility for CD/NCGS on the reproductive health of couples (13).

According to the World Health Organization, infertility and sterility affect millions of people worldwide and have a significant negative impact on both individuals and society (14, 15). There is no precise data on the prevalence of these pathologies, although it is estimated that only 30% of pregnancies reach term (16) and that between 2% and 5% of couples suffer recurrent miscarriages (17). The causes of these fertility problems can be multiple, including autoimmune alterations among others (anatomical, hormonal, infectious, genetic, and nutritional) (16). In approximately 50% of recurrent miscarriage cases, the underlying factor remains unidentified, highlighting the need for continued research into new causes, associations, and potential biomarkers.

In this study, we aimed to determine the prevalence of HLA-genetic susceptibility for CD and NCGS in a real-life cohort of patients with RRF. We also sought to better characterize this subgroup of patients and describe the potential beneficial effects of gluten withdrawal from the diet on reproductive success.

Materials and methods

Study design

This is a retrospective observational study, conducted at the Reproductive Immunology Unit, Clinical Immunology Department of the San Carlos Clinical University Hospital in Madrid. The data of 173 patients who were consecutively studied between February 2018 and April 2022 were analyzed. Recurrent pregnancy loss (RPL) was defined as the loss of two or more pregnancies, including non-visualized pregnancy losses, in accordance with the European Society for Human Reproduction and Embryology (ESHRE) Guidelines. Repeated implantation failure (RIF) was defined as the failure to achieve a clinical pregnancy after more than three high-quality embryo transfers or after the transfer of ≥10 embryos

in multiple transfers in women under 40 years old. Fetal death (FD) was defined as a composite outcome that included women with a history of late fetal loss (between 22 weeks and 28 weeks of pregnancy) and stillbirth (after 28 weeks gestational age).

The following data were collected from the clinical histories: age; personal history with special emphasis on the diagnosis of CD; gluten consumption; digestive, neurological, dermatological, gynecological, and endocrinological diseases or symptoms; smoking habit; previous clinical diagnoses; number of miscarriages; number of *in vitro* fertilization (IVF) cycles; and anatomical/infectious alterations detected by ultrasound or hysteroscopy. Endometriomas and adenomyosis were classified as endometriosis. Endometritis was diagnosed via hysteroscopy, supported by microbiological culture and biopsy confirming the presence of $CD138^+$ cells.

All patients underwent a baseline immunological study as part of the routine clinical workup for RRF, which included the detection of antinuclear antibodies, anti-thyroid antibodies (anti-thyroid peroxidase and anti-thyroglobulin antibodies), IgA anti-transglutaminase, IgG anti-deamidated gliadin and antiphospholipid antibodies (including IgM and IgG anti-cardiolipin and IgM and IgG anti-B2 glycoprotein antibodies), C3 and C4 complement levels, and relative and absolute values of NK cells in peripheral blood. HLA class II typing for identifying CD/NCGS- related genetic alleles (DQ2.2, DQ2.5, DQ8, and DQ7.5) was also performed. Additionally, other factors such as basic coagulation parameters, basal blood glucose and insulin, TSH and free T4 levels, and vitamin levels (B9, B12, and vitamin D) were collected and analyzed.

We analyzed the prevalence of alleles associated with susceptibility to CD/NCGS (HLA DQ2.2, DQ2.5, DQ8, and DQ7.5) and correlated these with analytical parameters and clinical data, such as alterations in the glucose profile, thyroid, presence of endometritis or endometriosis, gastrointestinal or neurological symptoms (specifically chronic migraines), skin alterations, polycystic ovary syndrome, presence of antiphospholipid antibodies, antinuclear antibodies, or expanded cytotoxic NK cells.

Alteration of the glycemic profile was defined as a basal glycemia higher than 100 mg/dL or a Homeostatic Model Assessment (HOMA) higher than 3. The HOMA was calculated using the formula: (glucose × insulin)/405.

Thyroid abnormalities were assessed when TSHs were above 2.5 uIU/ mL or when thyroid-specific autoantibodies were detected. Previous diagnosis of hypothyroidism was also considered (18–20).

Gestational success was defined as the birth of a live newborn at or beyond 37 weeks of pregnancy. The pregnancy success rate was determined in the next 12 months following the evaluation.

The Ethics Committee of our hospital approved the study protocol (FIS PI19/01450), and all subjects provided signed informed consent.

Laboratory tests

Autoimmunity tests were performed as routine samples in the Clinical Immunology laboratory using specific technologies and procedures. For anti-transglutaminase, anti-deamidated gliadin, and anti-peroxidase and anti-thyroglobulin antibodies determination, an ELISA (AESKU.GROUP, Wendelsheim, Germany) technique was used. The detection of antinuclear antibodies was performed by indirect immunofluorescence (AESKU.GROUP, Wendelsheim, Germany). The antiphospholipid antibodies were detected using Luminex technology (Bio- Rad Laboratories, Hercules, CA, USA), the evaluation of the complement system by turbidimetry (The Binding Site Group Ltd., Birmingham, UK), and the determination of NK ($CD3^-CD16^+CD56^+$) lymphocyte values by flow cytometry (Becton-Dickinson, San Jose, CA, USA). All techniques were performed and validated following the manufacturer's instructions.

BD MultiTEST™ CD3 fluorescein isothiocyanate (FITC)/ $CD16^+$CD56 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP)/CD19 allophycocyanin (APC) was used to study the NK cells. First, NK cells were gated by singlets and $CD45^+$ and side scatter appropriate for lymphocytes. Then were gated by $CD3^-$ and last for $CD16^+$ and $CD56^+$ and $CD19^-$. The absolute values of NK cells were calculated through the relative value obtained by flow cytometry and the absolute value of total lymphocytes in the blood count. The cutoff point used to consider pbNK cells expanded was 13%.

In order to extract the DNA from fresh peripheral blood leukocytes, we used MagNA Pure Compact Nucleic Acid Isolation Kit (Roche®, Darmstadt, Germany) following the manufacturer's procedures. All samples were genotyped for HLA-DRB1, HLA-DQA1, and HLA-DQB1 by polymerase chain reaction–sequence-specific oligonucleotide probe (PCR-SSOP) (Thermo Fisher, Waltham, USA), where PCR products were hybridized onto oligonucleotide probes attached to microspheres and labeled with streptavidin-conjugated phycoerythrin. These beads were analyzed with the Luminex® 100/200 TM System (Luminex Corp., Austin, TX, USA), which is based on flow cytometry and uses the principles of xMAP® Technology, as previously described (21).

The allelic results obtained were analyzed by an immunology specialist. The haplotypes were classified according to the following distribution: DQ2.5, DQA1 0501/0505 DQB1 0201/0202; DQ2.2, DQA 0201 DQB1 0201/0202; DQ8, DQA1 0301/0302 DQB1 0302; and DQ7.5, DQA1 0501/0505 DQB1 0301.

Statistical analysis

Descriptive data are presented as median ± standard deviation (SD). Statistical Product and Service Solutions (SPSS) software version 20 (Chicago, IL, USA) was used for descriptive and statistical data analysis. Comparisons between groups were made using the chi-square test (χ^2); median comparison were made using Student "t" test; $p \leq 0.05$ was considered statistically significant.

Results

Epidemiological and obstetric features

Of the 173 patients with recurrent reproductive failure analyzed, 112 had recurrent miscarriages (RMs), 51 had recurrent

10.3389/fimmu.2024.1451552

implantation failure (RIF), and five patients were referred preventively due to underlying immune pathology with risk of miscarriages or obstetric complications and five due to a history of previous fetal death. The mean age of the patients was 38.02 years.

Noteworthy, 121 patients out of 173 (69.94%) presented HLA-genetic susceptibility for CD/NCGS (HLA-DQ2.2, DQ2.5, DQ8, and/or DQ7.5), namely, HLA-risk group, while 52 patients (30.06%) did not present any of these alleles, HLA-negative group. No significant differences in age were found between both groups. Within the HLA-risk group, the distribution of the different haplotypes was as follows: DQ2.5 of 17.35%, DQ2.2 of 33.05%, DQ8 of 19.00%, and DQ7.5 of 12.39%. Additionally, 18.18% of the cohort had the presence of two different risk alleles.

In the HLA-risk group, 63.6% presented RM, 31.4% RIF, 2.48% history of FD, and 2.48% were referred due to underlying immune disorders associated with risk of RM. With respect to CD prevalence, 4 out of the 173 (2.3%) patients had been previously diagnosed with CD: three of them presented RM, and one previous FD. No statistical differences in obstetric morbidity were found compared to the HLA-negative group (Table 1).

Regarding clinical manifestations, the HLA-risk group presented significantly higher thyroid abnormalities compared to the HLA-negative group (48.76% vs. 26.92%, p=0.0368). However, no statistically significant differences were found between the HLA-risk group and the HLA-negative group for other parameters, such as glucose alterations, endometritis, endometriosis, dysmenorrhea, polycystic ovary syndrome, gastrointestinal symptoms, chronic migraines, presence of antiphospholipid antibodies, antinuclear antibodies, nor expanded percentages of cytotoxic NK lymphocytes (Table 1).

Only two patients were positive for anti-transglutaminase IgA antibodies and one for anti-deamidated gliadin IgG antibodies, all of them in the HLA-risk group.

In the HLA-risk group, successful pregnancy was achieved in 67 patients (55.37%). Two statistically significant differences were observed: first, gluten intake emerged as a key factor, with the success group having a higher percentage of patients on a gluten-free diet (p=0.01). The exclusion of gluten intake in HLA-risk patients was associated with an increased likelihood of gestational success, yielding an odds ratio (OR) of 2.791 (IC, 1.166–6.679; p=0.02). Second, the use for levothyroxine also showed a significant impact. Of the 56 (46.2%) patients with TSH levels above 2.5 who were treated with levothyroxine, 37 achieved a successful pregnancy (p=0.042), as detailed in Supplementary Table S1. However, no statistically significant differences were found in levothyroxine use when comparing the HLA-risk group with the non-HLA risk group (46.2% vs. 36.53%, p=0.17). Of these patients, 38 underwent IVF due to a prior diagnosis of RIF. For IVF patients, a gluten-free diet was recommended at least 1 month before the embryo transfer, whereas, in other cases, the diet was introduced immediately following their evaluation.

Within the HLA-risk group, 33 women withdrew the intake of gluten, of which 24 (72.72%) achieved a successful pregnancy. A total

TABLE 1 Epidemiological, clinical data and obstetric features of the HLA-susceptibility group and HLA-negative group patients.

Variable	HLA-risk group N = 121	HLA-negative group N = 52	*p*-value
Age (years)	38.14 ± 3.55	37.73 ± 4.35	0.25
Obstetric features			
RM	77 (63.63%)	35 (67.30%)	0.6
RIF	38 (31.4%)	13 (25.00%)	0.39
Fetal death	3 (3.84%)	2 (3.84%)	0.6
Previous miscarriages	1.86 ± 1.72	1.76 ± 1.35	0.71
Previous embryo transfer	1.72 ± 2.14	1.30 ± 1.75	0.21
Dysmenorrhea	73 (60.3%)	31 (59.6%)	0.92
Endometritis	14 (11.57%)	3 (5.76%)	0.23
Endometriosis	12 (9.91%)	5 (9.8%)	0.95
Clinical and analytical features			
Hypothyroidism	59 (48.76%)	17 (26.92%)	**0.03***
Expanded cytotoxic pbNK	43 (35%)	19 (36%)	0.76
Positive ANA	19 (15.7%)	12 (23%)	0.24
Positive antiphospholipid antibodies	23 (19%)	11 (21%)	0.74
Positive anti-thyroid antibodies	19 (15.7%)	5 (9.6%)	0.28
Chronic migraine	38 (31.40%)	18 (35%)	0.59

Values are expressed as median (SD) and absolute count (percentage). χ^2 test was performed. *p ≤ 0.05 was considered statistically significant. RMs, recurrent miscarriages; RIF, recurrent implantation failure; ANA, antinuclear antibodies ; pbNK, peripheral blood natural killer.
Bolded values indicate statistical significance with p < 0.05.

of 88 women did not make any change in their diet, and 43 (48.86%) of them achieved a successful pregnancy (p=0.019) (Table 2).

No statistical differences were observed regarding the use of levothyroxine in the HLA-risk group with gluten-free diet or normal diet (51% vs. 44%, p=0.47).

Discussion

In this real-life retrospective observational study, the first notable finding in our cohort is the high prevalence of HLA susceptibility alleles for CD/NCGS, 69.94%, almost doubling the 35%–40% prevalence reported in our population (22–25). Among patients in the HLA-risk group, a significantly higher incidence of thyroid pathology was observed. Additionally, it is noteworthy that both gluten withdrawal and levothyroxine replacement therapy, when necessary, were associated with a significantly higher rate of pregnancy success compared to patients who did not receive such treatment.

Thyroid disorders have also been described as a risk factor for pregnancy loss, and levothyroxine therapy is the standard treatment in these cases. Indeed, patients who received this drug in the HLA-risk group showed a higher pregnancy success rate. Few previous studies have explored the use of a gluten-free diet to treat patients with Hashimoto's thyroiditis with good results in hormone levels (26, 27). HLA-DQ2 and HLA-DQ8 present gluten peptides to $CD4^+$ T cells in the intestinal lamina propria, inducing immune activation and TH1 cell differentiation, which, in turn, drives an inflammatory process through the production of the inflammatory cytokines interferon (IFN)-γ and interleukin (IL)-21 (28). As previously mentioned, the line between non-celiac and seronegative gluten sensitivity is difficult to distinguish, especially since the pathophysiology is not yet fully understood. The data, *a priori*, suggest that the immune response is based more on the innate component, through toll-like receptors (TLR1 and TLR2) but presents an adaptive immunity factor through cytokines such as IFN-y and IL-15 (1, 5). It is important to highlight the role of NK cells during implantation of the fetus and placentation, as they play a fundamental part in the uterine tissue remodeling processes (29–31). The immunological alterations described preferentially affect this cell lineage (32–34). Although IL-15 is essential for the development of NK cells, continuous presence can lead to a functional defect in these cells due to exhaustion caused by a metabolic defect (35, 36).

This inflammatory response is not localized as in CD; however, since it is less intense, the effects may not be as visible or detectable. In fact, NCGS has also been associated with systemic autoimmunity symptoms such as psoriasis, thyroid disease, polyarthritis, antiphospholipid syndrome, rheumatoid arthritis, systemic sclerosis, and mixed connective tissue disease. Additionally, there have been cases where these diseases have improved significantly with the removal of gluten from the diet, even though they were refractory to immunosuppressive therapy (37).

There were no statistically significant differences in clinical manifestations between the two groups nor was the HLA-risk group associated with other typical alterations of CD such as endometritis, altered blood glucose, or the presence of ANAs or antiphospholipid antibodies (38–41).

CD and gluten-related conditions are prevalent in our environment and are on the rise due to various factors (1–3). Several studies have expressed concern that it may be an underdiagnosed disease (2, 3) and the importance of increasing awareness of atypical or extraintestinal manifestations, or even silent disease, which may progress to severe complications such as intestinal lymphoma.

Gluten-related disorders in human reproduction are still under investigation. Most studies have been conducted on patients with a clear diagnosis of CD. Among the observed alterations, amenorrhea, early menopause, recurrent miscarriages, lower pregnancy rate, placental dysfunction, low birth weight, intrauterine growth retardation, and increased risk of cesarean section have been reported (8–12).

More studies are necessary to determine the underlying pathophysiological mechanisms, which may be related to dysbiosis, inflammation, or even malabsorption, and the subsequent deficiency in micronutrients or a combination of these factors. Although the exact pathophysiological mechanisms by which these alterations occur are not completely known; in celiac patients, there is an improvement, as with the rest of the symptoms, with the withdrawal of gluten (11).

In recent years, the term polyautoimmunity has gained importance, which is defined as the presence of more than one well-characterized autoimmune disease in the same patient (42). As with other autoimmune diseases, where associations between multiple diseases with overlapping symptoms or laboratory abnormalities are observed, similar phenomena can be observed in celiac disease (1). A higher prevalence of autoimmune thyroid disease or type 1 diabetes mellitus in these patients is well documented and has been attributed to the genetic link between HLA-DQ2 and/or DQ8 and DR3 and DR4 (26). Additionally, the prevalence of autoimmune diseases in first-degree relatives is also increased, such as autoimmune thyroiditis or type 1 diabetes, as previously mentioned but also inflammatory bowel disease, Sjögren's syndrome, lupus, Addison's disease, autoimmune hepatitis, rheumatoid arthritis, primary biliary cirrhosis, and psoriasis among others (43).

CD has also been associated with the presence of antinuclear and antiphospholipid antibodies, known immunological factors of RRF (13, 41). This background of autoimmunity and inflammation has been described as an additional risk factor during pregnancy and the pursuit of pregnancy (44).

To date, in addition to classic CD, different forms of the spectrum have been described, such as potential CD (positive autoantibodies without mucosal lesion), silent CD (positive

TABLE 2 Gestational success and diet of HLA-risk group.

Pregnancy outcome	Gluten- free diet	Normal diet
No. (n=121)	33	88
Gestational success	**24 (72.72%)***	43 (48.86%)
Unsuccessful	9 (27.27%)	45 (51.13%)

Chi-square test value (χ^2)=5.531, *p=0.019 (p ≤ 0.05 was considered statistically significant). Bolded values indicate statistical significance with p < 0.05.

autoantibodies, presence of mucosal lesion but no symptoms), seronegative CD (absence of autoantibodies and mucosal lesion but with symptoms), wheat allergy (IgE-mediated pathology), and NCGS (1–3). However, some authors suggest greater complexity, suggesting that these diseases may represent expressions of a biological continuum (41).

The European guidelines for the study of CD include reproductive disorders as one of the extraintestinal symptoms of the disease. Furthermore, HLA class II typing is recommended when another immunological disease coexists (1). However, the current ESHRE guidelines do not recommend serology screening for this disease unless digestive symptoms are present, and they do not currently include HLA class II typing as part of the evaluation (45). The data obtained suggest that, in patients with suspected immunological alterations, HLA typing can provide clinically relevant information about the underlying cause and guide therapeutic measures, such as a gluten-free diet, the effectiveness of which was demonstrated in this study.

Conclusions

Immunological alterations derived from gluten intake affect a significant percentage of the population with genetic susceptibility. Patients seeking pregnancy, especially those with RRF, are of particular interest. Our study shows a significant association between the presence of class II susceptibility alleles for CD/NCGS and RRF, and hypothyroidism. A gluten-free diet has been shown to be an effective and safe therapeutic alternative for these patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Hospital Clínico San Carlos Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

EF-M: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. MF-A: Formal analysis, Methodology, Resources, Supervision, Validation, Writing – original draft. NS-I: Conceptualization, Investigation, Software, Writing – review & editing. KG-H: Formal analysis, Supervision, Validation, Visualization, Writing – review & editing. LS: Resources, Supervision, Validation, Writing – review & editing. RL: Data curation, Methodology, Writing – review & editing. MS: Data curation, Methodology, Writing – review & editing. JO-G: Investigation, Supervision, Validation, Writing – review & editing. MG-F: Investigation, Supervision, Validation, Writing – review & editing. ÁV: Data curation, Formal analysis, Investigation, Writing – review & editing. MM: Data curation, Writing – review & editing. NP: Resources, Validation, Writing – review & editing. RC: Resources, Supervision, Validation, Writing – review & editing. TG-H: Resources, Supervision, Validation, Writing – review & editing. MU: Resources, Validation, Writing – review & editing. IG: Resources, Supervision, Validation, Writing – review & editing. SS-R: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1451552/full#supplementary-material

SUPPLEMENTARY TABLE 1
Gestational success and levothyroxine of HLA-risk Group. Chi-square test value (X2)=4.1281, *P=0.042 ($p \leq 0.05$ was considered a statistically significant value).

References

1. Al-Toma A, Volta U, Auricchio R, Castillejo G, Sanders DS, Cellier C. European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. *United Eur Gastroenterol J*. (2019) 7:583–613. doi: 10.1177/2050640619844125

2. Raiteri A, Granito A, Giamperoli A, Catenaro T, Negrini G, Tovoli F. Current guidelines for the management of celiac disease: A systematic review with comparative analysis. *World J Gastroenterol*. (2022) 28:154–75. doi: 10.3748/wjg.v28.i1.154

3. Choung RS, Larson SA, Khaleghi S, Rubio-Tapia A, Ovsyannikova IG, King KS, et al. Prevalence and morbidity of undiagnosed celiac disease from a community-based study. *Gastroenterology*. (2017) 152:830–839.e5. doi: 10.1053/j.gastro.2016.11.043

4. Molina-Infante J, Santolaria S, Montoro M, Esteve M, Fernández-Bañares F. Sensibilidad al gluten no celiaca: una revisión crítica de la evidencia actual [Non-celiac gluten sensitivity: a critical review of current evidence. *Gastroenterol Hepatol*. (2014) 37:362–71. doi: 10.1016/j.gastrohep.2014.01.005

5. Elli L, Branchi F, Tomba C, Villalta D, Norsa L, Ferretti F, et al. Diagnosis of gluten related disorders: Celiac disease, wheat allergy and non-celiac gluten sensitivity. *World J Gastroenterol*. (2015) 21:7110–9. doi: 10.3748/wjg.v21.i23.7110

6. Roszkowska A, Pawlicka M, Mroczek A, Bałabuszek K, Nieradko-Iwanicka B. Non-celiac gluten sensitivity: A review. *Medicina*. (2019) 55:222. doi: 10.3390/medicina55060222

7. Farthing MJ, Edwards CR, Rees LH, Dawson AM. Male gonadal function in coeliac disease: 1. Sexual dysfunction, infertility, and semen quality. *Gut*. (1982) 23:608–14. doi: 10.1136/gut.23.7.608

8. Freeman HJ. Reproductive changes associated with celiac disease. *World J Gastroenterol*. (2010) 16:5810–4. doi: 10.3748/wjg.v16.i46.5810

9. Singh P, Arora S, Lal S, Strand TA, Makharia GK. Celiac disease in women with infertility: A meta-analysis. *J Clin Gastroenterol*. (2016) 50:33–9. doi: 10.1097/MCG.0000000000000285

10. Casella G, Orfanotti G, Giacomantonio L, Bella CD, Crisafulli V, Villanacci V, et al. Celiac disease and obstetrical-gynecological contribution. *Gastroenterol Hepatol Bed Bench*. (2016) 9:241–9.

11. Grode L, Bech BH, Plana-Ripoll O, Bliddal M, Agerholm IE, Humaidan P, et al. Reproductive life in women with celiac disease; a nationwide, population-based matched cohort study. *Hum Reprod*. (2018) 33:1538–47. doi: 10.1093/humrep/dey214

12. Saccone G, Berghella V, Sarno L, Maruotti GM, Cetin I, Greco L, et al. Celiac disease and obstetric complications: a systematic review and metaanalysis. *Am J Obstet Gynecol*. (2016) 214:225–34. doi: 10.1016/j.ajog.2015.09.080

13. Bold J, Rostami K. Non-coeliac gluten sensitivity and reproductive disorders. *Gastroenterol Hepatol Bed Bench*. (2015) 8:294–7.

14. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PloS Med*. (2012) 9:e1001356. doi: 10.1371/journal.pmed.1001356

15. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod (Oxford England)*. (2007) 22:1506–12. doi: 10.1093/humrep/dem046

16. Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis, and therapy. *Rev Obstet Gynecol*. (2009) 2:76–83.

17. El Hachem H, Crepaux V, May-Panloup P, Descamps P, Legendre G, Bouet PE. Recurrent pregnancy loss: current perspectives. *Int J Womens Health*. (2017) 9:331–45. doi: 10.2147/IJWH.S100817

18. Stagnaro-Green A, Abalovich M, Alexander E, Azizi F, Mestman J, Negro R, et al. American Thyroid Association Taskforce on Thyroid Disease During Pregnancy and Postpartum. Guidelines of the American Thyroid Association for the diagnosis and management of thyroid disease during pregnancy and postpartum. *Thyroid*. (2011) 21:1081–125. doi: 10.1089/thy.2011.0087

19. Lazarus J, Brown RS, Daumerie C, Hubalewska-Dydejczyk A, Negro R, Vaidya B. 2014 European thyroid association guidelines for the management of subclinical hypothyroidism in pregnancy and in children. *Eur Thyroid J*. (2014) 3:76–94. doi: 10.1159/000362597

20. Taylor PN, Minassian C, Rehman A, Iqbal A, Draman MS, Hamilton W, et al. and risk of miscarriage in women on long-term levothyroxine: a community-based study. *J Clin Endocrinol Metab*. (2014) 99:3895–902. doi: 10.1210/jc.2014-1954

21. Fernandez-Arquero M, Arroyo R, Rubio A, Martin C, Vigil P, Conejero L, et al. Primary association of a TNF gene polymorphism with susceptibility to multiple sclerosis. *Neurology*. (1999) 53:1361–3. doi: 10.1212/wnl.53.6.1361

22. Núñez C, Garrote J-A. Recommendations to report and interpret HLA genetic findings in coeliac disease. *Rev Española Enfermedades Digestivas*. (2018) 110:458–61. doi: 10.17235/reed.2018.5269/2017

23. Siddiqui K, Uqaili AA, Rafiq M, Bhutto MA. Human leukocyte antigen (HLA)-DQ2 and -DQ8 haplotypes in celiac, celiac with type 1 diabetic, and celiac suspected pediatric cases. *Medicine*. (2021) 100:e24954. doi: 10.1097/MD.0000000000024954

24. de Entresotos Villazán LD, García Calatayud S. Estudio de la Enfermedad Celiaca en la Población Pediátrica de cantabria y sus familiares de primer grado. *Gastroenterol y Hepatol*. (2008) 31:53–8. doi: 10.1157/13116070

25. Sollid LM, Lie BA. Celiac disease genetics: current concepts and practical applications. *Clin Gastroenterol Hepatol*. (2005) 3:843–51. doi: 10.1016/s1542-3565(05)00532-x

26. Krysiak R, Szkróbka W, Okopień B. The effect of gluten-free diet on thyroid autoimmunity in drug-naïve women with Hashimoto's thyroiditis: A pilot study. *Exp Clin Endocrinol Diab*. (2019) 127:417–22. doi: 10.1055/a-0653-7108

27. Ventura A, Neri E, Ughi C, Leopaldi A, Città A, Not T. Gluten-dependent diabetes-related and thyroid-related autoantibodies in patients with celiac disease. *J Pediatr*. (2000) 137:263–5. doi: 10.1067/mpd.2000.107160

28. Voisine J, Abadie V. Interplay between gluten, HLA, innate and adaptive immunity orchestrates the development of coeliac disease. *Front Immunol*. (2021) 12:674313. doi: 10.3389/fimmu.2021.674313

29. Sharma S. Natural killer cells and regulatory T cells in early pregnancy loss. *Int J Dev Biol*. (2014) 58:219–29. doi: 10.1387/ijdb.140109ss

30. Zhang X, Wei H. Role of decidual natural killer cells in human pregnancy and related pregnancy complications. *Front Immunol*. (2021) 12:728291. doi: 10.3389/fimmu.2021.728291

31. Shmeleva EV, Colucci F. Maternal natural killer cells at the intersection between reproduction and mucosal immunity. *Mucosal Immunol*. (2021) 14:991–1005. doi: 10.1038/s41385-020-00374-3

32. Martinez J, Huang X, Yang Y. Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection. *PloS Pathog*. (2010) 6:e1000811. doi: 10.1371/journal.ppat.1000811

33. Mah AY, Cooper MA. Metabolic regulation of natural killer cell IFN-γ Production. *Crit Rev Immunol*. (2016) 36:131–47. doi: 10.1615/CritRevImmunol.2016017387

34. Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, Paxton R, et al. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med*. (1994) 180:1395–403. doi: 10.1084/jem.180.4.1395

35. Wang X, Zhao X-Y. Transcription factors associated with IL-15 cytokine signaling during NK cell development. *Front Immunol*. (2021) 12:610789. doi: 10.3389/fimmu.2021.610789

36. Felices M, Lenvik AJ, McElmurry R, Chu S, Hinderlie P, Bendzick L, et al. Continuous treatment with IL-15 exhausts human NK cells via a metabolic defect. *JCI Insight*. (2018) 3:e96219. doi: 10.1172/jci.insight.96219

37. Lerner A, Freire de Carvalho J, Kotrova A, Shoenfeld Y. Gluten-free diet can ameliorate the symptoms of non-celiac autoimmune diseases. *Nutr Rev*. (2022) 80:525–43. doi: 10.1093/nutrit/nuab039

38. Isasi C, Tejerina E, Morán LM. Non-celiac gluten sensitivity and rheumatic diseases. *Reumatol Clin*. (2016) 12:4–10. doi: 10.1016/j.reuma.2015.03.001

39. Yu XB, Uhde M, Green PH, Alaedini A. Autoantibodies in the extraintestinal manifestations of celiac disease. *Nutrients*. (2018) 10:1123. doi: 10.3390/nu10081123

40. Laine O, Pitkänen K, Lindfors K, Huhtala H, Niemelä O, Collin P, et al. Elevated serum antiphospholipid antibodies in adults with celiac disease. *Dig Liver Dis*. (2018) 50:457–61. doi: 10.1016/j.dld.2017.11.018

41. Yang Y, Krishna K, Deshpande P, Ranganathan V, Jayaraman V, Wang T, et al. High frequency of extractable nuclear autoantibodies in wheat-related disorders. *biomark Insights*. (2018) 13:1177271918782893. doi: 10.1177/1177271918782893

42. Sarmiento-Monroy JC, Gómez-Puerta JA. Poliautoinmunidad en síndrome de Sjögren. *Rev Colombiana Reumatol*. (2020) 27:58–66. doi: 10.1016/j.rcreu.2020.07.003

43. Kahaly GJ, Frommer L, Schuppan D. Celiac disease and endocrine autoimmunity - the genetic link. *Autoimmun Rev*. (2018) 17:1169–75. doi: 10.1016/j.autrev.2018.05.013

44. Carp HJ, Selmi C, Shoenfeld Y. The autoimmune bases of infertility and pregnancy loss. *J Autoimmun*. (2012) 38:J266–74. doi: 10.1016/j.jaut.2011.11.016

45. Recurrent pregnancy loss (2017). Available online at: https://www.eshre.eu/Guidelines-and-Legal/Guidelines/Recurrent-pregnancy-loss (Accessed March 01, 2024).

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 13 December 2024
DOI 10.3389/fimmu.2024.1488127

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Cristina Wide Pissetti,
Federal University of Paraíba, Brazil
Athina Samara,
Karolinska Institutet (KI), Sweden

*CORRESPONDENCE
Maribel Diaz-Ricart
mdiaz@clinic.cat

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

RECEIVED 29 August 2024
ACCEPTED 28 November 2024
PUBLISHED 13 December 2024

CITATION
Ramos A, Youssef L, Molina P, Torramadé-Moix S, Martinez-Sanchez J, Moreno-Castaño AB, Blasco M, Guillén-Olmos E, De Moner B, Pino M, Tortajada M, Camacho M, Borrell M, Crovetto F, Ramirez-Bajo MJ, Ventura-Aguiar P, Banon-Maneus E, Rovira J, Escolar G, Carreras E, Gratacos E, Diaz-Ricart M, Crispi F and Palomo M (2024) Circulating extracellular vesicles and neutrophil extracellular traps contribute to endothelial dysfunction in preeclampsia.
Front. Immunol. 15:1488127.
doi: 10.3389/fimmu.2024.1488127

COPYRIGHT
© 2024 Ramos, Youssef, Molina, Torramadé-Moix, Martinez-Sanchez, Moreno-Castaño, Blasco, Guillén-Olmos, De Moner, Pino, Tortajada, Camacho, Borrell, Crovetto, Ramirez-Bajo, Ventura-Aguiar, Banon-Maneus, Rovira, Escolar, Carreras, Gratacos, Diaz-Ricart, Crispi and Palomo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Circulating extracellular vesicles and neutrophil extracellular traps contribute to endothelial dysfunction in preeclampsia

Alex Ramos[1,2†], Lina Youssef[3,4†], Patricia Molina[1], Sergi Torramadé-Moix[1], Julia Martinez-Sanchez[1,2], Ana Belen Moreno-Castaño[1], Miquel Blasco[5,6], Elena Guillén-Olmos[5], Blanca De Moner[1,2,4], Marc Pino[1], Marta Tortajada[3], Marta Camacho[3], Maria Borrell[3], Francesca Crovetto[3], Maria Jose Ramirez-Bajo[7], Pedro Ventura-Aguiar[5,7], Elisenda Banon-Maneus[7], Jordi Rovira[7], Gines Escolar[1], Enric Carreras[2,4], Eduard Gratacos[3,8], Maribel Diaz-Ricart[1,2*], Fatima Crispi[3,8‡] and Marta Palomo[2,9‡]

[1]Hemostasis and Erythropathology Laboratory, Hematopathology, Department of Pathology, Centre de Diagnòstic Biomèdic (CDB), Hospital Clínic de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain, [2]Barcelona Endothelium Team, Hospital Clínic de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain, [3]BCNatal | Barcelona Center for Maternal Fetal and Neonatal Medicine, Hospital Clínic and Hospital Sant Joan de Déu, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain, [4]Josep Carreras Leukaemia Research Institute, Hospital Clinic/University of Barcelona Campus, Barcelona, Spain, [5]Nephrology and Kidney Transplant Department, Center of Reference in Complex Glomerular Disease (CSUR), Hospital Clínic de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain, [6]Red de Investigación Cooperativa Orientada a Resultados en Salud RICORS2040, Universidad Autónoma de Madrid, Madrid, Spain, [7]Laboratori Experimental de Nefrologia i Trasplantament (LENIT), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain, [8]Centre for Biomedical Research on Rare Diseases (CIBER-ER), Instituto de Salud Carlos III, Madrid, Spain, [9]Hematology External Quality Assessment Laboratory, Biomedical Diagnostic Center, Hospital Clinic of Barcelona, Barcelona, Spain

Background: Preeclampsia (PE) is a pregnancy complication characterized by hypertension, proteinuria, endothelial dysfunction, and complement dysregulation. Placenta-derived extracellular vesicles (EVs), necessary in maternal–fetal communication, might contribute to PE pathogenesis. Moreover, neutrophil extracellular traps (NETs) play a pathogenic role in other complement-mediated pathologies, and their contribution in PE remains unexplored.

Materials and methods: EVs were isolated from PE (peEVs) and normotensive pregnant women sera. NETs were obtained incubating donor-pre-activated neutrophils with PE or control sera. Microvascular (HMEC) endothelial cells (ECs) were incubated with PE or control sera with or without (depleted sera) EVs or NETs, to assess changes in VCAM-1, ICAM-1, VE-cadherin, eNOS, VWF, ROS, and C5b-9 deposits. Results were expressed as fold increase vs. control.

10.3389/fimmu.2024.1488127

Results: VWF, VCAM-1, and ROS expression was significantly higher in cells exposed to PE sera vs. control (12.3 ± 8.1, 3.6 ± 2.3, and 1.8 ± 0.2, respectively, $p < 0.05$), though significantly lower in cells exposed to depleted PE (dPE) sera (6.1 ± 2.7, 0.7 ± 0.6, and 1.2 ± 0.1, respectively, vs. control, $p < 0.05$). EC exposure to depleted control sera supplemented with peEVs (dC+peEVs) significantly increased VWF, VCAM-1, and ROS compared to non-supplemented sera (4.5 ± 0.3, 2.8 ± 2.0, and 1.4 ± 0.2, respectively, $p < 0.05$). ICAM-1, VE-cadherin, and C5b-9 did not differ among groups. ECs incubated with PE-NETs increased VWF and VCAM-1 and decreased VE-cadherin expression vs. control (4 ± 1.6, 5.9 ± 1.2, and 0.5 ± 0.1, respectively, $p < 0.05$), and notably increased C5b-9 deposit (7.5 ± 2.9, $p < 0.05$). ICAM-1 and ROS did not differ.

Conclusions: Both circulating EVs and NETs from PE pregnant women exhibit a deleterious effect on ECs. Whereas EVs trigger a pro-oxidant and proinflammatory state, NETs potentiate the activation of the complement system, as already described in PE.

KEYWORDS

pre-eclampsia, exosome, neutrophil activation, endothelium, complement membrane attack complex, oxidative stress

Introduction

Preeclampsia (PE) is a pregnancy-specific complication that affects 2%–8% of all pregnancies and is the leading cause of maternal and neonatal mortality and morbidity. PE is characterized by new-onset hypertension after 20 weeks of gestation that is usually accompanied by proteinuria (1, 2). Although the etiology of this multifactorial disease remains unclear, endothelial dysfunction, complement dysregulation, and the imbalance of angiogenic factors have been postulated as key elements of this complication (3, 4).

Extracellular vesicles (EVs) originated from placental trophoblast are increasingly released into the maternal circulation. EVs contain RNAs, lipids, proteins, and DNA and play a key role in endocrine and paracrine communication in both physiologic and pathologic pregnancies (5). In PE, an increment of EVs has been reported. PE-EVs contain phosphatidylserine in their surface, resulting in widespread blood clot formation (6) and fibrin depositions (7), and contributing to a PE hypercoagulation state. Moreover, PE-EVs exhibit an increase of tissue factor (8, 9), which is involved in the activation of monocytes, macrophages, and the vascular endothelium (10).

These EVs are strongly related with NET formation as they could directly activate neutrophils leading to NETosis (11). Moreover, EVs interact with maternal immune cells (12) through fusion protein syncytin-1 and promote the release of proinflammatory cytokines [such as IFN-γ, interleukin (IL)-8, IL-12, and TNF-α] (13), which, in turn, induces neutrophil activation and the subsequent NET release. Finally, the ischemic situation occurring in PE placenta increases the production of reactive oxygen species (ROS) (14), which also contributes to the exacerbation of NETosis, increasing not only NETs' soluble levels (15) but also NETs' deposits on placenta (11). At the same time, the excessive production of NETs and their deposit on placenta hinders trophoblast migration and could contribute to defective placental development, scarce perfusion, and increased inflammation together with EV release (16).

NETs, composed of extracellular strings of DNA, histones, and enzymes such as elastase and myeloperoxidase, play an important role in the elimination of pathogens. The presence of circulating NETs has been described in healthy and pathologic pregnancies (17) and also in association with endothelial dysfunction (18), as extracellular histones activate NF-kB and the transcription of activator protein 1 (AP-1) via Toll-like receptor in vascular cells (19, 20), increasing the production of inflammatory cytokines and the expression of tissue factor favoring platelet activation and aggregation (16). Cytokines released in PE together with the C5a component of the complement system contribute to the upregulation of TLRs in neutrophils and the consequent NET release (21). This NET production increase (22) contributes to widespread damage of the maternal endothelium in PE (23) causing multiorgan failure, and especially affecting both liver and kidneys (24), together with innate immune system dysregulation.

Although the complement system activation is normal in pregnancy to protect against pathogens and to facilitate the

clearance of placental debris (25), the overactivation of the complement system has been related to the severity of the PE and to the development of PE and adverse pregnancy outcomes (26–28). This overactivation of the lytic complex was demonstrated by our group in an *in vitro* model exposing endothelial cell (EC) culture to activated plasma from PE pregnancy (29).

Together with the complement system activation, the endothelial damage has also been associated with PE (30), the pathophysiological mechanisms that connect these entities remain unknown. Therefore, the aim of the present study was to investigate the potential role of both EVs present in the sera of PE pregnant women and NETs as inductors of endothelial damage and the complement dysregulation occurring in PE. We hypothesize that the bidirectional relationship between the two components creates a vicious cycle that contributes to the clinical manifestations of PE increasing the proinflammatory and procoagulant state of the endothelium. The knowledge of the mechanisms involved may improve the management of these pregnancies by providing more targets for future therapeutic strategies.

Materials and methods

Study population

A total of 58 singleton pregnancies were prospectively enrolled for this study in the Department of Maternal–Fetal Medicine at Hospital Clinic, Barcelona, Spain, between 2016 and 2021. The study population comprised blood samples from two groups: normotensive mothers considered as controls (n = 23), and pregnancies complicated by PE (n = 35). PE was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg on two occasions, at least 4 h apart, and proteinuria (>300 mg/24 h or protein/creatinine ratio > 300 mg/g) developed after 20 weeks of gestation (31–33). Mothers under 18 years, twin pregnancies, congenital malformations, chromosomal anomalies, and intrauterine infections were excluded. Gestational age was calculated based on crown–rump length at the first-trimester ultrasound. This study was approved by the ethics committee of the Hospital Clinic (HCB/2020/0240) and conformed to the ethical guidelines of the Helsinki Declaration. All participants provided informed written consent before sample collection.

Sample collection and storage

Maternal blood samples were drawn at the time of diagnosis or at matched gestational age for controls and collected into citrated and non-anticoagulated tubes. Plasma and sera were separated by centrifugation at 1,500*g* for 10 min at 4°C, filtered through a 0.22-µm filter, and stored at −80°C until further use. All samples were enrolled in the National Register of Biobanks for biomedical research that conformed to Real Decree 1716/2011.

Study design

To explore EV and NET contribution to the endothelial damage associated with PE, an *in vitro* endothelial dysfunction model was used. To evaluate the role of EVs, ECs in culture were exposed to four different conditions: a pool of control sera (C), a pool of PE sera (PE), a pool of PE sera depleted from EVs (dPE), and a pool of control sera depleted from control EVs and supplemented with EVs from PE (dC+peEVs). Then, changes in different biomarkers of endothelial activation and damage were assessed. NETs were obtained from healthy pre-activated neutrophils exposed to control or PE sera (C-NETs and PE-NETs, respectively). ECs in culture were exposed to NETs to evaluate their effect on endothelial damage biomarkers. The effect of both peEVs and NETs on C5b-9 deposits on ECs was explored.

EV isolation

EVs and depleted serum were obtained from sera pools of patients in the study groups by differential centrifugation (34). Briefly, sera samples were centrifuged at 800*g* for 7 min and at 2000*g* for 15 min to remove cell debris. Subsequently, the supernatants were filtered through a 0.22-µm pore filter and ultracentrifuged (Optima L100XP, Beckman) at 100,000*g* for 2 h. Then, sera depleted from EVs were recovered and the EVs were subsequently washed with PBS and followed for a second ultracentrifugation. Pellets were suspended in PBS and stored at −80°C until further use.

EV nanoparticle tracking analysis

The size distribution and concentration of EVs were both measured using a NanoSight NS300 (Malvern Instruments Ltd., Malvern, UK), equipped with a 488-nm laser and camera—High-sensitivity sCMOS. Samples were diluted with PBS. For each sample, 5 videos of 50 s at camera level 10 and threshold 5 were captured. Analysis was done with nanoparticle tracking analysis (NTA) 3.4 Analytical software. Sample contamination was discarded as any sample showed the characteristic fog pattern of contaminated samples.

EV study by electron microscopy

EV characterization by electron microscopy was performed using a holey carbon support film on a 400-mesh copper grid. Three microliters of the EV sample was placed on a plunger (Leica EM GP). The suspension was vitrified by rapid immersion in liquid ethane (−179°C), and the grid was mounted on a Gatan 626 cryo-transfer system and inserted into the microscope. The images were taken using a cryo-electron microscope operating at 200 kV, recorded on a GatanUltrascan US1000 CCD camera, and analyzed with a Digital Micrograph 1.8 (n = 3 per group).

Flow cytometry analysis of EVs

The phenotypic characterization of EVs isolated from sera pools from patients included in the study and used in *in vitro* studies was done using the MACSPlex Exosome Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's recommendations. This kit enables the simultaneous detection of 37 surface epitopes (CD3, CD4, CD19, CD8, HLA-DR, CD56, CD105, CD2, CD1c, CD25, CD49e, ROR1, CD209, CD9, SSEA4, HLA-BC, CD63, CD40, CD62P, CD11c, CD81, MCSP1, CD146, CD41b, CD42a, CD24, CD86, CD44, CD326, CD133/1, CD29, CD69, CD142, CD45, CD31, CD20, and CD14) that are known to be present on different vesicles plus two isotype control beads (REA and IgG1). Briefly, 1×10^9 EVs (quantified by NTA) were diluted in 120 µL of MACSPLex buffer with 15 µL of beads and then were incubated under gentle agitation and protected from light on a rotor overnight at 4°C. After incubation and washing steps, APC antibodies against CD9, CD63, and CD81 were added and incubated for 1 h at RT under gentle agitation and protected from light. The samples were washed and detected using a BD LSRFortessaSORP cytometer analyzer (BD Bioscience, NJ, USA). MFI values of buffer control were subtracted, and subsequently, the MFI value of samples was normalized to the median fluorescence intensity of CD9/CD63/CD81. Data analysis was performed with FACS DIVA software (BD Biosciences, Heidelberg, Germany).

NET isolation

Citrated blood from a healthy human donor was mixed in the same proportion with Polymorphprep (Progen Biotechnik GmbH, Heidelberg, Germany) and centrifuged (500*g*, 35 min at RT) to obtain neutrophils. Then, isolated neutrophils were centrifuged with 25 mL of HBSS medium without Ca^{2+} and Mg^{2+} (400*g*, 10 min at RT) to obtain a pellet that was mixed with a hypotonic lysis solution (1 mL of H_2Od + 0.33 mL of NaCl 3.6% + 20 mL of HBSS medium without Ca^{2+} and Mg^{2+}) to remove red blood cells (250*g*, 5 min at RT). This pellet was resuspended with Ca^{2+} and Mg HBSS medium to obtain 1×10^7 neutrophils/mL.

Then, NET production was performed following Schreiber et al.'s protocol (35): 1 mL of 1×10^6 neutrophils/mL was seeded on pretreated coverslips (incubated with 2 mL of poly-L lysine 0.01% for 15 min at 37°C and 5% of CO_2). After 15 min, 4 µL of TNFalpha 2 ng/mL was added to preactivated neutrophils and the coverslips were incubated for 15 min (37°C, 5% CO_2). Then, neutrophils were exposed to 20 µL of 1/1,000 stock PMA (10 mg/µL) as positive control, to 500 µL of control sera from normotensive pregnant women (to obtain C-NETS), or to 500 µL of PE sera (to obtain PE_NETS) (3 h, 37°C, 5% CO_2). Finally, coverslips were washed with HBSS medium without Ca^{2+} and Mg^{2+}, incubated with 500 mL of DNase solution (20 UI/mL) for 30 min, and scratched to obtain NETs.

Coverslips of each group were stained with SYTOX green (2 µL of 1/100 of SYTOX green + 1 mL of HBSS medium without Ca^{2+} and Mg^{2+}, for 10 min, RT). Then, fluorescence was evaluated by light microscopy as previously described (36). The results were expressed as the percentage of the area covered by NETs (mean ± SD). Additionally, DNA concentration of the obtained NETs was measured in supernatants, after scratching the coverslips, by a nanodrop (ND-1000, Thermo Scientific).

Endothelial cell culture

Human microvascular endothelial cells (HMEC from ATCC, Manassas, USA) were grown with medium MCDB131 (Gibco-BRL, Madrid, Spain), supplemented with fetal bovine serum (Biowest, Nuaillé, France), L-glutamine, penicillin/streptavidin (Gibco-BRL, New York, USA), endothelial growth factor (BD Biosciences, Erembodegem, Belgium), and hydrocortisone (Sigma-Aldrich, Madrid, Spain). Microvascular (HMEC) ECs were maintained at 37°C in a CO_2 atmosphere (5%) and used at passages 5–12. ECs were seeded on pretreated 18×18 mm^2 coverslips, in 6-well plates (VWR, Radnor, USA). After 24 h, cells were exposed to the different conditions under study (for 48 h).

VWF, VCAM-1, ICAM-1, eNOS, and VE-cadherin expression in endothelial cells exposed to EVs and NETs

To evaluate the expression of VWF, VCAM-1, ICAM-1, VE-cadherin, and eNOS, ECs were exposed to EV groups (C, PE, dPE, dC+peEVs) and media containing 20% of NETs solution (C-NETs or PE-NETs). Then, cells were fixed with 4% paraformaldehyde (for 10 min), blocked with 2% BSA and incubated with a primary antibody against: VWF 1:2,000 (Dako/Agilent, Santa Clara, USA); VCAM-1 1:100 (GeneTex, Irvine, USA); ICAM1 1:50 (SantaCruz Biotech, Dallas, USA); eNOS dilution 1:50 (SantaCruz Biotech, Santa Cruz, USA), and VE-cadherin 1:500 (GeneTex, Irvine, USA), (1 h, RT) and a secondary antibody IgG conjugated with Alexa 488 or 594 (Molecular Probes, Eugene, USA) (dilution 1:500 for Alexa 488 and dilution 1:2,000 for Alexa 594), 1 h, at RT, and 4′,6-diamidino-2-phenylindole. Then, fluorescence was evaluated by light microscopy as previously described (29). The results were expressed in fold increase vs. control.

Reactive oxygen species production in endothelial cells exposed to EVs and NETs

Changes in the production of ROS were explored by immunofluorescence. EC seeded coverslips were preincubated with ROS detection reagent CM.H2DCFDA (Molecular Probes, New York, USA) at 37°C for 30 min. After three PBS washes, ECs were exposed to the different EV conditions and both NET groups (37°C, 30 min). ROS production was monitored by fluorescence microscopy (Leica DM4B, Barcelona, Spain) and 15 images of each sample were randomly captured through a video camera (Leica DFC9000GT, Barcelona, Spain). The fluorescence intensity of the images was analyzed by FIJI software (ImageJ Fiji, 10 Bethesda, Rockville, USA). The results were expressed in fold increase vs. control.

10.3389/fimmu.2024.1488127

C5b-9 deposition on endothelial cells exposed to NETs obtained from healthy pregnancies or preeclampsia

To evaluate C5b-9 deposition, a previous protocol was used (37), activated plasma was obtained by adding control sera to control citrated plasma (1:1), and then 6 µL of EVs or 10% of NET solution was added (n = 3 and n = 5, respectively). The area covered by the C5b-9 deposit was calculated and expressed as the average fold increase of each condition versus control.

Statistical analysis

Data normality was checked, and then parametric or non-parametric test was applied. Scheffe test was performed for homogeneity parametric results and *post-hoc* Games-Howell was carried out for non-homogeneity parametric results. Non-parametric results were evaluated by median test. Results were expressed as fold increase (mean ± SD) and differences were considered statistically significant when the p-value was <0.05.

Results

Baseline and perinatal characteristics of the study populations

Baseline characteristics of the study populations are summarized in Table 1. Maternal characteristics were similar between the two study groups. However, significant differences were detected in some parameters: PE pregnant women showed a higher body mass index compared to controls, and gestational age at delivery was earlier in 26 of 35 PE mothers (preterm deliveries). Furthermore, cesarean section was needed in 80% of PE deliveries, a significantly higher proportion compared to controls. In addition, the median birthweight of PE fetuses was below the 10th percentile with more than half of these fetuses having FGR (68.6%). To note, six PE patients developed HELLP syndrome.

TABLE 1 Baseline and perinatal characteristics of the study populations.

	Controls n = 23	Preeclampsia n = 35
Maternal characteristics		
Age (years)	34 (30.8–37.9)	34.9 (30.4–38.2)
Ethnicity		
White	13 (56.5)	23 (65.7)
African	1 (4.4)	3 (8.6)
Latin	5 (21.7)	4 (11.4)
Asian	4 (17.4)	5 (14.3)
Pre-gestational body mass index (kg/m^2)	21.3 (19.2–22.7)	25.1 (21.2–29.1)*
Nulliparity	12 (52.2)	15 (42.9)
Use of assisted reproductive technologies	0 (0)	6 (17.1)*
Smoking during pregnancy	2 (8.7)	3 (8.8)
Perinatal outcomes		
Gestational age at delivery (weeks)	40 (38.9–41)	34.1 (32.4–37.1)*
Preterm delivery#	1 (4.3)	26 (74.3)
Cesarean section	4 (17.4)	28 (80)*
Female gender	13 (56.5)	14 (41.2)
Birthweight (g)	3,262 (3,020–3,418)	1,778 (1,486–2,450)
Birthweight centile	38 (20–51)	2.5 (0–23)
Fetal growth restrictionΨ	0 (0)	24 (68.6)*
APGAR score 5 min <7	0 (0)	10 (28.6)*
Umbilical artery pH	7.19 (7.13–7.24)	7.22 (7.17–7.26)
Stillbirth	0 (0)	1 (2.9)

Data are median (interquartile range) or n (%) as appropriate.
#Preterm delivery defined as delivery occurring before 37 weeks of gestation.
ΨFetal growth restriction defined as birthweight below the 10th centile according to local standards.
*$p < 0.05$ by Mann–Whitney U test, Pearson χ^2 or Fisher exact tests as appropriate, compared to controls.

Differential composition of PE-EVs compared to control EVs

Through NTA and electron microscopy techniques, both presence of EVs and non-contamination of the samples were assessed (Figures 1A, B). No differences were observed regarding the concentration and the size of the PE and control EVs (Figures 1C, D). However, PE and control EVs characterized by MACSPlex exosome kit in conjunction with flow cytometry showed significant differences in their composition. PE-EVs showed a statistically significant decrease in the expression of CD63, CD9, CD29, CD42a, and CD41b compared to control EVs (CD63, CD29, and CD41b, $p < 0.05$; CD9 and CD42a, $p < 0.01$), and an increase in CD81 expression ($p < 0.01$) (Figures 1E, F).

EVs from PE pregnancies contribute to endothelial damage

As summarized in Figure 2, the exposure of ECs to PE sera induced an increase of VWF and VCAM-1 expression and ROS induction compared to control sera (fold change of 12.3 ± 8.1, 3.6 ± 2.3, and 1.8 ± 0.2, respectively, $p < 0.05$). Furthermore, these biomarkers (VWF, VCAM-1, and ROS) were significantly lower in ECs exposed to depleted PE sera with respect to ECs exposed to PE sera (fold change of 6.1 ± 2.7 vs. 12.3 ± 8.1, 0.7 ± 0.6 vs. 3.6 ± 2.3, and 1.2 ± 0.1 vs. 1.8 ± 0.2 respectively, $p < 0.05$). Finally, the exposure of ECs to control depleted sera supplemented with pdEVs from PE significantly increased VWF, VCAM-1, and ROS with respect to control sera (fold change 4.5 ± 0.3, 2.8 ± 2.0, and 1.4 ± 0.2, respectively, $p < 0.05$). No differences were observed between groups

10.3389/fimmu.2024.1488127

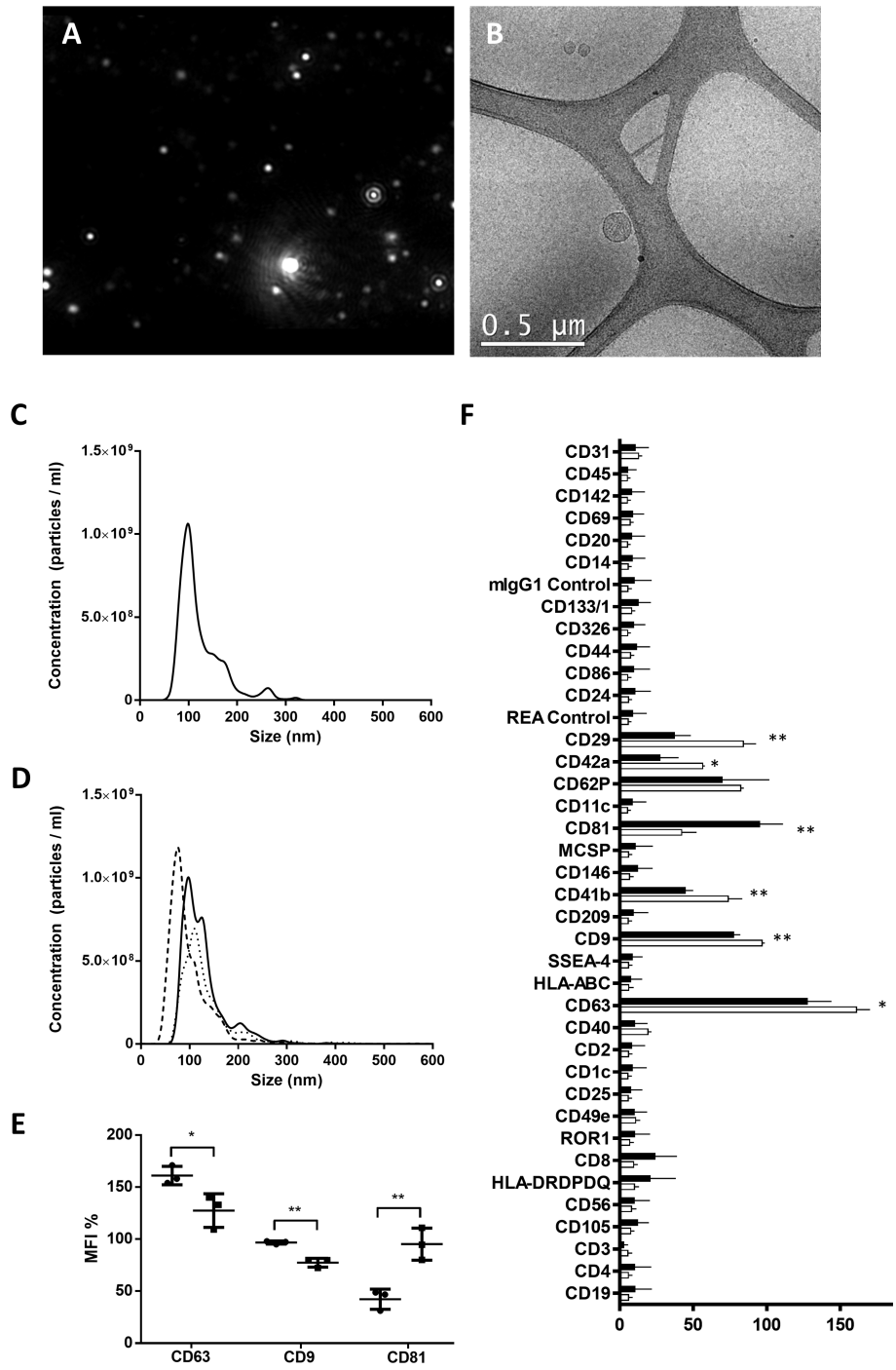


FIGURE 1
Characterization of EVs isolated from control and preeclampsia pool serums. Upper images show EV characterization by **(A)** nanotracking analysis with the absence of the characteristic fog pattern of contaminated samples and **(B)** electron microscopy (scale bars, 0.5 µm). Cryo-electron microscopy allows one to visualize the grid with an irregular distribution of hole sizes and shapes containing EVs of various sizes. **(C, D)** NTA particle concentration and size distribution of one control pool and three preeclampsia sera pools, respectively. **(E)** MFI of EV-markers CD9, CD63, and CD81 (control in black and PE in blue), obtained from MACSPlex analysis of EVs surface markers, and **(F)** phenotypic signature of EVs quantified by the MACSPlex Exosome Kit in conjunction with flow cytometry. Black bars correspond to PE pools and white bars correspond to control pools. *$p < 0.05$ and **$p < 0.01$ compared to the control group.

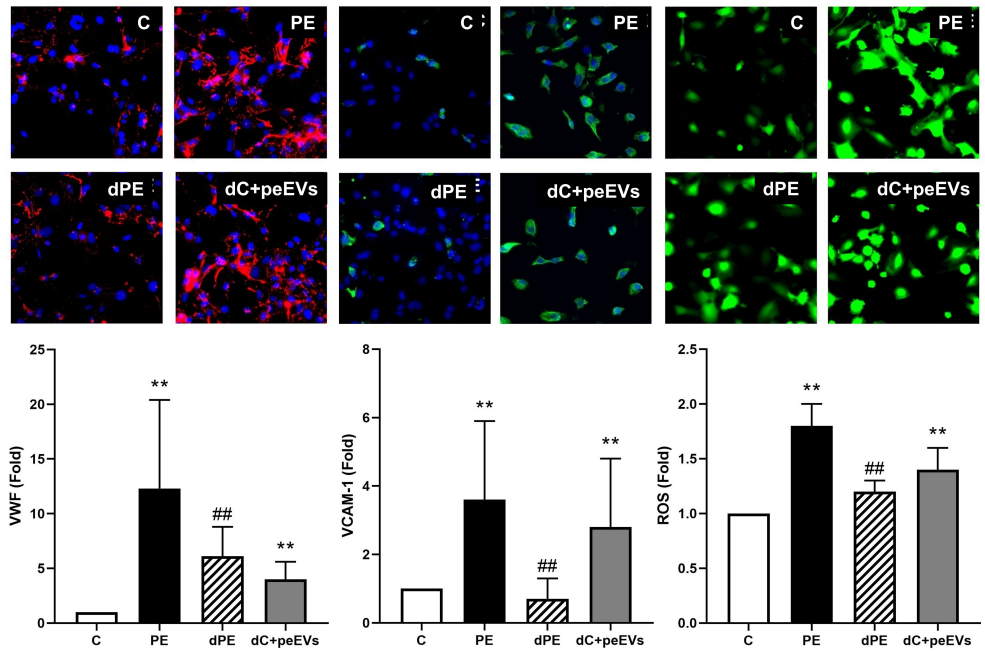


FIGURE 2
The exposure of endothelial cells to PE-EVs increased the expression of dysfunction endothelial markers. Representative microscopy image (40×) of VWF, VCAM-1, and ROS (red: VWF; ROS: green; and VCAM-1: green) on endothelial cells (4′,6-Diamidino-2-phenylindole-stained nuclei, blue) induced by exposure (48 h) to control, preeclampsia, depleted preeclampsia (without PE-EVs), and control sera pool supplemented with PE-EVs. The bar graph indicates the average fold increase of the different conditions compared to control. The vertical line indicates the standard deviation. $^{**}p < 0.01$ compared to the control condition, $\#\#p < 0.01$ compared to the PE condition.

for eNOS, ICAM-1, VE-cadherin (Supplementary Figure 1), and C5b-9 deposition (Figure 3).

PE sera increase NET production by neutrophils from a donor compared to control sera exposure

Donor neutrophils exposed to PE sera showed a marked NET production compared to control sera exposure (Figure 4). This increased NET formation was evaluated by both fluorescence microscopy with SYTOX green staining (fold increase of 2.24 vs. control, $p < 0.01$) and DNA quantification in the supernatant (17.26 ± 0.52 ng/mL for PE-NETs vs. 13.61 ± 0.47 ng/mL for C-NETs, $p < 0.01$).

PE-NETs induce a proinflammatory phenotype in endothelial cells in culture

A prothrombotic state was observed in ECs incubated with PE-NETs compared to the exposure to C-NETs triggered by an increase of VWF release (fold increase of 4.0 ± 1.6 vs. C-NETs, $p < 0.01$). Moreover, ECs incubated with PE-NETs showed higher expression of VCAM-1 on cell surface compared to C-NETs (fold increase of 5.9 ± 1.2 vs. C-NETs, $p < 0.01$), while the expression of VE-cadherin was significantly lower in ECs incubated with PE-NETs compared to C-NET incubation (fold increase 0.5 ± 0.1 vs. C-NETs, $p < 0.01$) (Figure 5). No differences were observed between groups in ICAM-1, eNOS, and ROS biomarkers ($p > 0.05$) (Supplementary Figure 2).

PE-NETs produce an increase in lytic complex C5b-9 deposition on ECs

Complement function was evaluated through the quantification of the lytic complex C5b-9 deposition on ECs. These deposits were significantly triggered by PE-NETS. C5b-9 fold increase was 7.5 ± 2.9 in ECs incubated with PE-NETs compared to ECs incubated with control activated plasma ($p < 0.01$) (Figure 6).

Discussion

The aim of the present study was to explore the contribution of EVs and NETs in the endothelial damage associated to PE using an *in vitro* model, and to explore their role as potential triggers of the complement system dysregulation described in these patients. Our results suggest that both elements play a pathogenic role in the endothelial phenotype described in PE, but activating different mechanisms. Moreover, NET overproduction in PE significantly contributes to complement system dysregulation, whereas no effect could be attributed to EVs.

Nowadays, no curative treatment has been established in PE, and the unique solution is pre-term labor to preserve mother and fetus health. Therefore, this disease is postulated as one of the most

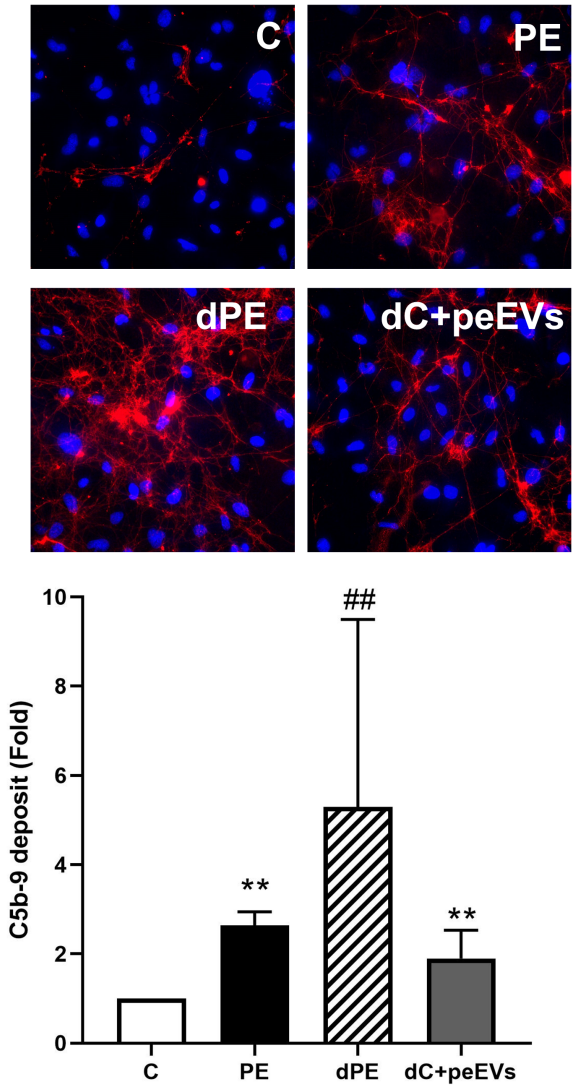


FIGURE 3
The complement system dysregulation is not mediated by EVs. Representative microscopy image (40×) of C5b-9 deposit (red) on endothelial cells (4′,6-diamidino-2-phenylindole-stained nuclei, blue) induced by exposure (4 h) to control, preeclampsia, depleted preeclampsia (without PE-EVs), and control sera pool supplemented with PE-EVs. The bar graph indicates the average fold increase of the different conditions under study compared to control. The vertical line indicates the standard deviation. $^{**}p < 0.01$ compared to control, $^{\#\#}p < 0.01$ compared to PE.

important complications during pregnancy. This pathology is intimately related to the endothelium. Although the PE patients of this study exhibited a slight overweight, its role in endothelial damage observed in this manuscript was discarded because none of these patients could be classified as obese. Our group has previously described high levels of soluble endothelial injury biomarkers such as VCAM-1 and VWF in these patients, and an increase of ICAM-1 and VWF expression in *in vitro* endothelial damage model in PE (30, 38). In addition, the overactivation of the complement system evaluated through an increase of C5b-9 deposition in ECs (37) has been proved in this pathology. Then, although endothelial damage and complement system dysregulation have been extensively demonstrated in this complication, the underlying mechanisms connecting these entities are unclear.

An abnormal placentation in the first trimester of pregnancy together with an imbalance of angiogenic factors (sFLT1 and PIGF), a pathophysiological immune activation (39), and the systemic activation of ECs of the maternal small arterioles in the late second or third trimester of pregnancy trigger a maternal endothelial crisis in PE. Moreover, there are some data relating this endothelial damage with placental-derived EVs (36); for instance, vasoconstriction and vascular endothelium damage observed in PE could be prevented and protected, respectively, by blocking the uptake of PE placenta-derived EVs (40). EVs released from the placenta to the blood exhibit a predominant role in paracrine and endocrine communication acting as homeostatic regulators in healthy pregnancies, but they also exhibit a potential implication in PE development (41–43). EVs contain proteins, mRNA, lipids, etc., surrounded by a lipid bilayer (44), and there are some studies suggesting differences between healthy pregnancy and PE-EVs (45). In our analysis, PE-EVs were not different from control EVs regarding size and concentration, but did present significant differences in their phenotype, especially in CD63, CD9, CD42a, and CD81 expression on their surface.

Both CD63 and CD9 are membrane markers expressed by platelets and immune cells that were found significantly lower in PE-EVs. CD63 has been proposed as a predictive PE biomarker (46) as it is increased in platelets from PE patients in the early stages of the complication but, to our knowledge, no evidence of CD63 and CD9 presence in PE-EVs has been described so far. Interestingly, we also found an increase of CD81 in PE-EVs compared to C-EVs, and this marker seems to be intimately related to PE pathogenesis (47). CD81, a member of the tetraspanin superfamily that plays significant roles in cell growth, adhesion, and motility, is significantly upregulated in sera from patients with early-onset severe PE. In addition, the exposure of ECs to a high dose of exogenous CD81 resulted in interrupted angiogenesis and EC activation (48). Regarding the detected lower expression of CD42a (or GPIX) in PE-EVs, another study observed a similar tendency but in platelets from PE pregnancies. This biomarker reflects severe PE progress and may be involved in its pathogenesis (49). Together with CD42a, CD29 and CD41b decrease was also observed in PE EVs. The EV composition described here could be responsible for their effect on the endothelial damage biomarkers evaluated in the present study.

In our *in vitro* model, the addition of PE-EVs to ECs in culture increased VWF and VCAM-1 expression and ROS production to similar levels to those observed when cells were exposed directly to PE sera, promoting the prothrombotic state and inflammatory phenotype previously described in these patients (50, 51). This increase in ROS production could activate NETosis as previous studies suggested (52) and be an additional mechanism to the

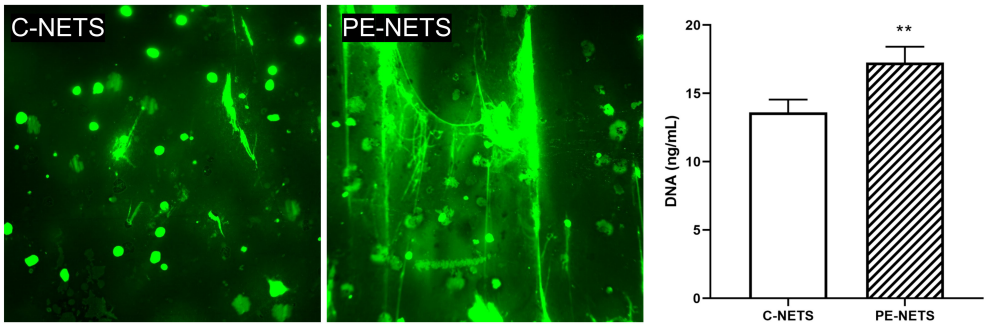


FIGURE 4
PE sera induce NET generation in neutrophils from a healthy donor. Micrographs of SYTOX green staining showed an increase in NET production by isolated donor neutrophils preactivated with TNFalpha incubated with PE sera compared to preactivated donor neutrophils incubated with sera from healthy pregnant women (micrographs taken at 40×). The bar graph indicates the result of DNA quantification (ng/mL). The vertical line depicts the standard deviation. $^{**}p < 0.01$ compared to the control group.

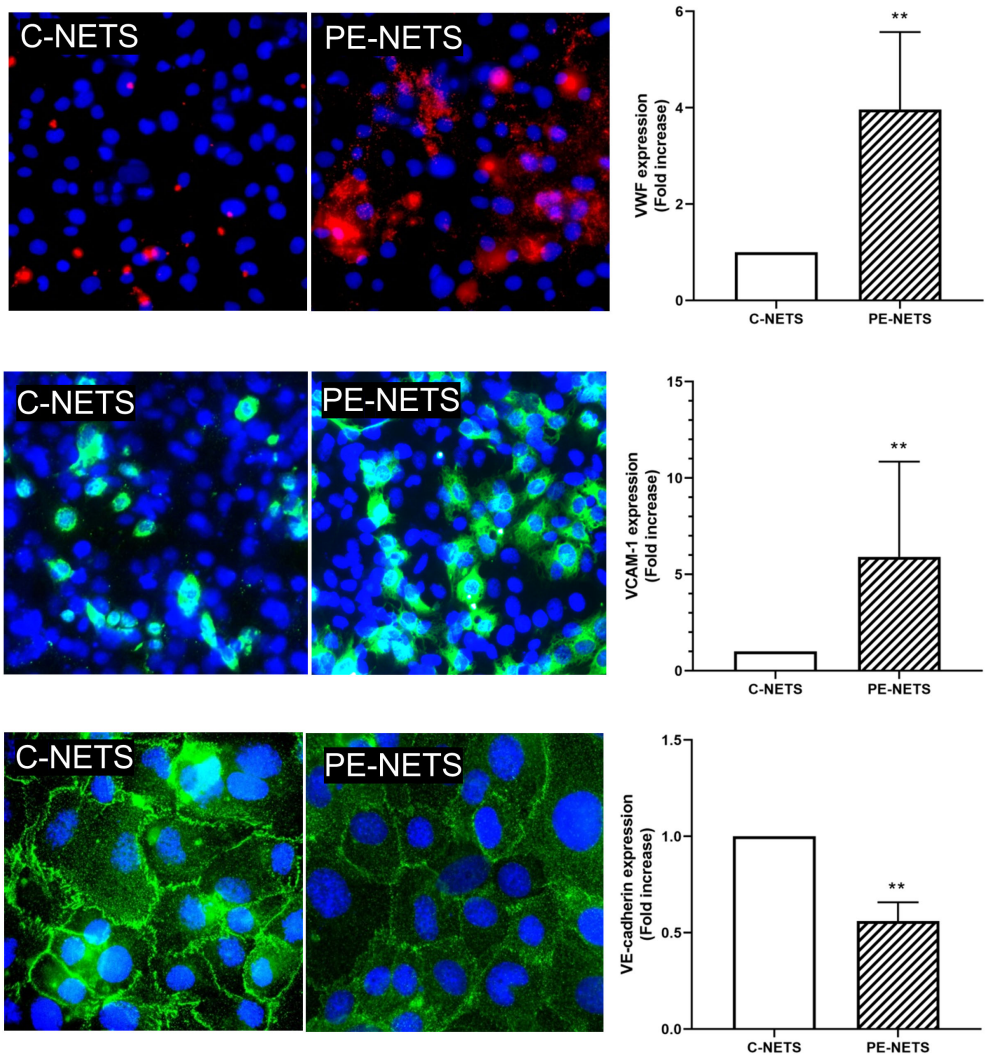


FIGURE 5
PE-NETs induce endothelial damage in the *in vitro* model compared to control NETs. Representative microscopy images of VWF (red, 40× micrographs), VCAM-1 (green, 40× micrographs), and VE-cadherin (100× micrographs) on endothelial cells (4′,6-diamidino-2-phenylindole-stained nuclei, blue) induced by exposure (48 h) to C-NETs and PE-NETS. The bar indicates the average fold increase compared to control. The vertical lines indicate the standard deviation. $^{**}p < 0.01$ compared to the control group.

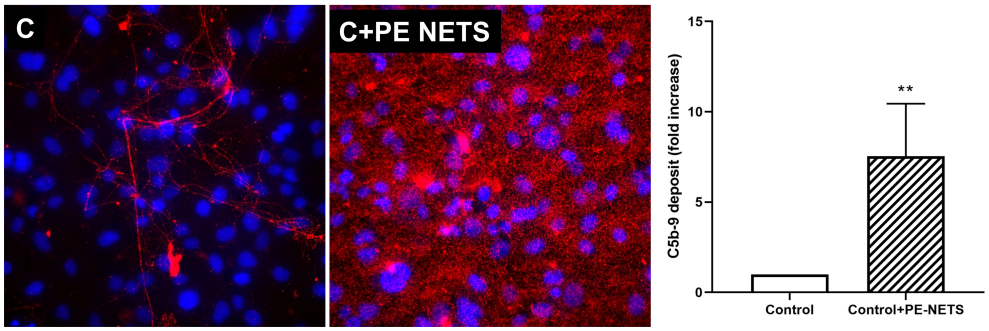


FIGURE 6
PE-NETs activate the complement system with an increase of the lytic complex C5b-9. Representative microscopy image (40×) of C5b-9 deposit stained with red fluorochrome on endothelial cells (4′,6-diamidino-2-phenylindole-stained nuclei, blue) induced by exposure (4 h) to control activated plasma **(C)** and this condition supplemented with PE-NETs (C+PE-NETS). The bar indicates the average fold increase compared to control. The vertical lines indicate the standard deviation. $^{**}p < 0.01$ compared to the control group.

presence of EVs and cytokines that activate neutrophils in a dose- and time-dependent manner (11). In contrast, PE-EVs alone did not induce a direct change in complement deposition. As expected, the exposure of cells to depleted-PE sera resulted in a milder effect on the endothelium, pointing to the deleterious effect of EVs. However, both VWF and ROS levels did not reach control levels in this condition, suggesting complementary mechanisms not related to EVs in the induction of the endothelial damage associated with PE.

PE sera dramatically increased NET production and release compared to control sera, in concordance with other studies (53). This phenomenon could be attributed to sera composition and maybe to the high interleukin levels, among other factors already reported in PE pregnancies. Like EV effects, PE-NETs also promote a prothrombotic and inflammatory phenotype state triggered by an increase of the VWF. NETs and VWF directly interact on the vascular wall and present a synergic effect promoting both the prothrombotic and proinflammatory phenotypes observed in PE. This proinflammatory phenotype was reflected in the detected overexpression of VCAM-1 in cells exposed *in vitro* to PE-NETs, in concordance with previous studies reporting that NETs increased VCAM-1 mRNA and protein expression in a time- and concentration-dependent manner (54). Although PE-NETs did not play a significant role in the induction of oxidative stress in our *in vitro* assays, ROS produced by EVs may be crucial for NET formation (55, 56). ROS overproduction found in PE, and triggered in our experiments by EVs, among other molecules, present the ability to activate transcription factors, such as NF-kB and AP-1 (57), promoting an overexpression of adhesion factors such as VCAM-1, and increasing IL-6 and IL-8 production (11, 48). This cytokine release is known to induce chemotaxis and neutrophil activation (58, 59), which, in turn, increases NET release (60).

Together with endothelial dysfunction and oxidative stress in PE, complement system dysregulation has been described as one of the most important pathophysiological mechanisms of this pregnancy complication (61, 62). Some evidence of the uncontrolled activation of the complement system in PE is the increment in C5b-9 membrane attack complex (MAC) deposition on ECs in *in vitro* assays (37), together with an increase of membrane attack complex at sites of villous injury in PE placental sections (63) and an increase in urinary excretion of C5b-9 (64) in these patients. The present study evidenced the direct effect of NETs in the overactivation of the complement system, causing a dramatic increase of C5b-9 deposition on ECs *in vitro* when added to the cell culture. Complement activation occurs not only on neutrophil membrane but also on released NETs (65), as NETs can directly activate an alternative complement pathway though properdin, factor B, and C3 (66, 67). Our results add new evidence to this intimate interaction between NETs and the complement system. Moreover, the potential linkage of severe PE to the most central complement gene, C3 (68, 69), makes this physical interaction between NETs and the complement system of interest for further investigations. In addition, it is known that the presence of complement split products, such as C3a and C5a (70), also contributes to hypertension and angiogenic imbalance in PE (71, 72). The terminal phase may be crucial in the management of PE, as complement inhibitors, such as anti-C5 (73) or antagonists of C5a receptor, reverted the angiogenic imbalance, prevented growth restriction and hypertension, and rescued pregnancies in an animal model (74). Moreover, the anti-C5 compound eculizumab has been used in pregnant women with satisfactory results (75–77). While the inhibition of the complement system may be a new treatment for PE, EVs and NETs may be a new target to improve the management of these patients.

Conclusion

In conclusion, the present study demonstrates the role of both EVs and NETs as endothelial damage and complement dysregulation factors in PE. While EVs specifically activated oxidative stress, NETs could be the main factor responsible for the complement system overactivation. Our data suggest that EVs

and NETs could be postulated as two critical elements participating in the pathophysiology of PE. Targeting the involved pathways by novel treatments that block the effect of EVs and NETs on ECs must be further explored in the management of PE. These treatments may potentially prolong the pregnancy and reduce maternal and perinatal complications.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Hospital Clinic (HCB/2020/0240). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AR: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. LY: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. PM: Methodology, Validation, Writing – review & editing. ST: Methodology, Validation, Writing – review & editing. JM: Methodology, Validation, Writing – review & editing. AM: Methodology, Validation, Writing – review & editing. MB: Conceptualization, Methodology, Writing – review & editing. EG: Conceptualization, Methodology, Writing – review & editing. BD: Methodology, Validation, Writing – review & editing. MPi: Methodology, Validation, Writing – review & editing. MT: Methodology, Validation, Writing – review & editing. MC: Methodology, Supervision, Writing – review & editing. MB: Methodology, Validation, Writing – review & editing. FC: Methodology, Validation, Writing – review & editing. MR: Methodology, Validation, Writing – review & editing. PV: Methodology, Validation, Writing – review & editing. EB: Methodology, Validation, Writing – review & editing. JR: Methodology, Validation, Writing – review & editing. GE: Funding acquisition, Project administration, Writing – review & editing. EC: Funding acquisition, Project administration, Writing – review & editing. EG: Funding acquisition, Project administration, Writing – review & editing. MD: Conceptualization, Funding acquisition, Resources, Writing – original draft. FC: Conceptualization, Funding acquisition, Resources, Writing – original draft. MPa: Conceptualization, Funding acquisition, Resources, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was partially supported by Fundació La Marató de TV3 (202026-10), Instituto de Salud Carlos III (ISCIII) (PI14/00226, PI17/00675, PI18/00073, PI20/00246, PI22/00684, PI22/00240, PI22/00109, and INT21/00027) cofinanciados por la Unión Europea, the Centro de Investigación Biomédica en Red de Enfermedades Raras (ERPR04G719/2016) (Spain), Cerebra Foundation for the Brain Injured Child (Carmarthen, Wales, UK), fundación Mutua Madrileña (AP180722022), and the Departament d'Innovació, Universitats i Empresa, Generalitat de Catalunya (2021-SGR-01422) (Spain). LY has received support from Juan de la Cierva grant FJC2021-048123-I, funded by MCIN/AEI/10.13039/501100011033 and by the European Union "NextGenerationEU"/PRTR. FC has received support from Fundación Jesus Serra del grupo Catalana Occident.

Acknowledgments

The authors are indebted to the Citomics core facility of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) for the technical help.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1488127/full#supplementary-material

References

1. ACOG Practice Bulletin no. 202: Gestational hypertension and preeclampsia. *Obstet Gynecol.* (2019) 133:1. doi: 10.1097/AOG.0000000000003018

2. Goel A, Maski MR, Bajracharya S, Wenger JB, Zhang D, Salahuddin S, et al. Epidemiology and mechanisms of DE Novo and persistent hypertension in the postpartum period. *Circulation*. (2015) 132:1726–33. doi: 10.1161/CIRCULATIONAHA.115.015721

3. Maynard S, Epstein FH, Karumanchi SA. Preeclampsia and angiogenic imbalance. *Annu Rev Med.* (2008) 59:61–78. doi: 10.1146/annurev.med.59.110106.214058

4. Rana S, Burke SD, Karumanchi SA. Imbalances in circulating angiogenic factors in the pathophysiology of preeclampsia and related disorders. *Am J Obstet Gynecol.* (2020) 226:S1019–34. doi: 10.1016/j.ajog.2020.10.022

5. Zhang J, Li S, Li L, Li M, Guo Chongye and Yao J, Mi S. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinf.* (2015) 13:17–24. doi: 10.1016/j.gpb.2015.02.001

6. Douglas AS, Mcnicol GP. Coagulation and fibrinolytic systems in pre-eclampsia and eclampsia. *Br Med J.* (1971) 2:12–6. doi: 10.1136/bmj.2.5752.12

7. Brown MA. The physiology of pre-eclampsia. *Clin Exp Pharmacol Physiol.* (1995) 22:781–91. doi: 10.1111/j.1440-1681.1995.tb01937.x

8. Gardiner C, Tannetta DS, Simms CA, Harrison P, Redman CWG, Sargent IL. Syncytiotrophoblast microvesicles released from pre-eclampsia placentae exhibit increased tissue factor activity. *PloS One.* (2011) 6:e26313. doi: 10.1371/journal.pone.0026313

9. Teng Y, Jiang R, Lin Q, Ding C, Ye Z. The relationship between plasma and placental tissue factor, and tissue factor pathway inhibitors in severe pre-eclampsia patients. *Thromb Res.* (2010) 126:e41–5. doi: 10.1016/j.thromres.2010.02.012

10. Chu AJ. Tissue factor, blood coagulation, and beyond: an overview. *Int J Inflam.* (2011) 2011:367284. doi: 10.4061/2011/367284

11. Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum Immunol.* (2006) 66:1146–54. doi: 10.1016/j.humimm.2005.11.003

12. Paul N, Sultana Z, Fisher JJ, Maiti K, Smith R. Extracellular vesicles- crucial players in human pregnancy. *Placenta*. (2023) 140:30–8. doi: 10.1016/j.placenta.2023.07.006

13. Hussain MT, Iqbal AJ, Norling LV. The role and impact of extracellular vesicles in the modulation and delivery of cytokines during autoimmunity. *Int J Mol Sci.* (2020) 21(19):7096. doi: 10.3390/ijms21197096

14. Aouache R, Biquard L, Vaiman D, Miralles F. Oxidative stress in preeclampsia and placental diseases. *Int J Mol Sci.* (2018) 19(5):1496. doi: 10.3390/ijms19051496

15. Hu Y, Li H, Yan R, Wang C, Wang Yun and Zhang C, Liu M, et al. Increased neutrophil activation and plasma DNA levels in patients with pre-eclampsia. *Thromb Haemost.* (2018) 118:2064–73. doi: 10.1055/s-0038-1675788

16. Calo G, Sabbione F, Pascuali N, Keitelman I, Vota D, Paparini D, et al. Interplay between neutrophils and trophoblast cells conditions trophoblast function and triggers vascular transformation signals. *J Cell Physiol.* (2019) 235:3592–603. doi: 10.1002/jcp.29247

17. Aslanian-Kalkhoran L, Mehdizadeh A, Aghebati-Maleki L, Danaii S, Shahmohammadi-Farid S, Yousefi M. The role of neutrophils and neutrophil extracellular traps (NETs) in stages, outcomes and pregnancy complications. *J Reprod Immunol.* (2024) 163:104237. doi: 10.1016/J.JRI.2024.104237

18. Qi H, Yang S, Zhang L. Neutrophil extracellular traps and endothelial dysfunction in atherosclerosis and thrombosis. *Front Immunol.* (2017) 8:2017.00928. doi: 10.3389/fimmu.2017.00928

19. Aldabbous L, Abdul-Salam V, McKinnon T, Duluc L, Pepke-Zaba J, Southwood M, et al. Neutrophil extracellular traps promote angiogenesis: Evidence from vascular pathology in pulmonary hypertension. *Arterioscler Thromb Vasc Biol.* (2016) 36:2078–87. doi: 10.1161/ATVBAHA.116.307634

20. Yang X, Li L, Liu J, Lv B, Chen F. Extracellular histones induce tissue factor expression in vascular endothelial cells via TLR and activation of NF-κB and AP-1. *Thromb Res.* (2015) 137:211–8. doi: 10.1016/j.thromres.2015.10.012

21. Huang Y-M, Wang H, Wang C, Chen M, Zhao M-H. Promotion of hypercoagulability in antineutrophil cytoplasmic antibody-associated vasculitis by C5a-induced tissue factor-expressing microparticles and neutrophil extracellular traps. *Arthritis Rheumatol.* (2015) 67:2780–90. doi: 10.1002/art.39239

22. Guillotin F, Fortier M, Portes M, Demattei C, Mousty E, Nouvellon E, et al. Vital NETosis vs. suicidal NETosis during normal pregnancy and preeclampsia. *Front Cell Dev Biol.* (2023) 10:2022.1099038. doi: 10.3389/fcell.2022.1099038

23. Powe CE, Levine RJ, Karumanchi SA. Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. *Circulation.* (2011) 123:2856–69. doi: 10.1161/CIRCULATIONAHA.109.853127

24. Haram K, Svendsen E, Abildgaard U. The HELLP syndrome: clinical issues and management. A Review. *BMC Pregnancy Childbirth.* (2009) 9:8. doi: 10.1186/1471-2393-9-8

25. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien Jyh Kae and Edwin S, et al. Normal pregnancy is characterized by systemic activation of the complement system. *J Matern Fetal Neonatal Med.* (2005) 17:239–45. doi: 10.1080/14767050500072722

26. Lynch AM, Gibbs RS, Murphy JR, Giclas PC, Salmon JE, Holers VM. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. *Obstet Gynecol.* (2011) 117:75–83. doi: 10.1097/AOG.0b013e3181fc3afa

27. Lynch AM, Gibbs RS, Murphy JR, Byers T, Neville MC, Giclas PC, et al. Complement activation fragment Bb in early pregnancy and spontaneous preterm birth. *Am J Obstet Gynecol.* (2008) 199:354.e1–8. doi: 10.1016/j.ajog.2008.07.044

28. Derzsy Z, Prohászka Z, Rigó J, Füst G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol.* (2010) 47:1500–6. doi: 10.1016/j.molimm.2010.01.021

29. Youssef L, Miranda J, Blasco M, Paules C, Crovetto F, Palomo M, et al. Complement and coagulation cascades activation is the main pathophysiological pathway in early-onset severe preeclampsia revealed by maternal proteomics. *Sci Rep.* (2021) 11:3048. doi: 10.1038/s41598-021-82733-z

30. Palomo M, Youssef L, Ramos A, Torramade-Moix S, Moreno-Castaño AB, Martinez-Sanchez J, et al. Differences and similarities in endothelial and angiogenic profiles of preeclampsia and COVID-19 in pregnancy. *Am J Obstet Gynecol.* (2022) 227:277.e1–277.e16. doi: 10.1016/j.ajog.2022.03.048

31. Roberts J, August P, Bakris G, Barton J, Bernstein I, Druzin M, et al. Hypertension in pregnancy. Report of the American college of obstetricians and gynecologists' task force on hypertension in pregnancy. *Obstet Gynecol.* (2013) 122:1122–31. doi: 10.1097/01.AOG.0000437382.03963.88

32. National Collaborating Centre for Women's, Health (UK) C. *Hypertension in pregnancy: The management of hypertensive disorders during pregnancy*. London: RCOG Press (2010).

33. Magee LA, Pels A, Helewa M, Rey E, von Dadelszen P, Canadian Hypertensive Disorders of Pregnancy (HDP) Working Group. Diagnosis, evaluation, and management of the hypertensive disorders of pregnancy. *Pregnancy Hypertens.* (2014) 4:105–45. doi: 10.1016/j.preghy.2014.01.003

34. Tubita V, Segui-Barber J, Lozano Juan José and Banon-Maneus E, Rovira J, Cucchiari D, Moya-Rull D, et al. Effect of immunosuppression in miRNAs from extracellular vesicles of colorectal cancer and their influence on the pre-metastatic niche. *Sci Rep.* (2019) 9:11177. doi: 10.1038/s41598-019-47581-y

35. Schreiber A, Rousselle A, Becker JU, von Mässenhausen A, Linkermann A, Kettritz R. Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. *Proc Natl Acad Sci U.S.A.* (2017) 114:E9618–25. doi: 10.1073/pnas.1708247114

36. Moreno-Castaño AB, Palomo M, Torramadé-Moix S, Martinez-Sanchez J, Ramos A, Molina P, et al. An endothelial proinflammatory phenotype precedes the development of the engraftment syndrome after autologous Hct. *Bone Marrow Transplant.* (2022) 57:721–8. doi: 10.1038/s41409-022-01610-z

37. Palomo M, Blasco M, Molina P, Lozano M, Praga M, Torramade-Moix S, et al. Complement activation and thrombotic microangiopathies. *Clin J Am Soc Nephrol.* (2019) 14:1719–32. doi: 10.2215/CJN.05830519

38. Many A, Hubel CA, Fisher SJ, Roberts JM, Zhou Y. Invasive cytotrophoblasts manifest evidence of oxidative stress in preeclampsia. *Am J Pathol.* (2000) 156:321–31. doi: 10.1016/S0002-9440(10)64733-5

39. Heydarlou H, Eghabl-Fard S, Ahmadi M, Aghebati-Maleki L, Dolati S, Movassaghpour AA, et al. Investigation of follicular helper T cells, as a novel player, in preeclampsia. *J Cell Biochem.* (2019) 120:3845–52. doi: 10.1002/JCB.27666

40. Erlandsson L, Ohlsson L, Masoumi Z, Rehnström M, Cronqvist T, Edvinsson L, et al. Preliminary evidence that blocking the uptake of placenta-derived preeclamptic extracellular vesicles protects the vascular endothelium and prevents vasoconstriction. *Sci Rep.* (2023) 13(1):18425. doi: 10.1038/S41598-023-45830-9

41. Gill M, Motta-Mejia C, Kandzija N, Cooke W, Zhang W, Cerdeira AS, et al. Placental syncytiotrophoblast-derived extracellular vesicles carry active NEP (neprilysin) and are increased in preeclampsia. *Hypertension.* (2019) 73:1112–9. doi: 10.1161/HYPERTENSIONAHA.119.12707

42. Chen Y, Huang P, Han C, Li J, Liu L, Zhao Z and Gao Y, et al. Association of placenta-derived extracellular vesicles with pre-eclampsia and associated hypercoagulability: a clinical observational study. *BJOG.* (2020) 128:1037–46. doi: 10.1111/1471-0528.16552

43. Han C, Wang C, Chen Y, Wang J, Xu X, Hilton T, et al. Placenta-derived extracellular vesicles induce preeclampsia in mouse models. *Haematologica.* (2019) 105:1686–94. doi: 10.3324/haematol.2019.226209

44. Mitchell MD, Peiris HN, Kobayashi M, Koh YQ, Duncombe G, Illanes SE, et al. Placental exosomes in normal and complicated pregnancy. *Am J Obstet Gynecol.* (2015) 213:S173–81. doi: 10.1016/j.ajog.2015.07.001

45. Tong M, Kleffmann T, Pradhan S, Johansson CL, DeSousa J, Stone PR, et al. Proteomic characterization of macro-, micro- and nano-extracellular vesicles derived from the same first trimester placenta: relevance for feto-maternal communication. *Hum Reprod.* (2016) 31:687–99. doi: 10.1093/humrep/dew004

46. Konijnenberg A, van der Post JA, Mol BW, Schaap MC, Lazarov R, Bleker OP, et al. Can flow cytometric detection of platelet activation early in pregnancy predict the

occurrence of preeclampsia? A prospective study. *Am J Obstet Gynecol.* (1997) 177:434–42. doi: 10.1016/s0002-9378(97)70212-6

47. Duan H, Hu Y. CD81, a new actor in the development of preeclampsia. *Cell Mol Immunol.* (2021) 18:2061. doi: 10.1038/s41423-021-00681-0

48. Shen L, Diao Z, Sun H-X, Yan G-J, Wang Z, Li R-T, et al. Up-regulation of CD81 inhibits cytotrophoblast invasion and mediates maternal endothelial cell dysfunction in preeclampsia. *Proc Natl Acad Sci U.S.A.* (2017) 114:1940–5. doi: 10.1073/pnas.1617601114

49. Wu F-J, Zheng M-Y, Liu L-L, Wang D-D, Ma X-Y. GPIbα reflects the development and progress of the patients with severe preeclampsia. *Int J Clin Exp Med.* (2015) 8:6039–45.

50. Kim S-Y, Ryu H-M, Yang JH, Kim M-Y, Ahn H-K, Lim H-J, et al. Maternal serum levels of VCAM-1, ICAM-1 and E-selectin in preeclampsia. *J Korean Med Sci.* (2004) 19:688–92. doi: 10.3346/jkms.2004.19.5.688

51. Parra-Cordero M, Bosco C, González Jaime and Gutiérrez R, Barja P, Rodrigo R. Immunohistochemical expression of von Willebrand factor in the preeclamptic placenta. *J Mol Histol.* (2011) 42:459–65. doi: 10.1007/s10735-011-9351-5

52. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: Mechanisms of NADPH oxidase activation and bacterial resistance. *Front Cell Infect Microbiol.* (2017) 7:2017.00373. doi: 10.3389/fcimb.2017.00373

53. Gupta AK, Hasler P, Holzgreve W, Hahn S. Neutrophil NETs: a novel contributor to preeclampsia-associated placental hypoxia? *Semin Immunopathol.* (2007) 29:163–7. doi: 10.1007/s00281-007-0073-4

54. Folco EJ, Mawson TL, Vromman A, Bernardes-Souza B, Franck G, Persson O, et al. Neutrophil extracellular traps induce endothelial cell activation and tissue factor production through interleukin-1α and cathepsin G. *Arterioscler Thromb Vasc Biol.* (2018) 38:1901–12. doi: 10.1161/ATVBAHA.118.311150

55. Kirchner T, Möller S, Klinger M, Solbach W, Laskay T, Behnen M. The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. *Mediators Inflammation.* (2012) 2012:849136. doi: 10.1155/2012/849136

56. Veenith T, Martin H, Le Breuilly M, Whitehouse T, Gao-Smith F, Duggal N, et al. High generation of reactive oxygen species from neutrophils in patients with severe COVID-19. *Sci Rep.* (2022) 12:10484. doi: 10.1038/s41598-022-13825-7

57. Dhar A, Young MR, Colburn NH. The role of AP-1, NF-kappaB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. *Mol Cell Biochem.* (2002) 234–235:185–93. doi: 10.1023/A:1015948505117

58. Huber AR, Kunkel SL, Todd 3RF, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Sci (1979).* (1991) 254:99–102. doi: 10.1126/science.1718038

59. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol.* (1994) 56:559–64. doi: 10.1002/jlb.56.5.559

60. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol.* (2013) 13:159–75. doi: 10.1038/nri3399

61. Balduit A, Agostinis C, Mangogna A, Zito G, Stampalija T, Ricci G, et al. Systematic review of the complement components as potential biomarkers of pre-eclampsia: pitfalls and opportunities. *Front Immunol.* (2024) 15:1419540. doi: 10.3389/FIMMU.2024.1419540

62. Burwick RM, Feinberg BB. Complement activation and regulation in preeclampsia and hemolysis, elevated liver enzymes, and low platelet count syndrome. *Am J Obstet Gynecol.* (2022) 226:S1059–70. doi: 10.1016/J.AJOG.2020.09.038

63. Rampersad R, Barton A, Sadovsky Y, Nelson DM. The C5b-9 membrane attack complex of complement activation localizes to villous trophoblast injury *in vivo* and modulates human trophoblast function in *vitro*. *Placenta.* (2008) 29:855–61. doi: 10.1016/j.placenta.2008.07.008

64. Guseh SH, Feinberg BB, Dawood HY, Yamamoto HS, Fichorova RN, Burwick RM. Urinary excretion of C5b-9 is associated with the anti-angiogenic state in severe preeclampsia. *Am J Reprod Immunol.* (2014) 73:437–44. doi: 10.1111/aji.12349

65. Wang H, Wang C, Zhao M-H, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. *Clin Exp Immunol.* (2015) 181:518–27. doi: 10.1111/cei.12654

66. O'Flynn J, Dixon KO, Faber Krol MC, Daha MR, van Kooten C. Myeloperoxidase directs properdin-mediated complement activation. *J Innate Immun.* (2013) 6:417–25. doi: 10.1159/000356980

67. Yuen J, Pluthero FG, Douda DN, Riedl M, Cherry A, Ulanova M, et al. NETosing neutrophils activate complement both on their own NETs and bacteria via alternative and non-alternative pathways. *Front Immunol.* (2016) 7:137. doi: 10.3389/fimmu.2016.00137

68. Teirilä L, Heikkinen-Eloranta J, Kotimaa J, Meri S, Lokki AI. Regulation of the complement system and immunological tolerance in pregnancy. *Semin Immunol.* (2019) 45:101337. doi: 10.1016/j.smim.2019.101337

69. Lokki AI, Kaartokallio T, Holmberg V, Onkamo P, Koskinen LLE, Saavalainen P, et al. Analysis of complement C3 gene reveals susceptibility to severe preeclampsia. *Front Immunol.* (2017) 8:2017.00589. doi: 10.3389/fimmu.2017.00589

70. Ye Y, Kong Y, Zhang Y. Complement split products C3a/C5a and receptors: Are they regulated by circulating angiotensin II type 1 receptor autoantibody in severe preeclampsia? *Gynecol Obstet Invest.* (2015) 81:28–33. doi: 10.1159/000440651

71. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med.* (2006) 203:2165–75. doi: 10.1084/jem.20061022

72. Wang W, Irani RA, Zhang Y, Ramin SM, Blackwell SC, Tao L, et al. Autoantibody-mediated complement C3a receptor activation contributes to the pathogenesis of preeclampsia. *Hypertension.* (2012) 60:712–21. doi: 10.1161/HYPERTENSIONAHA.112.191817

73. Lokki AI, Haapio M, Heikkinen-Eloranta J. Eculizumab treatment for postpartum HELLP syndrome and aHUS-case report. *Front Immunol.* (2020) 11:548. doi: 10.3389/FIMMU.2020.00548

74. Ahmed A, Singh J, Khan Y, Seshan SV, Girardi G. A new mouse model to explore therapies for preeclampsia. *PloS One.* (2010) 5:e13663. doi: 10.1371/journal.pone.0013663

75. Elabd H, Elkholi M, Steinberg L, Acharya A. Eculizumab, a novel potential treatment for acute kidney injury associated with preeclampsia/HELLP syndrome. *BMJ Case Rep.* (2019) 12(9):e228709. doi: 10.1136/bcr-2018-228709

76. Burwick RM, Feinberg BB. Eculizumab for the treatment of preeclampsia/HELLP syndrome. *Placenta.* (2012) 34:201–3. doi: 10.1016/j.placenta.2012.11.014

77. Lu AB, Lazarus B, Rolnik DL, Palmer KR. Pregnancy prolongation after eculizumab use in early-onset preeclampsia. *Obstet Gynecol.* (2019) 134:1215–8. doi: 10.1097/AOG.0000000000003570

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 19 February 2025
DOI 10.3389/fimmu.2025.1522346

Check for updates

OPEN ACCESS

EDITED BY
Marie-Pierre Piccinni,
University of Florence, Italy

REVIEWED BY
Yasmin Mohseni,
A2 Biotherapeutics, Inc., United States
Ahmad J.H. Albaghdadi,
Queen's University, Canada

*CORRESPONDENCE
Songcun Wang
songcunwang@fudan.edu.cn
Dajin Li
djli@shmu.edu.cn
Liyuan Cui
lycui15@fudan.edu.cn

†These authors have contributed equally to this work

RECEIVED 04 November 2024
ACCEPTED 04 February 2025
PUBLISHED 19 February 2025

CITATION
Meng X, Shi M, Qian J, Luo Y, Cui L, Li D and Wang S (2025) Tacrolimus (FK506) promotes placentation and maternal-fetal tolerance through modulating FASN-CEACAM1 pathway.
Front. Immunol. 16:1522346.
doi: 10.3389/fimmu.2025.1522346

COPYRIGHT
© 2025 Meng, Shi, Qian, Luo, Cui, Li and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Tacrolimus (FK506) promotes placentation and maternal-fetal tolerance through modulating FASN-CEACAM1 pathway

Xinhang Meng[1†], Minfeng Shi[2†], Jinfeng Qian[1], Yujie Luo[1], Liyuan Cui[1*], Dajin Li[1*] and Songcun Wang[1*]

[1]Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai, China, [2]Reproductive Medicine Center, Changhai Hospital, Naval Medical University, Shanghai, China

The establishment of placentation and maternal-fetal tolerance are important determinants of a successful pregnancy. Tacrolimus, also known as FK506, is a calcineurin inhibitor that has often been used for pregnant women after solid organ transplantation. Previous therapeutic interventions have shown the benefits of using the immuno-suppressive agent FK506 in improving clinical pregnancy and live birth rates and reducing the risk of spontaneous miscarriage. However, the mechanism(s) by which FK506 is involved in these processes have not been fully elucidated. To further characterize its function in early pregnancy, we explored the effect of FK506 on the human-derived first trimester extravillous trophoblast cells (HTR8/SVneo cells) and found that FK506 promoted invasion, tube formation and proliferation, but inhibited apoptosis of HTR8/SVneo cells. Based on the integrated metabolomics and transcriptomics analyses, the present study provided the cellular and molecular cues evidently showing that FK506 had positive effects on the placentation and maternal-fetal tolerance through modulating FASN-CEACAM1 pathway. The spontaneous-abortion-prone model gave further evidence that FK506 exerted a protective effect on pregnancy by regulating the FASN-CEACAM1 axis. These findings might provide a new fundamental mechanism and promising potential of low-dose FK506 in preventing pregnancy loss.

KEYWORDS

recurrent pregnancy loss, tacrolimus (FK506), placentation, HTR8/SVneo cells, maternal-fetal tolerance

1 Introduction

Pregnancy loss, defined as the spontaneous end of a pregnancy before the fetus reaches viability, is a common female reproductive disease. The prevalence of pregnancy loss increases with maternal age and even rises to 50% in women over 40 years old (1). Recurrent pregnancy loss (RPL, two or more consecutive miscarriages) account for 1%-5% of pregnant women (2, 3). RPL profoundly affects the quality of life of women and their partners. It can be argued that the

physical and psychological burden on women is compounded with each pregnancy loss they experience. In addition, women with a history of RPL are at a higher risk of subsequent pregnancy loss (4). Accordingly, it is imperative to explore the underlying mechanisms of RPL and identify its therapeutic targets.

Although several risk factors have been associated with RPL (2, 3), including chromosomal abnormalities, uterine anatomic abnormalities, endocrine abnormalities, metabolic abnormalities, infections and immune disorders, etc., approximately 50% of RPL causes are still unexplained (1). During pregnancy, human extravillous trophoblast cells (EVTs) invade into the maternal decidua and uterine blood vessels, dissolve the extracellular matrix, remodel the uterine vasculature and come into direct contact with the maternal decidual immune cells (5). Development of the allogeneic fetus in the maternal uterus requires the maternal immune system to accept the fetus expressing allogeneic paternal antigens as well as to provide competent responses to infections. Thus, the establishment of successful placentation and maternal-fetal tolerance are the basis of a successful pregnancy (6). Inadequate placental development and impaired induction of maternal-fetal tolerance have been found to be closely related to RPL (6, 7).

Tacrolimus, also known as FK506, was found as a 23-membered macrolide produced by *Streptomyces*. The complex of FK506 and its intracellular binding protein negatively affect the cytoplasmic nuclear factor of the activated T cell pathway by inhibiting calcineurin and interleukin (IL) -2 transcription, thereby inhibiting the production of IL-6, IL-1β and tumor necrosis factor (TNF) -α, and the proliferation of T cell-dependent B cells, resulting in a powerful immunosuppressive effect. Calcineurin inhibitors are widely used as immunosuppressants in solid organ transplant recipients and patients with autoimmune disorders. FK506 has been defined by the Food and Drug Administration as a Class C drug for pregnancy (8). FK506, in particular, has often been used for pregnant women after solid organ transplantation (9). FK506 could also have a potential efficacy against obstetrical complications associated with immune bias disorders, such as RPL, recurrent implantation failure (10–12).

Recently, FK506 was also reported to promote the migration and invasion of the human-derived first trimester extravillous trophoblast cells (HTR8/SVneo cells), suggesting an immune-independent action mode of FK506 in positively influencing placentation *in vitro* (13). To explore the mechanism by which FK506 functions on biological behaviors of EVTs, in the present study, FK506-treated HTR8/SVneo cells were collected and analyzed by transcriptomics and metabolomics. The involvement of FK506 in the maintenance of pregnancy and development of the placenta was also explored in a spontaneous-abortion-prone (SA) model.

2 Results

2.1 FK506 promoted the migration, invasion, proliferation and tube formation in HTR8/SVneo cells

To investigate the potential role of FK506 in placenta development, we studied the effect of FK506 on the HTR8/SVneo cell biological behaviors, including migration, invasion, tube forming ability, proliferation and apoptosis. As shown in Figure 1A, with the concentration range of 0.0001-1 μM, both 0.001 and 0.01 μM FK506 upregulated matrix metalloproteinase (MMP)-2 and MMP-9 expression of HTR8/SVneo cells, which reached a peak in the FK506 concentration of 0.01 μM. Thus, we used the concentration 0.01 μM in the follow-up experiments.

The gap of the wound was significantly reduced by migrating HTR8/SVneo cells after treated with 0.01 μM FK506 for 24 hours (Figure 1B). The numbers of HTR8/SVneo cells penetrating through the polycarbonate membranes coated with Matrigel and the tube formation by human umbilical vein endothelial cells (HUVECs) and HTR8/SVneo cells co-culture system increased under the treatment of FK506 (Figures 1C, D). FK506 also promoted proliferation (assessed by the Ki-67, Figure 1E), but inhibited apoptosis (according to the proportion of activated caspase-3^+ cells, Figure 1F) of HTR8/SVneo cells. These observations support previously published data indicating that FK506 has the potential to regulate biological behaviors of HTR8/SVneo cells and thus might play an important role in placentation (13, 14).

2.2 FK506 promoted the migration, invasion, proliferation and tube formation in HTR8/SVneo cells via inhibiting fatty acid synthase (FASN)

To explore the mechanism of FK506 function on the biological behaviors of EVTs, FK506-treated HTR8/SVneo cells were collected and detected by transcriptomics and metabolomics. Afterwards integrated analysis of differentially expressed genes and metabolites between FK506 exposure groups and control groups were conducted to systematically reveal the molecules and signaling pathways associated with the response to FK506 exposure. The top 100 differentially expressed genes and their correlations with the differential metabolites are shown in Figure 2A. To better understand the relationship between genes and metabolites, differentially expressed genes and differential metabolites caused by FK506 treatment of HTR8/SVneo cells were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map. The results indicated that these genes and metabolites were significantly enriched in pathways of fatty acid biosynthesis (Figures 2B, C). One key gene (*FASN*) and two metabolites (palmitic acid and myristic acid) were mapped simultaneously into the fatty acid biosynthesis pathway (Figure 2D). In addition, FK506 inhibited FASN expression in HTR8/SVneo cells (Figure 2E). The expression of FASN also increased in the villus of RPL patients (Figure 2F), we speculated that FK506 might promote the migration, invasion, proliferation and tube formation in HTR8/SVneo cells via inhibiting FASN.

As shown in Figure 3, *FASN* overexpression decreased FK506-induced MMP-2 and MMP-9 expression (Figure 3A) in HTR8/SVneo cells, inhibited the FK506-induced HTR8/SVneo cell migration (Figure 3B) and invasion (Figure 3C), reduced the tube formation of HUVECs and HTR8/SVneo cells co-culture system (Figure 3D), and also reversed the regulation of FK506 on the

proliferation (Figure 3E) and apoptosis (Figure 3F) of HTR8/SVneo cells.

2.3 Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) rescued the adverse effects of *FASN* overexpression on HTR8/SVneo cells

Gene-gene interaction studies, as analyzed using GeneMANIA Cytoscape program v.3.6.0 (https://genemania.org/) (15), demonstrated that *FASN* might interact with *CEACAM1* (Figure 4A), which serves important roles in trophoblast cell invasion (16) and immune response control (17). This attracted our interests as trophoblast cell invasion and maternal-fetal tolerance are the key aspects of successful pregnancy. FK506 increased CEACAM1 expression in HTR8/SVneo cells (Figure 4B). Compared to clinically normal first trimester pregnancies, the expression of CEACAM1 decreased in the villus of RPL patients (Figure 4C). CEACAM1 blockade reduced FK506-induced MMP-2 and MMP-9 expression (Supplementary Figure 2A) and also reversed the regulation of FK506 on the proliferation (Supplementary Figure 2B) and apoptosis (Supplementary Figure 2C) of HTR8/SVneo cells. In addition, *FASN* overexpression inhibited CEACAM1 expression, while *FASN* downregulation promoted CEACAM1 expression in HTR8/SVneo cells (Figure 4D, see also Supplementary Figures 1A, B).

With the intention of revealing the regulatory relationship between CEACAM1 and FASN, co-transfection of CEACAM1 and FASN in HTR8/SVneo cells was conducted. *CEACAM1* overexpression rescued the adverse effects of *FASN* overexpression on HTR8/SVneo cells. As shown in Figure 5, under the exposure of 0.01 μM FK506, *CEACAM1* overexpression reversed *FASN*-overexpression-induced MMP-2 and MMP-9 downregulation (Figure 5A) in HTR8/SVneo cells, promoted migration (Figure 5B) and invasion ability (Figure 5C) of HTR8/SVneo cells inhibited by *FASN* overexpression, increased the tube formation of HUVECs and *FASN*-transfected HTR8/SVneo cells co-culture system (Figure 5D), as well as reversed the regulation of *FASN* overexpression on the proliferation (Figure 5E) and apoptosis (Figure 5F) of HTR8/SVneo cells. Hence, there was a close regulatory relationship between CEACAM1 and FASN, and the trade-offs between them profoundly affected the biological behaviors of HTR8/SVneo cells.

2.4 FK506 alleviated fetal loss in SA model in a FASN-CEACAM1 dependent way

To further verify the therapeutic effect and mechanism of FK506 in pregnancy loss, we established a SA model (female

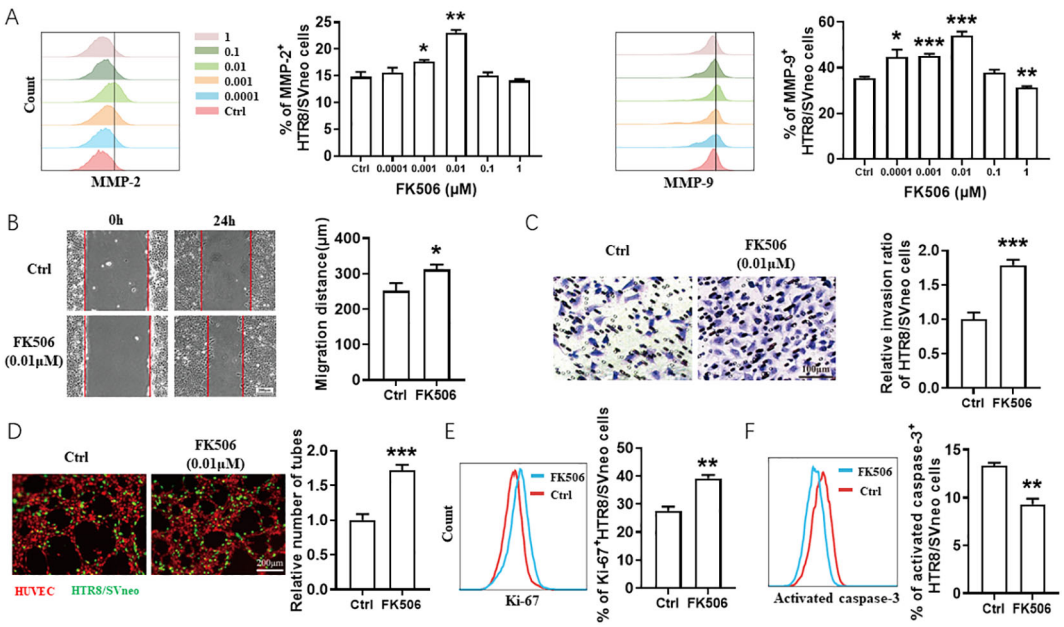


FIGURE 1
FK506 promoted the migration, invasion, proliferation and tube formation in HTR8/SVneo cells. **(A)** Flow cytometric analysis and quantitation of MMP-2 and MMP-9 expression on HTR8/SVneo cells treated with a range of concentrations of FK506 (0, 0.0001, 0.001, 0.01, 0.1, and 1 μM) for 48 hours. Ctrl, DMSO. **(B)** The wound-healing assays of HTR8/SVneo cells after treated with DMSO or 0.01 μM FK506 for 48 hours. Representative images obtained along the wounds at 0 and 24 hours. **(C)** Matrigel invasion assays of HTR8/SVneo cells after treated with DMSO or 0.01 μM FK506 for 48 hours. **(D)** Tube formation assay of HUVECs (red) and HTR8/SVneo cells (green) after treated with DMSO or 0.01 μM FK506 for 48 hours. Images are representative of three individual experiments. **(E)** Flow cytometric analysis and quantitation of Ki-67 expression on HTR8/SVneo cells treated with DMSO or 0.01 μM FK506 for 48 hours. **(F)** Flow cytometric analysis assessing the activated caspase-3 of HTR8/SVneo cells treated with DMSO or 0.01 μM FK506 for 48 hours. Data represent the mean ± SEM and are representative of three independent analyses. *p<0.05, **p<0.01, ***p<0.001.

10.3389/fimmu.2025.1522346

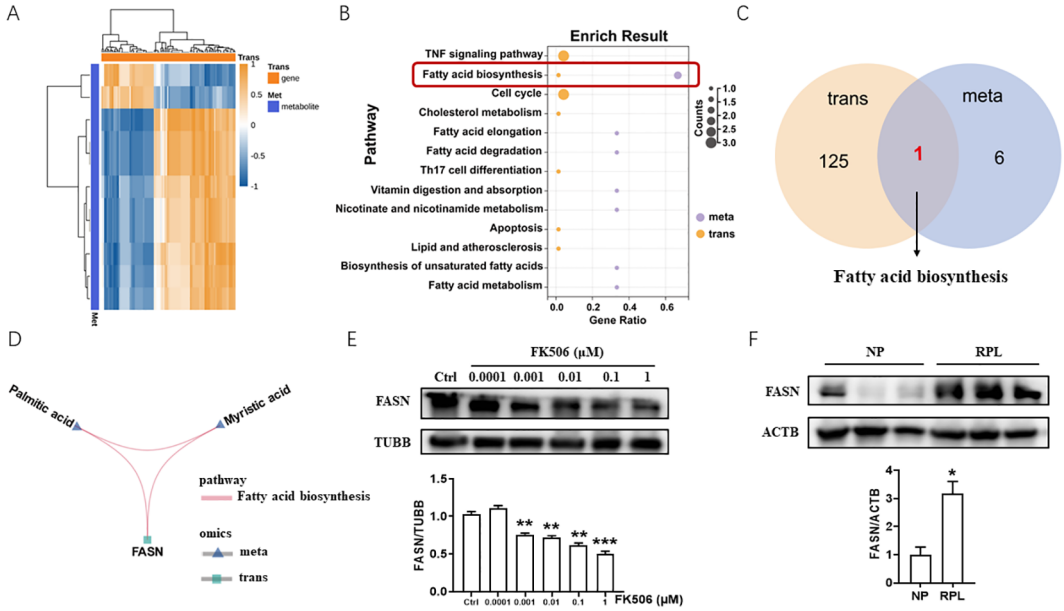


FIGURE 2
Integrated analysis of transcriptomics and metabolomics of FK506-treated HTR8/SVneo cells. **(A)** The top 100 differentially expressed genes and their correlations with the differential metabolites between 0.01 μM FK506 exposure and control group (DMSO). **(B)** KEGG analysis of differentially expressed genes and differential metabolites caused by FK506 treatment of HTR8/SVneo cells. **(C)** Differentially expressed genes and differential metabolites caused by FK506 were significantly enriched in pathways of fatty acid biosynthesis. **(D)** Among the differentially expressed genes and differential metabolites caused by FK506, one key gene (*FASN*) and two metabolites (palmitic acid and myristic acid) were mapped simultaneously into the fatty acid biosynthesis pathway. **(E)** The protein level of FASN in HTR8/SVneo cells treated with a range of concentrations of FK506 for 48 hours were examined by Western blot. Ctrl, DMSO. β-tubulin (TUBB) was used as an internal control. **(F)** The protein level of FASN in the placental villus from NP and RPL examined by Western blot. Images are representative of three individual experiments. β-actin (ACTB) was used as an internal control. Data represent the mean ± SEM and are representative of three independent analyses. *p<0.05, **p<0.01, ***p<0.001.

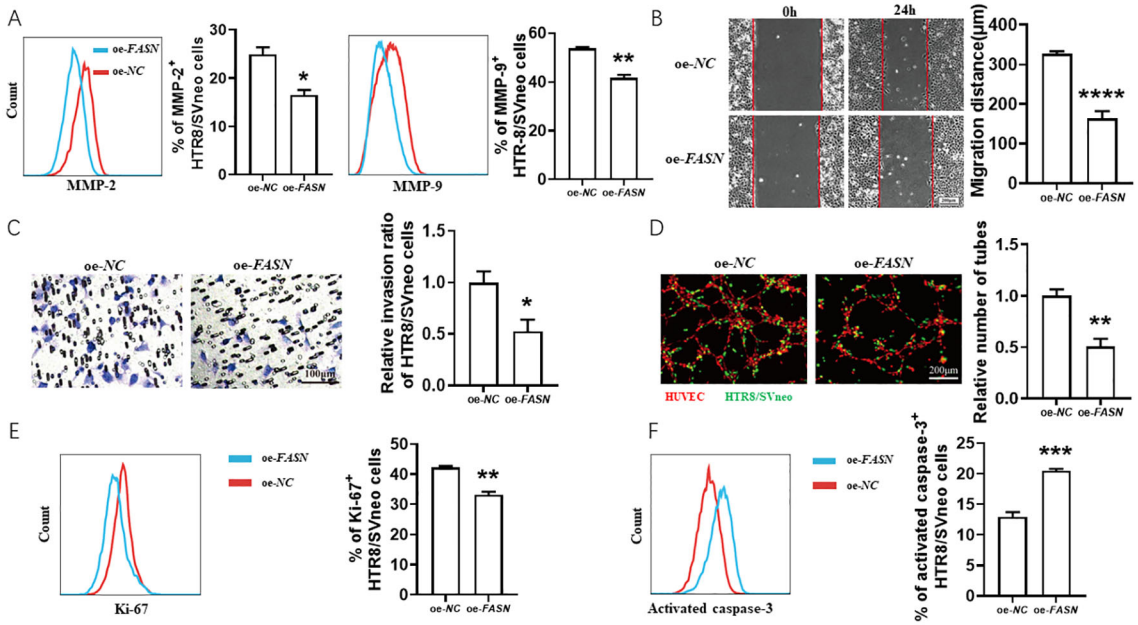


FIGURE 3
FK506 promoted the migration, invasion, proliferation and tube formation in HTR8/SVneo cells via inhibiting FASN. **(A)** Flow cytometric analysis and quantitation of MMP-2 and MMP-9 expression in HTR8/SVneo cells after transfected with empty vector or *FASN* overexpression plasmid in the exposure of 0.01 μM FK506 for 48 hours. oe, overexpression. *NC*, negative control. **(B–D)** The wound-healing assay **(B)**, Matrigel invasion assay **(C)** of HTR8/SVneo cells and tube formation assay of HUVECs (red) and HTR8/SVneo cells (green) co-culture system **(D)** with the indicated treatments. **(E, F)** Flow cytometric analysis and quantitation of Ki-67 **(E)** and activated caspase-3 **(F)** expression of HTR8/SVneo cells transfected with empty vector or FASN overexpression plasmid in the exposure of 0.01 μM FK506 for 48 hours. Images are representatives of three independent experiments. Flow cytometry plot is from one representative experiment. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

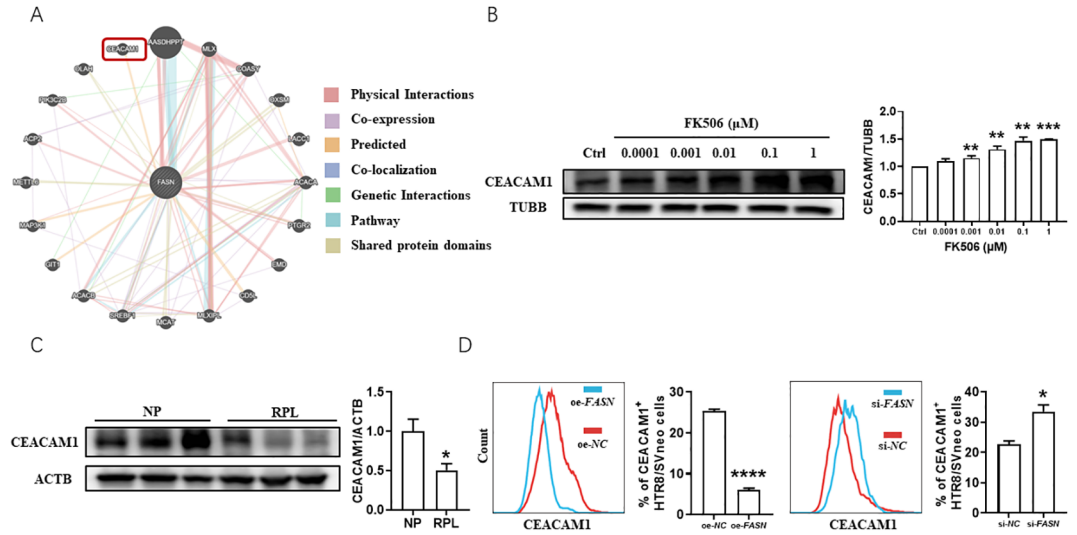


FIGURE 4
Evidence of a potential reciprocal interaction between FASN and CEACAM1 in the placenta of RPL patients and in HTR8/SVneo cells. **(A)** GeneMANIA Cytoscape program predicted that *FASN* might interact with *CEACAM1*. **(B)** The protein level of CEACAM1 in HTR8/SVneo cells treated with a range of concentrations of FK506 for 48 hours were examined by Western blot. β-tubulin (TUBB) was used as an internal control. **(C)** The protein level of CEACAM1 in the placental villus from NP and RPL examined by Western blot. β-actin (ACTB) was used as an internal control. **(D)** Flow cytometric analysis and quantitation of CEACAM1 expression of HTR8/SVneo cells after transfected with *FASN* overexpression plasmid or *FASN*-specific siRNA for 48h. *NC*, negative control. oe, overexpression. si, small interfering RNA. Images are representatives of three independent experiments. Flow cytometry plot is from one representative experiment. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

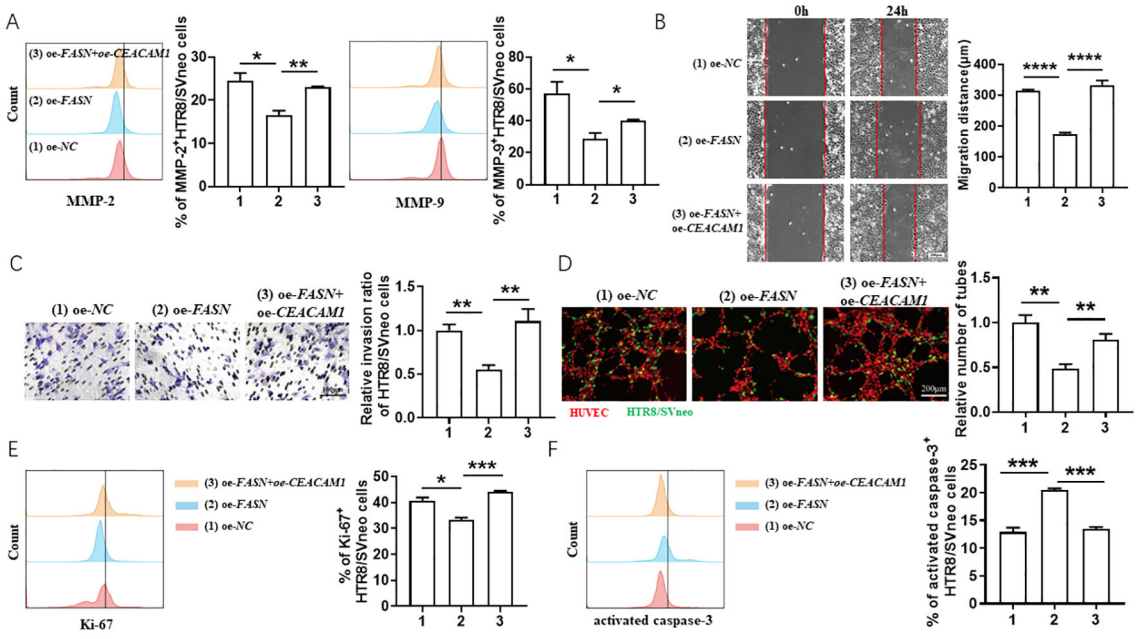


FIGURE 5
CEACAM1 overexpression rescued the adverse effects of *FASN* overexpression on HTR8/SVneo cells. **(A)** Flow cytometric analysis and quantitation of MMP-2 and MMP-9 expression in HTR8/SVneo cells after transfected with empty vector, *FASN* overexpression plasmid or co-transfected with *FASN* overexpression plasmid and *CEACAM1* overexpression plasmid in the exposure of 0.01 μM FK506 for 48 hours. **(B–D)** The wound-healing assays **(B)**, Matrigel invasion assays **(C)** of HTR8/SVneo cells and tube formation assay of HUVECs and HTR8/SVneo cells coculture system **(D)** with the indicated treatments. **(E, F)** Flow cytometric analysis and quantitation of Ki-67 **(E)** and activated caspase-3 **(F)** expression on HTR8/SVneo cells with the indicated treatments. Images are representatives of three independent experiments. Flow cytometry plot is from one representative experiment. *NC*, negative control, oe, overexpression. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p< 0.001, ****p< 0.0001.

CBA/J mice mated with male DBA/2 mice). FK506 intraperitoneal injection decreased the absorption rate of SA mice (Figure 6A, B), along with improvement of labyrinth region in placentae (Figure 6C), downregulation of FASN and upregulation of CEACAM1 (Figure 6D). However, the protection effects of FK506 were resisted by additional anti-CEACAM1 antibody treatment, which is reflected by the higher absorption rate and poorer development of labyrinth region in placentae compared with FK506 alone treatment (Figures 6A–C). In addition, the flow cytometry analysis revealed increased expression of Th2 and Treg cytokines (IL-4, transforming growth factor (TGF) -β1) but decreased expression of Th1 cytokines (TNF-α, interferon (IFN) -γ) in decidual $CD4^+$T cells in SA mice after FK506 treatment. Nonetheless, the Th2 and Treg bias in the pregnant uterus induced by FK506 was abrogated after CEACAM1 blockade (Figure 6E). Thus, blocking CEACAM1 counteracted the alleviation of adverse pregnancy outcome of SA mice by FK506.

3 Discussion

The embryo carries both maternal and paternal antigens, thus the development of the allogeneic fetus in the maternal uterus represents an allograft. Previous therapeutic interventions have shown the benefits of using the immuno-suppressive agent FK506 in mitigating the incidence of implantation failure and RPL, preventing placentation defects, and restoring proper spiral artery remodeling (10–12, 18, 19). However, the mechanism(s) by which FK506 is involved in these processes is yet to be fully comprehended. Based on the integrated metabolomics and transcriptomics analyses, the present study provided further cellular and molecular cues evidently demonstrating that FK506 can positively influence placentation and promote maternal-fetal tolerance at least in part through modulating FASN-CEACAM1 pathway (Figure 7).

Our studies on the FK506-induced promotion of migration, invasion, tube formation and proliferation, as well as inhibition of apoptosis of HTR8/SVneo cells supported recently reported immune-independent mechanism of FK506 in promoting the functional capacities of trophoblast cells during pregnancy *in vitro* (14). The improved maternal-fetal vascular network in the labyrinth of FK506-treated SA mice gave further insights that FK506 could positively influence placentation. The combined analysis of transcriptome and metabolome supported with Western blot indicated that FASN, a multifunctional enzyme necessary for the *de novo* synthesis of long-chain fatty acids (20), might be a downstream target of FK506 in influencing placentation.

The role of FASN during RPL remains poorly understood. Increased expression of FASN in male sperm of RPL couples has been reported (21, 22). Furthermore, placental *FASN* expression related negatively to placental weight (23). In the present study, we revealed that the expression of FASN was significantly increased in RPL villus. FK506 downregulated FASN expression in HTR8/SVneo cells, and functionally, *FASN* overexpression suppressed the migration, invasion and tube formation capacities of HTR8/SVneo cells induced by FK506. These data gave further clues that

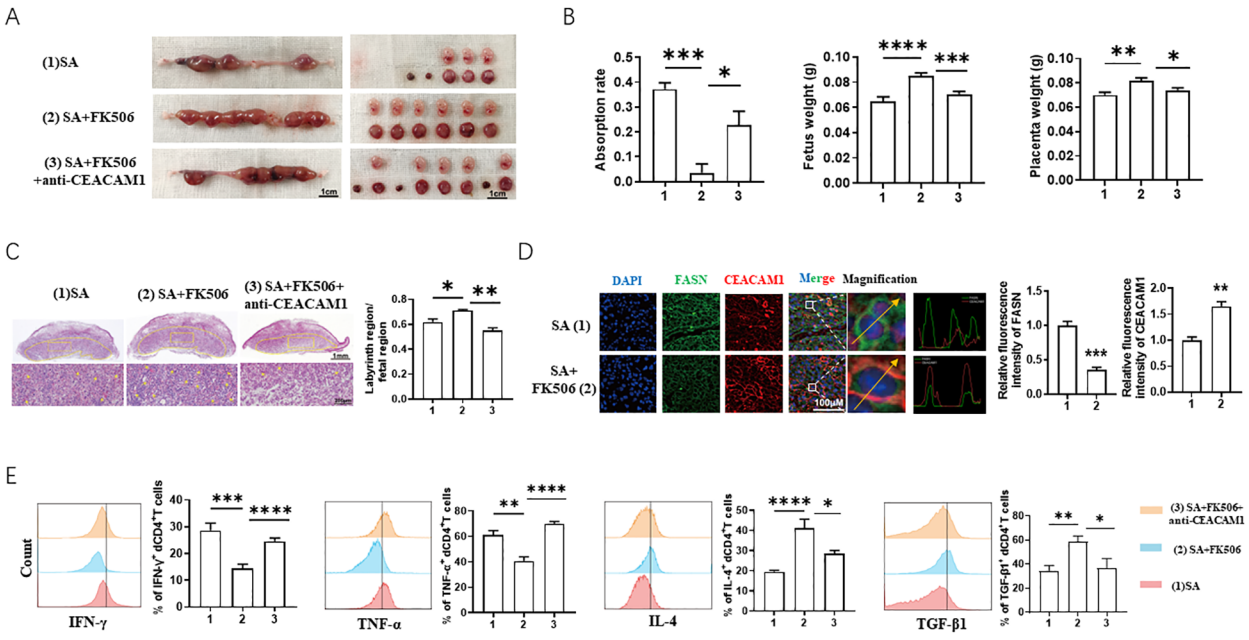


FIGURE 6
FK506 alleviated fetal loss of SA models in a FASN-CEACAM1 dependent way. **(A, B)** Representative images of uterus **(A)**, statistics of absorption rate, fetal weight and placental weight **(B)** of SA mice with the indicated treatments. **(C)** Representative images of placental hemi-sections (H&E-stained) of SA mice with the indicated treatments. **(D)** Immunofluorescence analysis and quantitation of placental FASN and CEACAM1 expression of SA mice with the indicated treatments. **(E)** Flow cytometric analysis and quantitation of IFN-γ, TNF-α, IL-4 and TGF-β1 expression on decidual $CD4^+$T cells of SA mice with the indicated treatments. Flow cytometry plot and images are representative of three individual experiments. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p< 0.001, ****p< 0.0001.

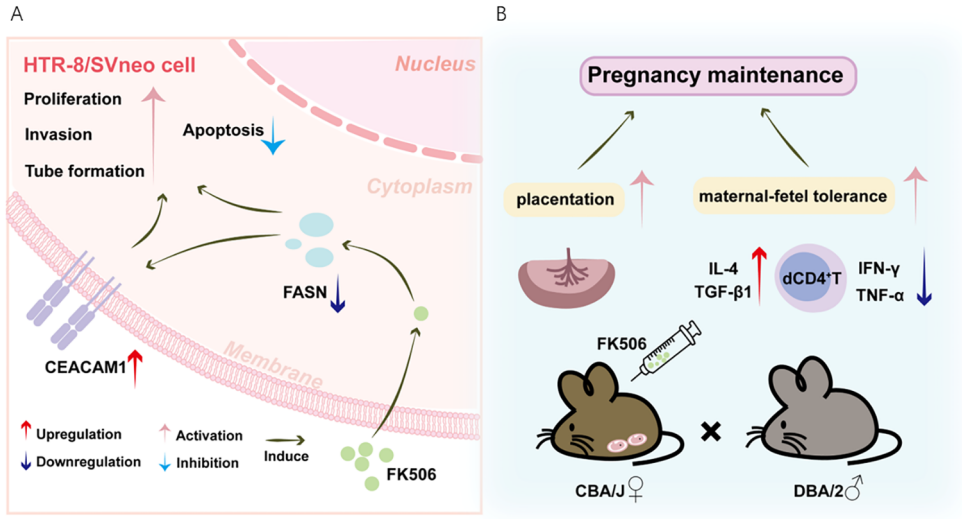


FIGURE 7
Schematic diagram of FK506-mediated positive effects on the placentation and maternal-fetal tolerance through modulating FASN-CEACAM1 pathway. **(A)** FK506 promoted invasion, tube formation and proliferation, but inhibited apoptosis of HTR8/SVneo cells. Based on the integrated metabolomics and transcriptomics analyses, FK506 might regulate biological behaviors of HTR8/SVneo cells via inhibiting FASN, which might interact with CEACAM1. FK506 increased CEACAM1 expression on HTR8/SVneo cells. In turn, CEACAM1 rescued the adverse effects of FASN on HTR8/SVneo cells. **(B)** The SA mouse model gave further evidence that FK506 exerted a protective effect on pregnancy by promoting placentation and inducing Th2/Treg bias in the pregnant uterus to maintain maternal-fetal tolerance through regulating the FASN-CEACAM1 axis.

the relatively low expression of FASN might be important for the maintenance of a healthy pregnancy, as there was a potential correlation between high levels of FASN and RPL. However, FK506 could induce dyslipidemia by upregulating FASN during liver transplantation (24). This seems to be contrary to our findings and may be due to the higher dosage of FK506 used during organ transplantation (25), further reminding us to use low-dose FK506 during pregnancy and pay attention to its effective concentration range and adverse reactions. As placental *FASN* expression also related positively to circulating FASN, studies to address changes in FASN in plasma would be informative and might have more clinical value that FASN might act as a potential biomarker for RPL diagnosis and therapy (23).

Gene-gene interaction studies predicted that *FASN* might interact with *CEACAM1*. CEACAM1 is a member of the immunoglobulin as the ligand of T-cell immunoglobulin mucin-3 (Tim-3) (26). CEACAM1 not only suppresses the inflammatory response but also initiates extracellular matrix remodeling during tumor development (27). Notably, CEACAM1 also plays important roles in trophoblast cell invasion (16). It has been reported that CEACAM1 could interact with FASN to promote hepatic insulin clearance so as to maintain normo-insulinemia and insulin sensitivity (28, 29). We also found that there might be a colocalization between FASN and CEACAM1 in placenta (Figure 6D). Nevertheless, the molecular mechanisms between them need to be further investigated. In the present study, CEACAM1 expression was decreased in RPL villus, and *CEACAM1* overexpression could rescue the adverse effects of *FASN* overexpression on HTR8/SVneo cells. Thus, the relative higher CEACAM1 expression might be beneficial to the maintenance of pregnancy. CEACAM1 expression was downregulated in diabetes mellitus and high-fat diet mouse model, therefore, controlling diet to maintain glucose and lipid metabolism balance might be also a potential therapeutic strategy in RPL (30). FK506 upregulated CEACAM1 expression on HTR8/SVneo cells and placenta, and anti-CEACAM1 canceled the protective effect of FK506 on HTR8/SVneo cells, as well as placental development and maternal-fetal tolerance of SA mice, suggesting that FK506 exerted a protective effect on pregnancy by regulating the FASN-CEACAM1 axis.

Though HTR8/SVneo cells have been proven effective for recapitulating key aspects of EVTs (31), questions remain regarding the validity of using immortalized cell lines to represent the *in vivo* environment. Data obtained in the present study further extend the notion that the underlying mechanisms of FK506 capacity for regulating trophoblast functions and maternal-fetal tolerance are associated with FASN-CEACAM1 axis, highlighting the promising potential of low-dose FK506 (0.01 μM in the present study) in preventing pregnancy loss. Nonetheless, the functional regulation of FK506 on primary trophoblasts and the related mechanisms still require further study.

4 Materials and methods

4.1 Human samples

The use and collection of human samples were approved by the Human Research Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University (No. Kyy2021-11). All participants provided written informed consent. All the methods were carried out in accordance with the approved guidelines. Human

villi from human first-trimester pregnancies were obtained from RPL patients (RPL group) and clinically normal pregnancies (NP group, terminated for non-medical reasons, had at least one successful pregnancy and no history of spontaneous abortions). Subjects with RPL included those undergoing spontaneous abortion and who also had a history of two or more consecutive spontaneous abortions without known causes (including parental or embryonic karyotype anomalies, uterine anatomic abnormalities, infection-associated factors, endocrine disorders and antiphospholipid syndrome, etc.). None of the subjects had any history of autoimmune diseases or immunotherapy, hormone therapy, renal or liver diseases, alcohol addiction, smoking or vaccination within 3 months before sample collection.

4.2 Cell treatment

HTR8/SVneo cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B at 37°C in 5% CO_2. HTR8/SVneo cells were cultured 12 hours in complete medium and further incubated in serum-free medium for 12 hours, followed by treatment with a range of concentrations of FK506 (F138016, Aladdin, China, dissolved in Dimethyl sulfoxide (DMSO), 0, 0.0001, 0.001, 0.01, 0.1, and 1 μM) for 48 hours. In some experiments, HTR8/SVneo cells were dealt with anti-human CEACAM1 neutralizing antibody (Biocampare, ABIN5684089), *FASN*-specific small interfering RNA (siRNA) (si-*FASN*, 5'-GGAGCGUAUCUGUGAGAAAT-3', 5'-UUUCUCACAGAUAC GCUCCTT-3'), *FASN* overexpression plasmid and/or *CEACAM1* overexpression plasmid (Public Protein/Plasmid Library, PPL, China) in the exposure of 0.01 μM FK506 for 48 hours using transfection reagent (L3000015, Thermo Fisher Scientific, U.S.A or Polyethylenimine Linear (PEI) MW40000 (49553-93-7, Yeasen Biotechnology, Shanghai, China), according to the manufacturer's instructions. Notably, 0.01 μM is the optimal concentration of FK506 to promote the invasion in HTR8/SVneo cells, which is equivalent to 8.2203 ng/mL (as the molar mass of Tacrolimus Monohydrate is 822.03) and close to 10 ng/mL in the previous publications (13, 14).

4.3 Western blot

The tissue and cell samples were lysed with cold radioimmunoprecipitation buffer (Beyotime Biotechnology, China) supplemented with a protease inhibitor cocktail (C0001, TargetMol, China). Protein concentrations were determined by the BCA Protein Assay Kit (WB6501, ncmbiotech, China). Lysates were heated at 95°C for 10 minutes, and then loaded on 7.5% gels (Bio-Rad, U.S.A) for SDS-polyacrylamide gel electrophoresis. After electrophoretic separation, the proteins were transferred onto 0.45 μm PVDF membranes (ipvh00010, Millipore, Germany), blocked with 5% defatted milk, and incubated overnight at 4°C with the primary antibodies (anti-FASN (ab128870, Abcam, U.S.A), anti-CEACAM1 (ab300061, Abcam, U.S.A), anti-CEACAM1(283340, Invitrogen, U.S.A), anti-β-Tubulin (ab6046, Abcam, U.S.A) and anti-Actin (ab179467, Abcam, U.S.A)). Membranes were washed and incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson, U.S.A) at room temperature for 1 hour. The antibody-labeled proteins were detected by chemiluminescence instrument (Amersham™ Imager 600, GE Healthcare, U.S.A) using Chemiluminescent Kit (Share-bio, China).

4.4 Wound-healing assay

HTR8/SVneo cell migration was measured by determining the ability of the cells to move into an acellular space, as described previously (32). Briefly, the linear scratches were made when HTR8/SVneo cells with indicated treatment were grown to 90% confluence. Then, the cells were gently washed with phosphate-buffered saline (PBS) for three times to remove medium with FK506. The cells were allowed to grow in serum-free medium, and the width of the wound was monitored at the indicated time of wound healing after treatment under the phase-contrast microscope. Photographs were taken and the relative distance traveled by the cells at the acellular front was measured at 5 different marked crossed locations per well.

4.5 Matrigel invasion assay

Cell culture inserts were precoated with Matrigel (Corning, U.S.A.) and placed in a 24-well plate. HTR8/SVneo cells (2×10^4 in 200 μL of serum-free medium) pre-treated with FK506, specific siRNA or overexpression plasmid for 48 hours were plated in the upper chamber. The lower chamber was filled with 500 μL of DMEM/F12 with 10% FBS. The cells were incubated at 37°C for 48 hours. After the inserts fixed with 4% paraformaldehyde (PFA) (G1101, Servicebio, China) and stained with crystal violet solution (G1063, Solarbio, China), the Matrigel and non-invading cells were removed from the upper surface of the filter by wiping with a cotton swab gently. Cells were observed using an Olympus BX51tDP70 fluorescence microscope (Olympus, Japan). Cells that had migrated to the lower surface were counted at a magnification of 100×. Each experiment was carried out in triplicate and repeated three times independently.

4.6 Tube formation assay

In vitro cell tube formation was assayed by determining the ability of the cells to form tubes on a 3D Matrigel scaffold. Matrigel (356234, BD Biosciences, San Jose, CA, USA) was plated into 96-well plates at 50 μL per well and polymerized for 30 minutes at 37 °C. HUVECs stained with cell tracker red fluorescent probe (C2925, Invitrogen, CA, USA) were plated at 2×10^4 cells per well and HTR8/SVneo cells (pre-treated with FK506, specific siRNA or overexpression plasmid for 48 hours as described earlier), which were stained with cell tracker green fluorescent probe (C2927,

Invitrogen, CA, USA), were seeded at 2×10^4 cells per well in 100 µL DMEM/F12 with 10% FBS. Tube formation was visualized under a fluorescence microscope after 4 hours.

4.7 Combined metabolomics and transcriptomics analysis

HTR8/SVneo cells treated with DMSO or 0.01 µM FK506 for 48 hours were set up for transcriptomics and metabolomics sequencing. The number of sequenced reads falling in the exons of this meta-gene was calculated using feature Counts tool in subread, and the differential expression analysis was conducted by DE-Seq. $P < 0.001$ were regarded as the significance threshold. Differential genes were enriched by the KEGG in gene set enrichment analysis. The metabolites with the variable importance in the projection≥1 and P-value of Student's t-test ≤0.05 were considered as significantly changed. The KEGG database was used to annotate and display the differential metabolites. Other analyses included partial least- squares discriminant analysis and pathway enrichment.

4.8 Mice

CBA/J female and DBA/2 male mice were purchased from Slac laboratory animal Co. (Shanghai, China) and Huafukang bioscience Co. (Beijing, China) and maintained in an animal facility according to institutional and National Institutes of Health Guidelines. Eight-week-old CBA/J females were mated to DBA/2 males to establish SA models (33). All the CBA/J females were inspected every morning for vaginal plugs. The day of visualization of a plug was designated as day 0.5 of pregnancy. Pregnant mice were divided into 3 groups: SA group, SA group receiving injections of FK506 on day 4.5, SA group receiving injections of FK506 (0.05 mg/kg) on day 4.5 and anti-CEACAM1 antibody (Clone: MAb-CC1, 134534, Biolegend, U.S.A) at doses of 250 µg, 125 µg, and 125 µg on days 4.5, 6.5, and 8.5, respectively. All pregnant mice were monitored at day 13.5 of pregnancy. The percentage of fetal loss (the embryo absorption rate) was calculated as following: % of absorption = R/(R + V) × 100, where R represents the number of hemorrhagic implantation (sites of fetal loss) and V stands for the number of viable, surviving fetuses.

4.9 Preparation of mouse cells

Uteri from pregnant mice were dissected free from the mesometrium and removed by cuts at the ovaries and cervix. The fetal and placental tissues were carefully removed and washed in PBS. Minced uteri were digested in RPMI 1640 medium supplemented with collagenase type IV and DNase I for 45 minutes at 37°C with gentle agitation. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 µg/mL amphotericin B at 37°C in 5% CO_2 for 4 hours to remove adherent stromal cells. Anti-mouse CD3 (5 µg/mL, Clone: 145-2C11, 100340, Biolegend, U.S.A), anti-mouse CD28 (1 µg/mL, Clone: 37.51,102116, Biolegend, U.S.A), phorbol 12-myrstate 13-acetate (50 ng/mL, Biolegend, U.S.A.), ionomycin (1 µg/mL, Biolegend, U.S.A.) and brefeldin A (10 mg/mL, BioLegend, U.S.A.) were added 4 hours for intracellular cytokine analysis.

4.10 Hematoxylin and eosin (H&E) staining of placental hemi-sections

Mouse placentae were fixed in 4% PFA overnight and embedded in paraffin. Serial 3 µm thick mid-sagittal sections were cut and used for H&E staining. Labyrinth vascular areas were observed using an Olympus BX51tDP70 fluorescence microscope (Olympus, Japan). In all cases, the central region of the placenta was examined. An average of five nonconsecutive sagittal sections at an interval of at least 40 µm for each placenta was used for the measurement.

4.11 Immunofluorescence staining

Mouse placentae were fixed in 4% PFA overnight and embedded in paraffin. Serial 3 µm thick mid-sagittal sections were cut and used for immunofluorescence staining. The tissue sections were dewaxed and rehydrated in xylene and ethanol gradients. Then, the slides were immersed in citric acid antigen repair buffer (pH 6.0, G1219-1L, Servicebio, China) and heated at 98 °C for 15 minutes for antigen retrieval. After cooling to room temperature, the sections were blocked with 10% donkey serum (SL050, solarbio, China) for 1 hour at room temperature before incubation with primary antibodies (anti-FASN (ab128870, Abcam, 1:400), anti-CEACAM1(283340, Invitrogen, U.S.A, 1:200), anti-CEACAM1 (A25475, ABclonal, China, 1:100)) overnight at 4 °C. The next day, the slides were placed in PBS and washed three times on a decolorizing shaker before incubation with Alexa Fluor-conjugated secondary antibodies (AS027, AS039, AS053, AS054, ABclonal, China, 1:100) for 1 hour at room temperature. The sections were subsequently counterstained with 4′,6-diamidino-2-phenylindole (DAPI, G1012, Servicebio, China) and mounted with anti-fade mounting medium. Images were captured on a THUNDER Imaging Systems (Leica, Germany). Mean fluorescence intensities was determined by Image J software and the relative fluorescence intensities of FASN and CEACAM1 in the placentae was calculated as following: Relative fluorescence intensity = X/S, where S represents the mean fluorescence intensities of all cells of three fields of view of the placentae from SA mice, and X stands for the mean fluorescence intensities of cells in each field of view of the placentae from SA mice and SA mice with FK506 administration. The fluorescence curves represented the fluorescence density value along the direction of the arrow.

4.12 Flow cytometry

Cell surface molecular expression and intracellular cytokine production were evaluated using flow cytometry. AlexaeFluor® 488-conjugated anti-mouse IL-4 (Biolegend, U.S.A); APC-conjugated anti-human MMP-9 (Biolegend, U.S.A); PE-conjugated anti-human MMP-2 (R&D, U.S.A) or anti-human active caspase-3 (BD Biosciences, U.S.A); PE/CY7-conjugated anti-mouse TNF-α (Biolegend, U.S.A); PE-TexasRed-conjugated anti-mouse IFN-γ; Brilliant Violet 421-conjugated anti-human Ki-67 or anti-mouse TGF-β1; Brilliant Violet 510-conjugated anti-mouse CD4 (Biolegend, U.S.A). For intracellular staining, cells were fixed and permeabilized using the Fix/Perm kit (Biolegend, U.S.A). Isotype antibodies were used for setting gates. Flow cytometry was performed on a Beckman-Coulter CyAn ADP cytometer and analyzed with FlowJo software (Tree Star, U.S.A.).

4.13 Statistical analysis

All variables were normally distributed in this study. Thus, variables were presented as means and standard error of mean (SEM). Significance of differences between two groups was determined by Student's *t*-test. Multiple groups were analyzed by one-way ANOVA with Bonferroni posttests or the *post-hoc* Dunnett *t*-test using Prism Version 8 software (GraphPad, San Diego, CA, USA). For all statistical tests, *p*- values <0.05 were considered statistically significant.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Human Research Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Research Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SW: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft. XM: Data curation, Investigation, Methodology, Writing – review & editing. MS: Data curation, Investigation, Methodology, Resources, Writing – review & editing. JQ: Investigation, Resources, Validation, Writing – review & editing. YL: Methodology, Software, Validation, Writing – review & editing. LC: Funding acquisition, Methodology, Project administration, Software, Supervision, Writing – review & editing. DL: Conceptualization, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by grant from the National Key Research and Development Program of China (2023YFC2705403 to SW and LC), National Nature Science Foundation of China (NSFC) (32470992 to SW, 82201852 to LC), the Special Youth Project for Clinical Research in Health Industry of Shanghai Municipal Health Commission (20224Y0005 to SW).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1522346/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The protein level of FASN and CEACAM1 in HTR8/SVneo cells after indicated treatment examined by Western blot. β-actin (ACTB) was used as an internal control.

SUPPLEMENTARY FIGURE 2

(A) Flow cytometric analysis and quantitation of MMP-2 and MMP-9 expression in HTR8/SVneo cells after CEACAM1 blockade in the exposure of 0.01 μM FK506 for 48 hours. **(B, C)** Flow cytometric analysis and quantitation of Ki-67 **(B)** and activated caspase-3 **(C)** expression on HTR8/SVneo cells with the indicated treatments. Images are representatives of three independent experiments. Flow cytometry plot is from one representative experiment. Ctrl, DMSO. Data represent mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p< 0.001$.

References

1. El Hachem H, Crepaux V, May-Panloup P, Descamps P, Legendre G, Bouet PE. Recurrent pregnancy loss: current perspectives. *Int J Womens Health*. (2017) 9:331–45. doi: 10.2147/IJWH.S100817

2. Regan L, Rai R, Saravelos S, Li TC, Obstetricians RC. Recurrent miscarriage green-top guideline no. 17. *Bjog-Int J Obstet Gy*. (2023) 130:E9–E39. doi: 10.1111/1471-0528.17515

3. Dimitriadis E, Menkhorst E, Saito S, Kutteh WH, Brosens JJ. Recurrent pregnancy loss. *Nat Rev Dis Primers*. (2020) 6:98. doi: 10.1038/s41572-020-00228-z

4. Rasmark Roepke E, Christiansen OB, Kallen K, Hansson SR. Women with a history of recurrent pregnancy loss are a high-risk population for adverse obstetrical outcome: A retrospective cohort study. *J Clin Med*. (2021) 10. doi: 10.3390/jcm10020179

5. Kojima J, Ono M, Kuji N, Nishi H. Human chorionic villous differentiation and placental development. *Int J Mol Sci*. (2022) 23(14):8003. doi: 10.3390/ijms23148003

6. Arck PC, Hecher K. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat Med*. (2013) 19:548–56. doi: 10.1038/nm.3160

7. Haram K, Mortensen JH, Myking O, Roald B, Magann EF, Morrison JC. Early development of the human placenta and pregnancy complications. *J Matern Fetal Neonatal Med*. (2020) 33:3538–45. doi: 10.1080/14767058.2019.1578745

8. Nevers W, Pupco A, Koren G, Bozzo P. Safety of tacrolimus in pregnancy. *Can Fam Physician*. (2014) 60:905–6.

9. Kainz A, Harabacz I, Cowlrick IS, Gadgi SD, Hagiwara D. Review of the course and outcome of 100 pregnancies in 84 women treated with tacrolimus. *Transplantation*. (2000) 70:1718–21. doi: 10.1097/00007890-200012270-00010

10. Liu J, Li M, Fu J, Yuan G, Li N, Fu Y, et al. Tacrolimus improved the pregnancy outcomes of patients with refractory recurrent spontaneous abortion and immune bias disorders: a randomized controlled trial. *Eur J Clin Pharmacol*. (2023) 79:627–34. doi: 10.1007/s00228-023-03473-9

11. Hisano M, Nakagawa K, Ono M, Yoshino O, Saito T, Hirota Y, et al. Multicenter, 2-dose single-group controlled trial of tacrolimus for the severe infertility patients. *Medicine*. (2023) 102:e34317. doi: 10.1097/MD.0000000000034317

12. Shen P, Zhang T, Han R, Xie H, Lv Q. Co-administration of tacrolimus and low molecular weight heparin in patients with a history of implantation failure and elevated peripheral blood natural killer cell proportion. *J Obstetrics Gynaecology Res*. (2022) 49:649–57. doi: 10.1111/jog.15500

13. Albaghdadi AJH, Coyle K, Kan FWK. Low-dose tacrolimus promotes the migration and invasion and nitric oxide production in the human-derived first trimester extravillous trophoblast cells *in vitro*. *Int J Mol Sci*. (2022) 23(15):8426. doi: 10.3390/ijms23158426

14. Albaghdadi AJH, Xu W, Kan FWK. An immune-independent mode of action of tacrolimus in promoting human extravillous trophoblast migration involves intracellular calcium release and F-actin cytoskeletal reorganization. *Int J Mol Sci*. (2024) 25(22):12090. doi: 10.3390/ijms252212090

15. Carbon S, Douglass E, Dunn N, Good B, Harris NL, Lewis SE, et al. The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Res*. (2019) 47:D330–D8. doi: 10.1093/nar/gky1055

16. Briese J, Oberndörfer M, Pätschenik C, Schulte HM, Makrigiannakis A, Löning T, et al. Osteopontin is colocalized with the adhesion molecule CEACAM1 in the extravillous trophoblast of the human placenta and enhances invasion of CEACAM1-expressing placental cells. *J Clin Endocrinol Metab*. (2005) 90:5407–13. doi: 10.1210/jc.2004-2434

17. Memaj P, Ouzerara Z, Jornayvaz FR. Role of oxidative stress and carcinoembryonic antigen-related cell adhesion molecule 1 in nonalcoholic fatty liver disease. *Int J Mol Sci*. (2023) 24:11271. doi: 10.3390/ijms241411271

18. Albaghdadi AJH, Kan FWK. Immunosuppression with tacrolimus improved implantation and rescued expression of uterine progesterone receptor and its co-regulators FKBP52 and PIASy at nidation in the obese and diabetic mice: Comparative studies with metformin. *Mol Cell Endocrinol*. (2018) 460:73–84. doi: 10.1016/j.mce.2017.07.007

19. Albaghdadi AJH, Hewitt MA, Putos SM, Wells M, Ozolins TRS, Kan FWK. Tacrolimus in the prevention of adverse pregnancy outcomes and diabetes-associated embryopathies in obese and diabetic mice. *J Transl Med*. (2017) 1515(1):32. doi: 10.1186/s12967-017-1137-4

20. Xiao Y, Yang Y, Xiong H, Dong G. The implications of FASN in immune cell biology and related diseases. *Cell Death Disease*. (2024) 15(1):88. doi: 10.1038/s41419-024-06463-6

21. Thapliyal A, Tomar AK, Chandra KB, Naglot S, Dhiman S, Singh N, et al. Differential sperm proteomics reveals the significance of fatty acid synthase and clusterin in idiopathic recurrent pregnancy loss. *Reprod Sci*. (2023) 30:3456–68. doi: 10.1007/s43032-023-01288-8

22. Xue D, Zhang Y, Wang Y, Wang J, An F, Sun X, et al. Quantitative proteomic analysis of sperm in unexplained recurrent pregnancy loss. *Reprod Biol Endocrinol*. (2019) 17(1):52. doi: 10.1186/s12958-019-0496-5

23. Carreras-Badosa G, Prats-Puig A, Puig T, Vázquez-Ruíz M, Bruel M, Mendoza E, et al. Circulating Fatty Acid Synthase in pregnant women: Relationship to blood pressure, maternal metabolism and newborn parameters. *Sci Rep-Uk*. (2016) 6:24167. doi: 10.1038/srep24167

24. Chen L-J, Xin Y, Yuan M-X, Ji C-Y, Peng Y-M, Yin Q. CircFOXN2 alleviates glucocorticoid- and tacrolimus-induced dyslipidemia by reducing FASN mRNA stability by binding to PTBP1 during liver transplantation. *Am J Physiology-Cell Physiol*. (2023) 325:C796–806. doi: 10.1152/ajpcell.00462.2022

25. Yoon SB, Lee JM, Jung CW, Suh KS, Lee KW, Yi NJ, et al. Machine-learning model to predict the tacrolimus concentration and suggest optimal dose in liver transplantation recipients: a multicenter retrospective cohort study. *Sci Rep*. (2024) 14:19996. doi: 10.1038/s41598-024-71032-y

26. Sauer N, Janicka N, Szlasa W, Skinderowicz B, Kolodzinska K, Dwernicka W, et al. TIM-3 as a promising target for cancer immunotherapy in a wide range of tumors. *Cancer Immunol Immun*. (2023) 72:3405–25. doi: 10.1007/s00262-023-03516-1

27. Gerstel D, Wegwitz F, Jannasch K, Ludewig P, Scheike K, Alves F, et al. CEACAM1 creates a pro-angiogenic tumor microenvironment that supports tumor vessel maturation. *Oncogene*. (2011) 30:4275–88. doi: 10.1038/onc.2011.146

28. Zhang K. CEACAM1: Shielding the liver against fibrosis. *Eur J Clin Invest*. (2024) 54(8):e14182. doi: 10.1111/eci.14182

29. Najjar SM, Yang Y, Fernström MA, Lee S-J, DeAngelis AM, Rjaily GAA, et al. Insulin acutely decreases hepatic fatty acid synthase activity. *Cell Metab*. (2005) 2:43–53. doi: 10.1016/j.cmet.2005.06.001

30. Yu J, Liu H, Chen Y, Wang L, Chen P, Zhao Y, et al. miR-449a disturbs atherosclerotic plaque stability in streptozotocin and high-fat diet-induced diabetic mice by targeting CEACAM1. *Diabetol Metab Syndrome*. (2024) 16. doi: 10.1186/s13098-024-01322-y

31. Shan N, Zhang X, Xiao X, Zhang H, Tong C, Luo X, et al. Laminin alpha4 (LAMA4) expression promotes trophoblast cell invasion, migration, and angiogenesis, and is lowered in preeclamptic placentas. *Placenta*. (2015) 36:809–20. doi: 10.1016/j.placenta.2015.04.008

32. Wang SC, Yu M, Li YH, Piao HL, Tang CL, Sun C, et al. Cyclosporin A promotes proliferating cell nuclear antigen expression and migration of human cytotrophoblast cells via the mitgen-activated protein kinase-3/1-mediated nuclear factor-κB signaling pathways. *Int J Clin Exp Patho*. (2013) 6:1999–2010.

33. Li Y, Zhang D, Xu L, Dong L, Zheng J, Lin Y, et al. Cell-cell contact with proinflammatory macrophages enhances the immunotherapeutic effect of mesenchymal stem cells in two abortion models. *Cell Mol Immunol*. (2019) 16:908–20. doi: 10.1038/s41423-019-0204-6

frontiers | Frontiers in Immunology

TYPE Clinical Trial
PUBLISHED 24 February 2025
DOI 10.3389/fimmu.2025.1523871

Check for updates

OPEN ACCESS

EDITED BY
Marie-Pierre Piccinni,
University of Florence, Italy

REVIEWED BY
Soledad Gori,
Universidad de Buenos Aires, Argentina
Raj Raghupathy,
Kuwait University, Kuwait
Ricardo Barini,
UNICAMP, Brazil

*CORRESPONDENCE
Nathalie Lédée
nathalie-ledee@orange.fr

RECEIVED 06 November 2024
ACCEPTED 04 February 2025
PUBLISHED 24 February 2025

CITATION
Lédée N, Petitbarat M, Dray G, Chevrier L, Kazhalawi A, Rahmati M, Vicaut E, Diallo A, Cassuto NG, Ruoso L and Prat-Ellenberg L (2025) Endometrial immune profiling and precision therapy increase live birth rate after embryo transfer: a randomised controlled trial.
Front. Immunol. 16:1523871.
doi: 10.3389/fimmu.2025.1523871

COPYRIGHT
© 2025 Lédée, Petitbarat, Dray, Chevrier, Kazhalawi, Rahmati, Vicaut, Diallo, Cassuto, Ruoso and Prat-Ellenberg. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Endometrial immune profiling and precision therapy increase live birth rate after embryo transfer: a randomised controlled trial

Nathalie Lédée[1,2,3]*, Marie Petitbarat[2], Geraldine Dray[1], Lucie Chevrier[2], Alaa Kazhalawi[2], Mona Rahmati[4], Eric Vicaut[5], Abdourahmane Diallo[5], Nino Guy Cassuto[6], Lea Ruoso[6] and Laura Prat-Ellenberg[2]

[1]MatriceLab Innove Laboratory, Immeuble Les Gemeaux, Creteil, France, [2]Centre d'Assistance Médicale à la Procréation, Hôpital des Bluets, Paris, France, [3]Université Versailles- St Quentin en Yvelines (UVSQ), Unité de Formation et Recherche (UFR), Versailles, France, [4]London Women's Clinic, London, United Kingdom, [5]Unité de Recherche Clinique, Hôpital Fernand Widal, (APHP), Université Paris-Diderot Paris 7, Paris, France, [6]Laboratoire Drouot, Paris, France

Introduction: Despite advancements in assisted reproductive treatments, 70% of transferred embryos fail to implant successfully, yielding significant personal and global repercussions. One promising avenue of research is to take into account the individual's immune uterine profile in order to tailor treatment and optimise outcomes. This randomised controlled trial represents the initial exploration into the consequences of disregarding the state of the uterine immune environment in infertile women embarking on IVF/ICSI.

Materials and methods: This randomised controlled open two-arm trial included IVF patients, with assessment of immune endometrial environment and precision therapy before embryo transfer (ET). 493 patients were enrolled from October 2015 to February2023. Endometrial biopsies were collected during the mid-luteal phase. Endometrial immune profiling involves the analysis of cytokine biomarkers in the endometrium. If an immune endometrial dysregulation was diagnosed, a computerised randomisation assigned patients to either a conventional ET (disregarding the immune profile) or a personalised ET (with a precision therapy adapted to the immune profile). The primary analysis focussed on demonstrating the superiority of precision treatments using the modified intent-to-treat population (mITT), excluding patients without ET. The primary endpoint was the live birth rate (LBR) following the first attempt of ET.

Results: Among the population, 78% had an endometrial immune dysregulation and were randomised. The mITT analysis showed a significant increase in LBR with precision care compared to conventional care (29.7% vs. 41.4%; OR: 1.68 [1.04-2.73], p=0.036). The positive impact of precision care was particularly

noticeable in patients with morphologically suboptimal embryos (LBR: 21.2% vs. 39.6%; OR: 2.12 [1.02-4.41]) or those with a history of two or more failed ET (LBR: 23.4% vs. 48.1%; OR: 3.03 [1.17-7.85]).

Limitations and reasons for caution: The data should be interpreted with caution due to inherent structural limitations of human IVF trials. Generalising and empowering our findings would rely on the replication of controlled trials by independent research teams possibly integrating the usage of optimised embryo quality with PGT-A.

Conclusion: The regulation of the endometrial immune environment emerges as one of the leading innovative strategies to facilitate embryo implantation and enhance the overall performance of assisted reproductive therapies (ART). Based on these findings, endometrial immune profiling could become an essential part of routine ART practice.

Clinical trial registration: clinicaltrials.gov, identifier NCT02262117.

KEYWORDS

endometrial immune profiling, uterine immune regulation, precision care, IVF, pregnancy, randomised controlled trial

1 Introduction

Assisted reproductive technologies (ART) have made significant progress in the last few decades and have become a widely accepted therapy for infertility. According to WHO, 15% of couples having unprotected sex, representing 48 million couples and 186 million people, suffer from infertility worldwide (1). However, despite the improvements in ART, the success rates remain relatively low. Indeed, the live birth rate per initiated cycle is approximately 30% for women under 35 years old (equal to 70% of failure) and decreases drastically with age (2). This leads to emotional, psychological, and financial stress with significant social and economic consequences, including loss of productivity and a decline in the quality of life for couples (3). Therefore, there is an urgent need for research and innovation to improve success rates and reduce the emotional and financial burden associated with conventional treatment.

Endometrial immune profiling is an innovative strategy involving the analysis of functional immune biomarkers in the endometrium. This approach aims to identify immune disturbances contributing to embryo implantation failures or pregnancy loss and guide the development of personalised treatment plans to increase embryo implantation rates. Incorporating uterine immunity as a key factor in routine practice for designing effective reproductive treatments has not been undertaken thus far. Human pregnancy is a precisely timed (4) semi-allograft that needs to be tolerated by the maternal immune system to survive in physiological conditions. (5) The maternal immune system itself has to be reprogrammed towards tolerance (6, 7). An increasing number of clinical studies also report the essential role of immune cells in endometrial receptivity to embryo implantation and early placental development (8, 9). The Uterine Immune Profile offers a simplified representation of the complex immune processes involved in implantation. The clinical objective is to create a tool that helps clinicians apply precision medicine by integrating this essential local immune response. Previous extensive cohort studies, focussing on individuals with a history of repeated unexplained implantation failures or unexplained recurrent miscarriages using endometrial immune profiling, have revealed that 75-80% of these infertile patients have uterine dysregulations impeding the implantation process. Personalising care to address observed dysregulations has yielded significant benefits, with a relative increase of 40-50% in live birth rates observed compared to the ones expected in these populations (10–12).

Human implantation involves the synchronised interaction of the embryo and the endometrium. The window of implantation (WOI) defines the crucial time frame of uterine receptivity when the endometrium undergoes changes in response to hormonal signals from the ovary (mainly progesterone), preparing it to receive and support an embryo (i.e. decidualisation) (13). Endometrial immune cells play a critical role in the process, as they contribute to the establishment of a receptive environment for the embryo to implant and develop (14, 15). During this window, a crucial shift from adaptive immunity to innate immunity takes place in the endometrium (16). This shift creates an immunologically tolerant and fruitful environment for the developing embryo, which is a

semi-allograft. The balance between Th1 and Th2 cytokines, initially described by Tom Wegmann thirty years ago, plays an essential role in the success of implantation (17). The shift to a Th2-dominant immune environment influences the differentiation of immune cells, including macrophages, dendritic cells, uterine NK cells, and regulatory cells, either positively or negatively, thereby promoting or inhibiting implantation and placentation (7, 18). The quantification of RNA expression levels of five biomarkers gave key information regarding the immunoregulated Th-2/Th-1 local balance, the destabilisation of spiral arteries, and the mobilisation and maturation of the very specific uterine natural killer (uNK) cells (19). Interleukin-18 (IL-18) is a pro-inflammatory cytokine crucial for immune regulation in reproduction, playing key roles in embryo implantation, trophoblast invasion, NK cell modulation, and placental vascularisation (20–22) Interleukin-15 (IL-15) supports embryo implantation and placentation by promoting uterine natural killer cell maturation, function and cytokine production essential for reproductive processes (23–25). In the context of embryo implantation, TWEAK/Fn-14 signalling has been shown to regulate the cytotoxicity of uNK cells, which is important for controlling trophoblast invasion and preventing foetal rejection (26, 27). Hence, the ratio of IL-18/TWEAK mRNA was used as a biomarker that served as an indicator of both angiogenesis and the Th1/Th2 balance. IL-18/TWEAK provided insights into the local immune environment and the potential presence of an immune deviation towards Th1 cytokines, which can affect the implantation process (11). On the other hand, IL-15/Fn-14 mRNA was used as a biomarker to assess the activation and maturation status of uterine natural killer (uNK) cells, along with the evaluation of uNK-CD56 cell count (11). By quantifying these targets, we established the endometrial immune profile during the mid-luteal phase, aiming to understand how the endometrium is prepared for successful implantation and to identify dysregulations that may hinder this process (28).

One promising avenue of research is the use of precision medicine approaches, considering an individual's unique immune endometrial profile to tailor treatment and optimise outcomes. Specific immune cell biomarkers identified through endometrial profiling can guide the selection of appropriate immune-modulating therapies to improve pregnancy outcomes. By gaining a deeper understanding of the endometrial immune profile, we may be able to offer more effective and individualised care to patients struggling with infertility.

In the present randomised controlled trial (RCT), the endometrial immune profile was performed in IVF patients before their embryo transfer. If a local immune dysregulation was diagnosed, computerised randomisation allocated the patient to either a conventional embryo transfer (disregarding the immune profile) or a personalised embryo transfer (considering the immune profile and a proposed plan to correct the dysregulation). The primary outcome was the live birth rate following embryo transfer among dysregulated patients with conventional versus precision care. This RCT explored the consequences of not considering the endometrial immune environment in patients during IVF treatment.

2 Methods

2.1 Study design

The study protocol was approved by Institutional Review Boards at the University Paris Diderot (clinicaltrials.gov NCT02262117) and our trial followed the extended CONSORT guidelines.

The study has been designed as an open RCT including infertile patients below 38 years with no ovarian insufficiency involved in assisted reproductive treatment and for which documentation of their immune endometrial environment has been performed before a scheduled embryo transfer.

Patients with a diagnosed endometrial dysregulation were randomised: half of the patients received conventional medical care (disregarding the immune profile) while the other half received precision medical care (according to their immune endometrial profile). The primary analysis was based on the modified intention-to-treat population (excluding patients without ET) and the primary efficacy endpoint was the live birth rate (LBR).

This randomised controlled trial was spread over 10 years because we had to revise our initial approach. From October 2014 to August 2016, we started this RCT with the same inclusion criteria and the same mITT but the randomisation was between endometrial profiling vs. no endometrial profiling, although all patients had a biopsy at enrolment. We decided to abandon this study design because in the case of randomisation to the 'no endometrial profiling' arm, we would have missed some important information such as the presence or absence of endometrial dysregulation and its type, which is essential for further analysis. 12 Patients included in the group "no immune profiling" group who had an endometrial biopsy stored but not analysed were re-included in the this RCT. Major amendment was applied for this new design and a new electronic list of randomisation was generated.

The first patient was included on October 30th, 2015, the last patient on February 8th, 2023.

A Data and Safety Monitoring Board (DSMB) reviewed interim results periodically throughout the study. No change was made to the personal treatment design during the study, except enlargement of inclusion criteria. Amendments were applied in 2017 to enlarge criteria of inclusion to a range of three oocytes pick up (initially set a 2) and to accept patients using frozen embryos (initially only fresh ET). This new design required more patients to be included. The protocol was suspended during the COVID-19 pandemic in 2020.

As two authors (NL, MPB) hold the patent related to the presented innovation, measures were implemented to ensure the impartiality of the study and address potential bias. To this end, patient inclusion and follow-up until birth or not were independently inspected by a clinical research associate from the independent clinical research unit of the University of Paris Diderot. Furthermore, statistical analyses were conducted by a statistician from the same independent research unit to ensure objectivity in data interpretation.

All patients were followed at the same ART Unit ("Pierre Rouques Les Bluets" Hospital in Paris, France) for the endometrial biopsy, the oocyte retrieval and the embryo transfer.

The study flow is illustrated in Figure 1.

2.1.1 Stage 0: consent, inclusion criteria and information

The inclusion criteria were infertile patients with an indication to perform either an IVF with or without ICSI. The indication for IVF were tubal infertility, endometriosis, ovarian dysovulation or idiopathic infertility after IUI failure. The indication for ICSI was male infertility (oligo-astheno-teratospermy) or previous failure of oocyte fertilisation in IVF. Patients were younger than 38 years old (age < or equal to 38 years at the time of inclusion), with no ovarian insufficiency (AMH>1.5ng/ml, FSH<10 IU/l on day-3, antral follicles count (AFC) over 6 on day-3 of the cycle by ultrasound). The range of previous oocyte pick-up for IVF attempts were strictly lower than 3. If a previous live birth had occurred in the past by IVF, the range of the new attempt was 1. Patient had signed an informed consent form and had medical insurance.

The exclusion criteria were azoospermia or cryptozoospermy for the partner, a uterine malformation, an IVF attempt scheduled in another ART unit or contraindication to the use of corticoids, HCG or slow intralipid perfusion.

If the inclusion criteria have been met and exclusion criteria were absent, the clinician informedthe patient of the proposed protocol. If patients agreed to participate, an endometrial biopsy was scheduled in the mild luteal phase.

2.1.2 Stage 1: endometrial biopsy, collection and analysis

In order to target the mid-luteal phase and avoid problems associated with cycle fluctuations, 90% of patients were prepared in a substituted cycle and samples were taken exactly 7 days after the introduction of progesterone.10% were evaluated on a monitored natural cycle and samples were taken 9 days after the LH surge with progesterone dosed 48 hours before sampling. The endometrial fragment was gently aspirated by rotating a Cornier Pipelle within the endometrial cavity (Leclair et al., 2011). The Pipelle content was divided into two parts: the first part was placed in 4% formaldehyde (QPath Formol 4% buffered, VWR Chemicals, Fontenay-sous-Bois, France) for endometrial datation (29), by a histological test to determine the phase of the cycle, and CD-56 immuno-labelling. The second part was placed in RNAlater stabilisation solution for immunological analysis (MatriceLab Innove, France). The samples were sent at room temperature by postal services.

2.1.2.1 RNA extraction and reverse transcription

After confirmation of the histological dating, RNA was extracted from the biopsy sample conserved in RNAlater (Qiagen). RNA extraction was performed on Biomek1.5 using Kit RNAdvance Tissue (Beckman-Coulter). The RNA was reverse-transcribed into cDNA with the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Meylan, France), according to the manufacturer's instructions. The cDNAs were stored at –20°C until use.

2.1.2.2 Quantitative RT-PCR

Quantitative RT-PCR was performed with a Light Cycler 480 instrument (Roche Diagnostic) and the Light Cycler 480 SYBR Green I Master mix (Roche Diagnostic). Final concentrations for reaction set-up were 0.5 µM of sense and anti-sense primers and 1/20 of diluted cDNA. Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantitation (95°C for 10 s, 60°C for 10 s and 72°C for 15 s) repeated 40 times, a melting curve program (65-95°C with a ramp rate of 2.2°C/s) and a cooling step to 4°C. Each quantitative RT-PCR assay included a solution without cDNA and inter-run calibrator (IRC)

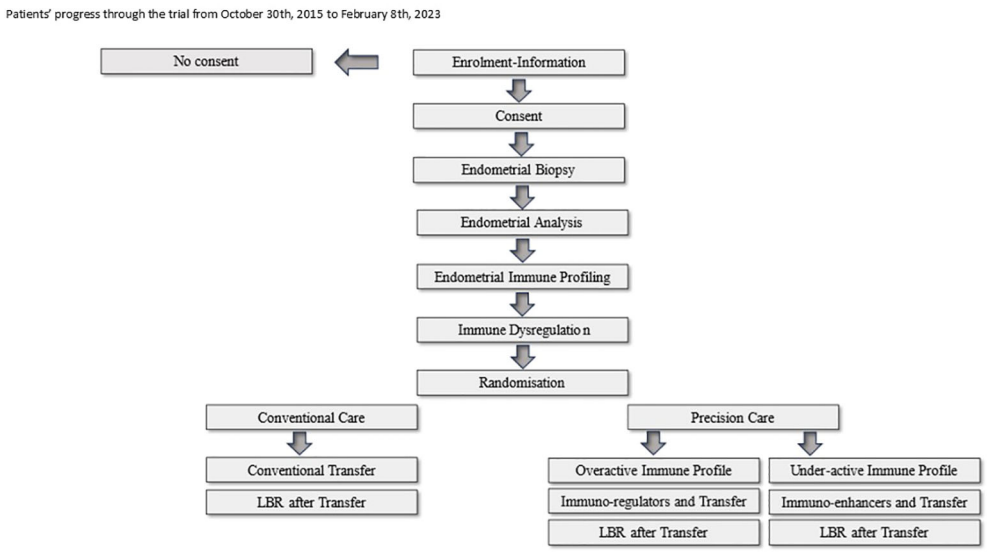


FIGURE 1
Patients' progress through the trial from October 30th, 2015 to February 8th, 2023.

samples as negative and positive controls. The IRC for all the primers (IL18, IL15, TWEAK, Fn14 and CD56) was obtained from pools of RNA endometrial samples. The IRC cDNA, after dilution by a factor of 20, underwent the same quantitative RT-PCR protocol as the unknown samples. PCR efficiency for each quantified target and reference was calculated with known serial dilutions of each specific cDNA. LightCycler®480 Software release 1.5.0 was used to analyse data, and each specific target transcription level was normalised to the geometric mean of the transcription level of the reference gene, with the software's advanced relative quantification workflow. Gene amplification efficiency was specifically determined. For each sample, the results were expressed as the ratio of target/reference cDNA.

2.1.2.3 Immunohistochemistry (IHC) of uNK cells

IHC was performed on the biopsy sample tissue conserved in 4% formol on 5-µm thick slides, with an automated streptavidin-biotin method (Benchmark GX, Ventana Medical Systems). The prediluted anti-CD56 (clone 123C3) murine monoclonal primary antibody (Ventana Medical Systems®, Roche Diagnostics) was applied according to the manufacturer's instructions. Briefly, after deparaffinization of the slides, antigen retrieval was performed for 60 minutes in a pH 8.4 Cell Conditioning 1 solution. The CD56 primary antibody was then applied for 32 min. Slides for negative controls were prepared by replacing the primary antiserum with non-immune IgG. Slides were then incubated for 8 min with a biotinylated anti-mouse secondary antibody. Diaminobenzidine or 3-amino-9-ethylcarbazole was used as the chromogen (iVIEW DAB detection kit, Ventana Medical Systems) and slides were counterstained with haematoxylin for 2 min, incubated in bluing reagent (for 2 min), and mounted. Between each step, slides were rinsed with reaction buffer. The uNK cell count was measured as the mean of CD56+ cells in 4 representative fields at ×400 magnification.

To establish the endometrial immune profile, a step-by-step procedure first considered the IL-18/TWEAK mRNA ratio (reflecting local angiogenesis and possibly a Th1 deviation), then the CD56+ cell count (reflecting uNK cell mobilisation), and finally the IL-15/Fn-14 mRNA ratio (indicative of uNK cell maturation and uNK cytotoxic activation).

2.1.3 Stage 2: interpretation of analysis

Using standardised RT-qPCR method, the expression norms of our biomarkers were previously established in a fertile cohort. In particular, we documented that an immune profile was reproducible from one cycle to the next over a six-month period if no surgery or pregnancy had occurred in the interim.

Endometrial immune profiles was classified into four types:

1. A balanced endometrial immune activation profile, which is characterised by IL-18/TWEAK and IL-15/Fn-14 mRNA ratios and a CD56+ cell count within the same range as previously defined in the fertile cohort. This profile suggested that the endometrium was ready to go through the following steps of implantation, including apposition, adhesion, and invasion Patients presenting with this endotype were not randomised and excluded from the study.

The three other subgroups represented patients with immune dysregulation who were randomised via the electronic server (Cleanweb- APHP).

2. An under-activated endometrial immune profile was defined by low IL-15/Fn-14 (reflecting immature uNK cells) and/or low IL-18/TWEAK mRNA ratios as well as low CD56+ cell expression.

This profile suggested thatthe endometrium was not fully effective for adhesion and promoting adequate immunotrophism during initial placentation.

3. An over-activated endometrial immune profile was characterised by high IL-18/TWEAK and/or IL-15/Fn-14 mRNA ratios and/or a high CD56+ cell expression.

4. A mixed endometrial immune profile was distinguished by a high IL-18/TWEAK (excess Th-1 cytokines) mRNA ratio and a low IL-15/Fn-14 mRNA ratio (reflecting immature NK cells) and/or low CD56+ expression.

For over-activated and mixed profiles, their profiles suggested that the endometrium were not prepared for the crucial step of trophoblast invasion and may be in a state that can reject the embryo because of a cytotoxic activation of uNK cells in LAKs (lymphocyte-activated killer cells) (30). A test under therapy (glucocorticoids or intralipids) was proposed if the patient was randomised in the personalised arm.

A report, describing the presence or absence of endometrial immune dysregulation was generated and included in the patient's medical file.

2.1.4 Stage 3: randomisation

Randomisation by blocks was made using the electronic server (Cleanweb- APHP) which allocated patients in a 1:1 ratio to the groups "dysregulated - conventional care" or "dysregulated - precision care" once histological and immune results confirmed the mid-luteal phase and the validity of the endometrial immune profile. Only patients with diagnosed endometrial immune dysregulation were randomised.

If the patient has been randomised for precision care, the report described the suggested treatment plan to apply for the embryo transfer.

2.1.4.1 In case of randomisation: "dysregulated - conventional care"

The patient had a standard fresh or frozen embryo transfer without scratching, or adjunction of corticoids, intralipids, chorionic gonadotropins or double sequential embryo transfer. If the attempt fails, the clinician could decide to personalize the patient's attempt at the second embryo transfer when the patient ended her participation in the present study.

2.1.4.2 In case of randomisation: "dysregulated - precision care"

Once randomised to the Precision Care group, the treatment they received depended on their individual immune profile- For patients diagnosed with under-active immune profile: the treatment strategy was directed to stimulate mobilisation of immune cells and expression of adhesion molecules.

The precision care was characterised.

- by a endometrial scratching in the mild luteal phase of the cycle preceding the embryo transfer. The objective was to trigger the expression of adhesion molecules and interleukin-15 (31–34).
- by supplementing with chorionic gonadotropins the luteal phase, to trigger local angiogenesis and uNK cells mobilisation (35, 36).
- by advising to have sexual intercourse after the embryo transfer to stimulate the local mobilisation and expression of immune cells (37, 38).

If the patient is over 35 years old with at least one previous ET failure, a double sequential transfer of one embryo on day 3 and one embryo on day 5 was proposed to stimulate the local embryo-endometrium dialogue before implantation (39).

Micronized progesterone for luteal support was usually prescribed at 200 mg three times a day.

- For patients diagnosed with an over-active immune profile or a mixed profile: the strategy aims to down-regulate the local activity of local immune cells.

In this subgroup, immunosuppressive therapy was introduced, aiming at controlling the dysregulated Th-1/Th-2 ratio evaluated by IL-18/TWEAK mRNA expression levels which were elevated in this subgroup. We previously documented using micro-histoculture endometrial models that a high IL-18/TWEAK ratio revealed an underlying cytotoxic activation of uterine NK cells (30). Glucocorticoids was prescribed as a first line of treatment (40, 41) (42) and slow perfusion of intralipids as a second line of treatment in case of resistance to glucocorticoids (43).

The dose of micronized progesterone for luteal support was increased to 400 mg three times a day for its documented immunosuppressive properties (44, 45).

A cycle test under therapy was proposed to evaluate if corticoids or intralipids were able to normalise the endometrial profile. If the endometrial profile was normalised, then the therapy tested was considered as efficient and added for the next embryo transfer. If the endometrial profile was not normalised under corticoids, intralipids was used. If the endometrial profile was not normalised under intralipids, corticoids was used.

If the over-activated profile was associated with, a low uNK cell mobilisation (<10/field) or immature uNK cells (mixed profile), endometrial scratching was added to the cycle preceding embryo transfer and chorionic gonadotropins was used in the luteal phase after the transfer cycle.

Of note, if the patient did not want to have a test of her sensitivity to corticoids, corticoids or intralipids was administered by default.

2.1.4.3 Treatments suggested

Regarding the immune profile, intralipids or/and corticoids and/or chorionic gonadotropins were administered with a variable dosage.

- Glucocorticoid tablets (20 mg daily) was taken by the patient from the third day of the cycle until the pregnancy test and continued for 2 months if pregnant (from 21 days to 3 months) after the endometrial immune analysis. Glucocorticoids was gradually weaned off and stopped in case of negative pregnancy test or after 10 weeks of pregnancy.
- Intralipids (Intralipid 20g/100mL diluted in 400mL of NaCl 0.9%) was administered by slow perfusion during ovarian stimulation (around Day 8 of the cycle) and repeated if pregnant, at 5 weeks and 9 weeks.
- 250µg/0.5 ml of Ovitrelle was administered by subcutaneous route in the luteal phase, 4, 6 and 8 days after the egg collection or the introduction of progesterone (46). This treatment was not prescribed if more than 11 oocytes have been collected or if the oestradiol blood levels on the day of triggering the ovulation was over 3000 pg/ml to avoid ovarian hyperstimulation syndrome.

2.1.5 Stage 4: preparation for the embryo transfer

IVF after a monitored ovarian hyper-stimulation for a fresh embryo transfer as well as endometrial preparation for frozen embryos were conducted as per common protocols.

The delay between the last endometrial immune analysis and the embryo transfer must not exceed 9 months for the mITT analysis. If a spontaneous pregnancy or a gynaecological surgery occured between the biopsy and embryo transfer, patients were excluded from the mITT analysis.

Before 2018, embryo culture until day-5 was not applied as a first-line policy of transfer and day 2-3 embryos were mainly transferred. For the initial transfer, one day-3 embryo was used if the patient was below 30 years old, but in the majority of cases, two day-3 embryos were transferred.

After 2018, the embryo transfer policy has been to favour prolonged culture of embryos until day-5 to promote singletons and prevent multiple pregnancies. Day-3 transfers were only performed if less than 2 embryos were available on day 3 or in case of previous prolonged culture failure.

Endometrial immune profiling did not impact the classical embryo transfer policy except for patients with under-active immune profiles. For these patients, a specific policy of transfer was in place, if they were over 35 years old or previously failed with standard embryo transfer. In such a profile, a sequential double transfer was proposed, the first embryo was transferred on day 2-3 and the second one on day 5-6.

To evaluate the impact of embryo quality on subsequent pregnancy rate, embryo transfers were organised into 2 classes ("top" transfer or "no top" transfer) according to the embryologist's observations on the day of transfer (47, 48). Each embryo transfer included in the study has been classified anonymously by two distinct embryologists (LR, GC).

On days 2-3, the standard BLEFCO classification (49) was used to evaluate the embryos and on day-5 the Gardner classification (47) was used for the evaluation of blastocyst quality. On day 2-3,

"top" grade A high quality embryo was defined as an embryo with typically equal-sized blastomeres or unequal-sized blastomeres according to the number of cells with less than 10% fragmentation. On day-2 the embryo should have 2 to 4 cells, and 6 to 10 on day-3. On day-5, "top" grade A excellent quality Blastocyst was defined as a blastocyst with large, fully expanded blastocoel, inner cell mass, and trophectoderm tightly packed and clearly defined [B5AA-B5AB-B5BA-B4AA-B4AB-B4BA].

Top transfers were defined as the transfer of top quality embryos. If two embryos were transferred, the two embryos have been evaluated as "top". All the other combinations were classified as "no top" transfer.

Patients for whom a decision to transform the IVF attempt to IUI was taken were included in the mITT analysis as the ITT has been applied and patients were equally represented among the groups. This decision was made because the ovarian response to stimulation was too low (less than 3 follicles) to decide to retrieve oocytes, despite potential fertility on both the male and female side.

2.1.6 Stage 5: outcomes, analysis of the embryo transfer attempts

The live birth rate was defined by the birth of a living baby and was the primary outcome of the mITT analysis. Secondary outcomes were the ongoing pregnancy rate, the clinical pregnancy rate and the miscarriage rate.

The ongoing pregnancy rate was defined by a scan attesting the presence of a gestational sac with an embryonic cardiac activity, that had progressed beyond the first trimester (12 weeks) and was continuing. The clinical pregnancy rate was defined by a BhCG over 100 IU/l in the serum 12 to 10 days after the embryo transfer. Miscarriage referred to the loss of a pregnancy that had occurred after embryo transfer, at any stage of pregnancy, from implantation to the end of the first trimester (12 weeks gestation). Miscarriage did not include biochemical pregnancies that were considered as no pregnancy in our analysis but included early pregnancy losses (gestational sac seen on ultrasound but no heartbeat).

2.2 Statistical methodology

Categorical data were presented as numbers (percentages). Continuous variables were presented as means with standard deviations (SDs) and medians with interquartile ranges (interquartile range IQR described as 25th and 75th percentile) for normal and skewed distributions, respectively.

The primary analysis was based on the modified intention-to-treat population (excluding patients without ET) and the primary efficacy endpoint was the live birth rate. The primary efficacy endpoint was compared between the personalised and conventional care using a binary logistic regression.

A logistic regression model was performed including prior known risk factors as covariates (age class, embryo quality, embryo transfer and endometrial immune profile). The 95% two-sided CI for odds ratio (OR) was computed using the bias-corrected and accelerated (BCa) bootstrap interval, OR was presented together with a two-sided 95% BCa confidence interval and associated p-values.

All secondary analyses were based on the mITT population. The secondary binary endpoints were analysed using the same methods as the primary endpoint. For secondary continuous endpoints that were normally distributed with a homogeneity of variance across groups, a t-test was used. For secondary continuous endpoints that were normally or asymptotically normally distributed and heteroscedastic, the Welch t-test was used. For secondary continuous endpoints that are heavy-tailed and skewed, the Mann–Whitney U test was used.

Pre-specified subgroup analyses to evaluate variations in treatment effect were done using logistic regression models, with terms for treatment, subgroup, and interaction of treatment with subgroup. All reported subgroup analyses were pre-specified.

Assuming a 25% birth rate per embryo transfer with conventional care and a 40% relative increase in birth rate with precision care, a sample size of 152 patients per group was needed to achieve 80% power to detect this difference using a chi-square test at a two-sided 5% significance level. Given an anticipated 25% exclusion rate post-randomisation, a total of 380 dysregulated patients (190 per group) had to be randomised. To reach this target, approximately 500 patients were screened since 20% were expected not to be dysregulated. All statistical tests were two-sided and were performed at the 0.05 level. All tests were performed using SAS version 9.4 or later.

3 Results

3.1 Flow-chart of the study participants

The Figure 2 illustrates how patients progressed through the trial. 493 patients were included in this study from October 30th, 2015 to February 8th, 2023. The immune profiling analysis, however, was successfully performed only for 484 patients.

Out of the 484 patients, 78% (378) had a dysregulated endometrial immune profile.

Among deregulated patients, 190 were randomised to receive conventional treatment, and 188 were randomised to receive precision treatment according to their type of immune dysregulation.

14 patients gave up the protocol before the embryo transfer. In 8 cases, surgery was indicated before the embryo transfer and one patient developed endometritis, invalidating the endometrial immune profiling. 3 patients developed azoospermia or ovarian premature failure (exclusion criteria) and 2 couples postponed IVF due to significant health issue. 27 patients were lost to follow-up and did not contact the IVF unit for their embryo transfer after having performed the endometrial immune profiling and 3 patients withdrew their consent.

Finally, 17 patients became pregnant spontaneously. These patients will be treated separately in the analysis because some of these pregnancies seemed to be the direct consequence of identified and treated endometrial immune dysregulation.

Overall, 317 patients have been scheduled for an embryo transfer.

10.3389/fimmu.2025.1523871

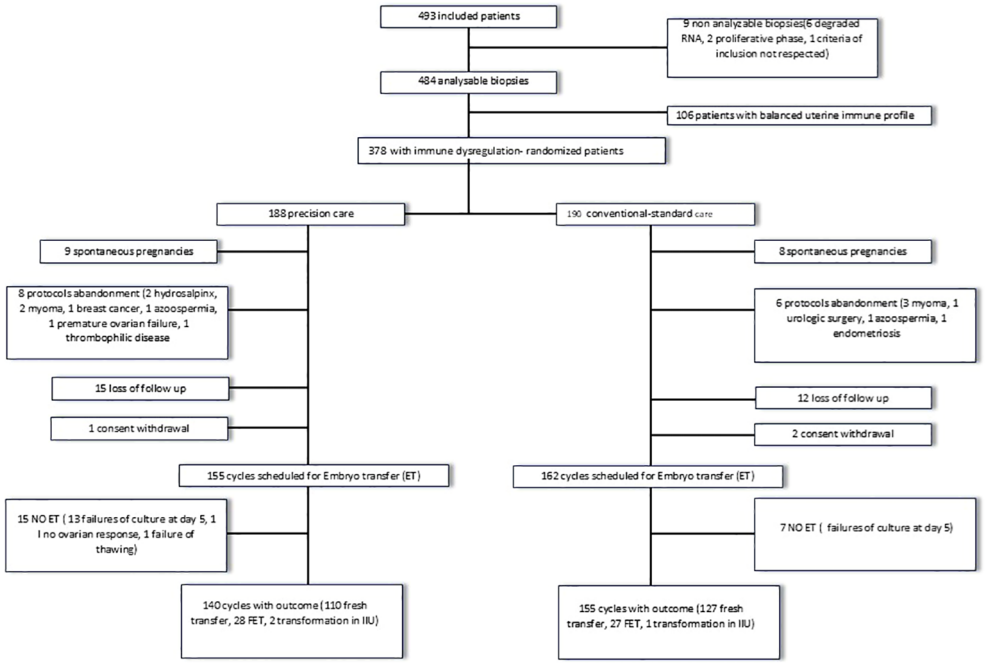


FIGURE 2
Flow chart of the study.

No embryo transfer could be performed in 22 patients (failure of embryo culture until day-5 in 20 cases, no ovarian response in 1 case, failure of embryo thawing in 1 case).

An outcome was available for 295 dysregulated patients: 140 dysregulated with precision care and 155 dysregulated with conventional care.

240 patients were scheduled for a fresh embryo transfer, and among those, 3 were converted to intra-uterine insemination. 58 patients were scheduled for a frozen embryo transfer.

For fresh ET, 80% were stimulated using an antagonist protocol and 20% a long agonist protocol. For frozen transfers, 42% were prepared through natural cycles, 10% with FSH mild stimulation and 8% were prepared with a substituted cycle.

3.2 Demographic characteristics, aetiology and past history of study participants

Table 1A summarises the clinical and demographic data of patients randomised in conventional versus precision care.

The mean age of the cohort was 33 years old. 64.7% were below 35 years old and 35.3% were over 35 years old.

The main cause of infertility among dysregulated patients was male infertility in 36%, a tubal-related pathology in 20%, an ovulatory problem in 14%, endometriosis in 10%, idiopathic in 10% and recurrent miscarriages in 1%. 9% of the infertility was mixed with male and female factors.

At the time of inclusion, 33% (98/295) never had oocyte retrieval, 50.5% (149/295) failed to be pregnant despite 1 oocyte pick-up and 16% (48/295) failed to be pregnant despite 2 oocyte pick-ups.

Of the 295 patients randomised for whom an outcome was available, 33.2% (98/295) never had any ET before, 21.7% (64/295) previously failed one ET, 27.7% (82/295) previously failed two ET and 17.3% (51/295) failed more than 2 ET (3-5).

According to the new definition of repeated implantation failures (RIF) edited by the ESHRE committee (50) in 2023, 5% (15/295) of the patients randomised with outcome could have been classified as RIF patients in this cohort.

3.3 Endometrial immune profiling among study participants

106 patients had a balanced endometrial immune profile, comprising 22% of the cohort, while 378 patients had dysregulated profile, making up 78% of the cohort. No significant differences were observed between the conventional and precision groups with regard to age, previous embryo transfers, fresh or frozen transfers, protocols used, transfer quality, or the distribution of different immune profiles (Tables 1A, B). Nor did they differ between dysregulated and non-dysregulated women.

Among dysregulated patients, 30% had under-active profiles, 47% had over-active profiles, and 13.8% had mixed profiles (Table 1B).

Therapy testing (glucocorticoids or intralipids) was suggested for patients with overactivation or mixed profiles in the precision group. 83 patients underwent a therapy testing prior ET to verify the immunosuppressive efficacy of the treatment on the diagnosed dysregulation. After testing, 26 patients received glucocorticoids, 44 patients received intralipids (resistance to GC), and 18 received combined intralipids and corticoids. 14 patients with over-

10.3389/fimmu.2025.1523871

TABLE 1A Descriptive clinical data of patients included in this study.

	Total N=295	Precision Care N=140	Conventional Care N=155
Age, years			
n (miss.)	295 (0)	140 (0)	155 (0)
Mean ± sd	33.3 ± 3.3	33.5 ± 3.4	33.2 ± 3.2
Median (Q1;Q3)	33.5 (31.1;36.1)	33.7 (31.5;36.1)	33.4 (31.0;36.1)
Min, Max	23.1, 38.7	23.1, 38.7	24.4, 38.5
Age, class — no. (%)			
≥ 35	104 (35.3%)	54 (38.6%)	50 (32.3%)
< 35	191 (64.7%)	86 (61.4%)	105 (67.7%)
All	295 (100.0%)	140 (100.0%)	155 (100.0%)
AMH ng/ml	3.16 (2.27;4.70)	3.30 (2.40;4.49)	2.95 (2.21;4.86)
Previous ET failure, two levels — no. (%)			
At least 1 ET failure	197 (66.8%)	95 (67.9%)	102 (65.8%)
No previous ET	98 (33.2%)	45 (32.1%)	53 (34.2%)
All	295 (100.0%)	140 (100.0%)	155 (100.0%)
Previous ET failure, three levels — no. (%)			
Two or more transfer failed	133 (45.1%)	69 (49.3%)	64 (41.3%)
One transfer failed	64 (21.7%)	26 (18.6%)	38 (24.5%)
No previous ET	98 (33.2%)	45 (32.1%)	53 (34.2%)
All	295 (100.0%)	140 (100.0%)	155 (100.0%)
Number of previous embryos transferred			
n (miss.)	295 (0)	140 (0)	155 (0)
Mean ± sd	1.4 ± 1.3	1.5 ± 1.3	1.3 ± 1.2
Median (Q1;Q3)	1 (0;2)	1 (0;2)	1 (0;2)
Min, Max	0, 5	0, 5	0, 5

TABLE 1B Summary of type of immune imbalance and type of embryo transfer.

	Total N=295	Precision Care N=l40	Conventional Care N=l55
Uterine immune profile - no. (%)			
Over activation	145/295 (49.2%)	711140 (50.7%)	74/155 (47.7%)
Under activation	111/295 (37.6%)	46/140 (32 .9%)	65/155 (41.9%)
Mixt	39/295 (13.2%)	23/140 (16.4%)	161155 (10.3%)
All	295/295 (100.0%)	140/140 (100.0%)	155/ 155 (100.0%)
Number of ET			
n (miss.)	291 (4)	138 (2)	153 (2)
Mean ± sd	1.3 ± 0.5	1.3 ± 0.5	1.2 ± 0.4
Median (Ql ;Q3)	1 (1;2)	1 (1;2)	1 (1;1)
Min, Max	1, 3	1, 3	1, 2
All	290/290 (100.0%)	137/ 137 (100.0%)	153/153 (100.0%)

(Continued)

10.3389/fimmu.2025.1523871

TABLE 1B Continued

	Total N=295	Precision Care N=l40	Conventional Care N=l55
Stage and number of ET - no. (%)			
1 Day 2/3	(0.68%)	1/138 (0.73%)	1/153 (0.65%)
2 Day 2/3	*521290* (17.9%)	26/138 (18.9%)	261153 (16.9%)
1 Day 5/6	*2061290* (71%)	89/ 138 (64%)	117/153 (76%)
2 Day 5/6	16/290 (5.5%)	7/ 138 (5.1%)	9/153 (5.8%)
DSET	15/290 (5.1%)	15/ 138 (10.9%)	0 (0%)
All	*2901290* (100.0%)	138 (100.0%)	153 (100.0%)
Quality of ET - no. (%)			
No Top	190/291 (65.3%)	91/138 (65.9%)	99/153 (64.7%)
Top	101/291 (34.7%)	47/ 138 (34.1%)	54/153 (35 .3%)
All	291/291 (100.0%)	138/ 138 (100.0%)	153/153 (100%)
Embryo transfer - no. (%)			
Fresh Transfer	222/290 (76.6%)	103/ 137 (75.2%)	119/ 153 (77.8%)
Fet	68/290 (23.4%)	34/ 137 (24.8%)	34/153 (22.2%)
All	290/290 (100.0%)	137/ 137 (100.0%)	153/ 153 (100.0%)

activation declined therapy testing, with 10 having the transfer under glucocorticoids and 4 under intralipids. 5 patients with mixed profiles declined testing and received glucocorticoids with additional support.

No side effects or significant adverse events were in the present cohort related to glucocorticoids, slow perfusion of intralipids, or HCG supplementation.

3.4 Live birth rates in dysregulated patients randomised to precision versus conventional care

Comparing the LBR between dysregulated patients randomised to conventional versus personalised care constituted the primary endpoint of this study. The modified intention-to-treat (mITT) analysis revealed a significant increase in LBR with precision medical care, rising from 29.7% to 41.4%. The unadjusted odds ratio (OR) was 1.68 [1.04-2.73], p=0.036. Notably, the OR adjusted for age class, embryo quality at transfer, fresh or frozen transfer, and endometrial immune profile type was 1.75 [1.04-2.92], p=0.03).

In terms of the secondary endpoints assessed in the mITT analysis, both clinical pregnancy and ongoing pregnancy rates were consistently elevated with precision care (50.7% and 41.4%, p=0.04) when compared to conventional care (39.4% and 30.4%), as demonstrated through both unadjusted and adjusted analyses. No difference was observed regarding the miscarriage rate between conventional and precision groups. mITT analysis with primary and secondary endpoints are summarised in Table 2.

Subgroup analyses unveiled two particular subgroups which experienced substantial benefits from precision care (Figure 3). Firstly, patients with morphologically sub-optimal embryos for transfer ("no top" transfer) exhibited a significant increase in their LBR with precision care (21.2% with conventional care versus 39.6% with precision care, OR: 2.43 [1.28-4.61]). Conversely, for embryos with optimal morphology, immune dysregulation had no discernible impact (46% with conventional care, 43% with precision care).

Furthermore, the subgroup of patients who had previously undergone two or more embryo transfers and experienced failures also significantly benefited from personalisation, resulting in a substantial increase in their LBR (25.5% with conventional care versus 41% with precision care, OR: 2.66 [1.26-5.73]).

In terms of the specific type of immune dysregulation, it appeared that patients with either immune over-activation or immune under-activation benefitted from precision care, while those with a mixed profile did not show the same level of improvement.

Patients with an overactive immune profile who received precision therapy had a significant increase in LBR compared to those who received conventional standard therapy (47% versus 27%, odds ratio [OR]: 2.34 [1.2-4.8]). Notably, the differences were even more pronounced when focussing on patients who underwent the therapy test to assess their sensitivity to the immunosuppressive agent (51% versus 27%, OR: 2.8 [1.34-5.8]). Among patients with both over-active and mixed profiles, those with the therapy test had a significantly higher LBR than those without the therapy test (41% versus 27.7%, p=0.01).

For patients with immune under-activation, the live birth rate (LBR) showed a non-significant increase with precision care compared to conventional care (43% vs. 32%; OR: 1.61 [0.74–3.5], p = 0.23). However, it is essential to highlight as described

10.3389/fimmu.2025.1523871

TABLE 2 Primary and Secondary EndPoints, ITT population.

	Total N=295	Precision Care N=140	Conventional Care N=155	Absolute Difference* (95% CI)	Odds Ratio* (95% CI)	P-value[£]	Absolute adjusted Difference** (95% CI)	Odds Ratio** (95% CI)	P-value[£££]
Live Birth rate	104/295 (35.3%)	58/140 (41.4%)	46/155 (29.7%)	11.8 (1.0 ; 22.6)[b]	1.68 (1.04 to 2.73)[b]	0.036	11.6 (0.4 ; 22.2)[b]	1.75 (1.04; 2.92)[b]	0.03
Ongoing pregnancy rate	105/295 (35.6%)	58/140 (41.4%)	47/155 (30.3%)	11.1 (0.40 ; 22.0)[b]	1.63 (1.01; 2.65)[b]	0.048	11.0 (-0.07 ; 21.7)[b]	1.68 (1.00; 2.81)[b]	0.043
Clinical pregnancy rate	132/295 (44.7%)	71/140 (50.7%)	61/155 (39.4%)	11.4 (-0.05 ; 22.5)[b]	1.59 (1.00; 2.52)[b]	0.052	11.6 (-0.08 ; 22.7)[b]	1.66 (0.99; 2.70)[b]	0.041
Early miscarriage rate	27/133 (20.3%)	13/71 (18.3%)	14/62 (22.6%)	-4.27 (-18.3 ; 9.43)[b]	0.77 (0.31; 1.87)[b]	0.543	-2.34 (-17.6 ; 11.9)[b]	0.80 (0.31; 2.04)[b]	0.608

Values are no. (%) or no./total no. (%). CI denotes confidence interval.
*Unadjusted model.
**Adjusted model for Age class, Embryos quality, embryo transfer and Uterine immune profile.
[£]Unadjusted pvalue from Type 3 analysis.
[£££]Adjusted pvalue from Type 3 analysis.
[b]Bias-Corrected and Accelerated bootstrap (BCa) confidence interval from 10,000 replications.

below that within this subgroup, 8% of patients achieved spontaneous pregnancy after the biopsy, indicating an additional factor contributing to successful outcomes.

3.5 Spontaneous pregnancies occurring before the embryo transfer and related to the endometrial immune profile

As the endometrial biopsy performed to collect the endometrium for the endometrial immune profiling is an equivalent of the scratching recommended in an under-activated immune profile to trigger the maturation of uNK by stimulating IL-15 local expression (25), 8% (9 patients) with under-immune activation had a natural pregnancy after the biopsy. 8% (5/64) were randomised in the conventional group and 8% (4/45) were randomised in the precision group among which 87.5% successfully delivered and one had a miscarriage 4 spontaneous pregnancies occurred in the precision group after the test under therapy, 2 resulted in miscarriage and 2 in live birth. The two miscarriages occurred in cases showing an absence of normalisation of the profile under therapy.

2 patients, one over-activated and one with a mixed immune profile included in the conventional group, became spontaneously pregnant and had a miscarriage.

4 Discussion

The findings of the present study are novel and of paramount importance in terms of potential implications for routine ART practice. Because of their importance, the caveats and limitations of our study are detailed separately below. In the past, precision interventions targeting the endometrial immune dysregulation were usually dedicated to patients with a history of repeated implantation failure (RIF) and Recurrent Miscarriages (RM) (28). The present study has revealed that an unbalanced endometrial environment may be a significant contributing factor in certain instances of IVF failure among women experiencing infertility, even in the absence of a history of RIF. It is noteworthy that even in this relatively favourable prognosis group undergoing IVF, the proportion and pattern of endometrial immune dysregulation closely resembled those observed in patients with RIF, with 78% showing such dysregulations. This considerable percentage indicates that the endometrial immune profile may not necessarily indicate a pathological condition in the uterus, but may alternatively suggest a less receptive immune environment for implantation. A “less receptive environment” refers to inadequate uterine immune preparation that may impede successful embryo attachment and implantation if the embryo cannot independently correct the local imbalance. To our knowledge, documenting uterine immune equilibrium remains the only method to detect such imbalances, as no tools currently exist to assess immune expression on the embryo’s side. The fact that 30% of patients, despite having an endometrial immune dysregulation, successfully delivered after ET with conventional

10.3389/fimmu.2025.1523871

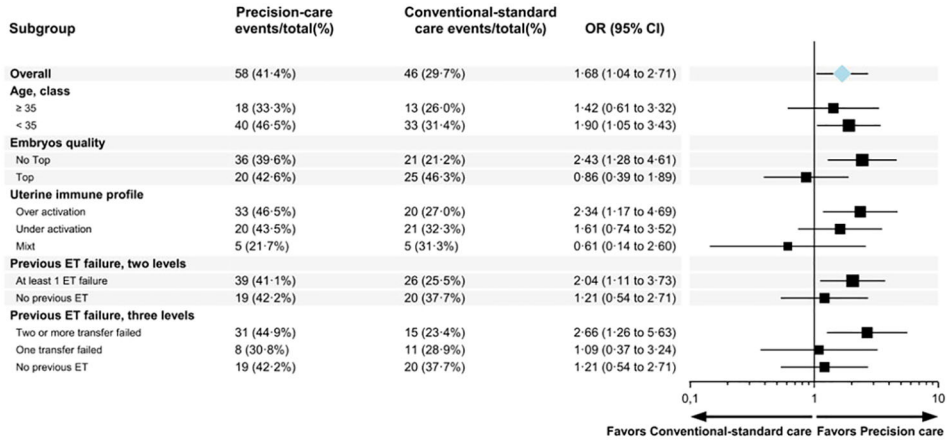


FIGURE 3
Forest plots describing the odds ratio of live birth comparing precision vs conventional-standard cares.

care provides additional support for this hypothesis. The impact of endometrial immune dysregulation appeared to be negligible when the embryo exhibits optimal morphological quality probably because of its ability to trigger adhesion or control the activation of immune cells if necessary. However, for most patients who have experienced two or more previous implantation failures or have embryos with suboptimal morphology (as noted in 65% of the reported cases), precision treatment may become essential to improve success rates. There is compelling evidence that the endometrium acts as a biosensor, selecting embryos based on their quality for implantation (51). While the embryo itself has the capacity to correct any identified dysregulations, the endometrium may play a pivotal role in rescuing some embryos and enabling their successful implantation. For decades, there has been an ongoing debate about establishing a threshold for the number of previously transferred embryos to define cases of RIF (50, 52). This threshold was the starting point to trigger further research, in particular investigations on the endometrium. This study suggests that the integration of endometrial immune profiling should be taken into consideration at an earlier stage in the patient's treatment journey. Defining RIF without taking into account the endometrial immune environment and its immune dysregulation as a parameter overlooks a crucial piece of the puzzle.

This study underscores the importance of accurately identifying the specific type of dysregulation through endometrial immune profiling, which in turn determines the most appropriate treatment option. Endometrial immune profiling can provide a basis to identify the most appropriate additional therapies to address local immune dysregulation. The absence of an established immune diagnosis makes the evaluation of specific procedures, such as the random use of steroids or endometrial scratching (53–55) almost impossible. As shown with scratching, a procedure based on immune diagnosis can favour pregnancy, even spontaneous pregnancy (56). In this RCT, 6.8% of infertile patients scheduled for IVF with under-activation became spontaneously pregnant after endometrial biopsy (equivalent to scratching), while only 2.8% of the non-deregulated group became spontaneously pregnant. In contrast, in patients with overactivation, spontaneous pregnancies occurred only after an effective test under treatment, while the other half resulted in miscarriage, suggesting a probable negative effect of the biopsy.

Another effective aspect of our study is the establishment of an endometrial diagnosis coupled with treatment testing to document drug resistance or sensitivity. In most cases, simple, well-known interventions are sufficient to re-establish the local immune balance. However, we also report some cases of corticosteroid resistance, where perfusion of intralipids may have promising outcomes. Clearly, a one-size-fits-all approach is inadequate in the context of infertility, underlining the necessity for precision medicine. It is imperative to use an integrated model considering both the embryo and the endometrium to enhance overall the overall outcomes. The observation that an endometrial immune dysregulation has no impact on pregnancy rates when embryos with optimal morphological features are transferred indicates that a competent embryo is capable of effectively regulating endometrial dysregulation independently. These findings are consistent with previous research indicating that a competent embryo releases pro-adhesive molecules to initiate adhesion (57) and produces immunosuppressive agents to prevent rejection ((58, 59). Nevertheless, in numerous instances of infertility, the embryos produced may not be fully competent from an immune standpoint. Consequently, addressing diagnosed endometrial dysregulation could exert beneficial effects. As observed by Leese et al., an optimal range of metabolic activity on the embryonic side – referred to as the 'Goldilocks zone' – is crucial for maximizing embryonic developmental potential (60, 61). Similarly, it is plausible that a corresponding 'immune Goldilocks zone' of regulation may exist on the maternal side as well. Pregnancy relies on a delicate early immune dialogue between the embryo and the endometrium, a process that remains not fully understood (62). The endometrial immune environment, often overlooked in routine clinical practice, is emerging as a crucial factor in improving ART outcomes. Documenting the endometrial immune environment before IVF is straightforward but requires advance planning. It involves collecting a mid-luteal phase endometrial sample via aspiration and analysing it using a patented semi-automated method for

precise biomarker quantification. This approach, which complements efforts to enhance embryo quality, could benefit most of patients. Further investigations are needed to evaluate its relevance for routine use, even before IVF. Rebalancing the local immune environment might also facilitate natural conception. Overall, 5% of patients could potentially avoid costly ART through simple, well-known treatment options.

5 Limitations

The data presented in this study should be interpreted with caution due to inherent structural limitations that are almost impossible to avoid in human IVF trials. This study was an open-label study with a modified trial design because we had to revise our approach. During the eight years of recruitment, there were several changes in IVF practice, such as a shift from cleaved embryo transfer to blastocyst transfer and the introduction of the 'freeze-all' strategy. As a result, the eligibility criteria were expanded to maintain adequate statistical power and to adapt the randomised clinical trial to these changing practices. The single-centre design has its advantages and disadvantages: it minimises variability in practice over time, but also limits the generalisability of the results to other settings. Since this RCT mixed Day-3, Day-5, single or doble embryo transfer, the clear influence of the endometrial environment on the embryo itself also need further investigation. For that reason, we decided to launch a pair matched trial selecting the population who exclusively received a single Day-5 embryo transfer (SET) and benefitted of a uterine immune profiling. This population will be matched to a population. who did not have uterine immune profiling prior to the single Day 5 embryo transfer (NCT06503952). The strength and applicability of our findings depend on replication by independent research teams with an independent validation of these biomarkers, possibly using techniques such as preimplantation genetic testing for aneuploidy (PGT-A) to ensure optimal embryo quality. In addition, replication of these results in patients with RIF and older populations requires careful consideration of potential confounding factors, such as the ploidy of transferred embryos. Conducting randomised controlled trials in the context of IVF failure is essential but challenging, as patients with repeated and unexplained previous failures are often reluctant to participate in randomisation. One possible approach could be to focus on patients who have failed only two embryo transfers. In terms of uterine immune profiling itself, the lack of immune profiling for other key immune cells such as macrophages and dendritic cells and T regulatory cells is a limitation, as these cells are also known to be critical in the implantation process. Moreover, quantifying the individual impact of each of these factors is hard, and assessing their combined effect is even more complex.

6 Conclusion

In conclusion, this extended open randomised controlled trial showed that 78% of a standard infertile population undergoing IVF had an immune imbalance in the endometrium at the predicted time of implantation, as identified by endometrial immune profiling. Rebalancing the immune environment with precision therapies led to a significant increase in live birth rates, particularly in patients with previous implantation failures or morphologically sub-optimal embryos. This study provides new evidence for reproductive immunology - a field largely overlooked and underestimated in reproductive medicine - by highlighting the potentially promising role of immune tolerance development in successful pregnancy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCT02262117- Data supporting the findings of this study will be made available upon reasonable request.

Ethics statement

The studies involving humans were approved by Institutional Review Boards at the University Paris Diderot (clinicaltrials.gov NCT02262117). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

NL: Conceptualization, Investigation, Supervision, Validation, Writing – original draft. MP: Conceptualization, Writing – review & editing. GD: Investigation, Writing – review & editing. LC: Formal Analysis, Writing – review & editing. AK: Formal Analysis, Writing – review & editing. MR: Conceptualization, Writing – review & editing. EV: Conceptualization, Methodology, Project administration, Validation, Writing – review & editing. AD: Methodology, Writing – review & editing. NC: Writing – review & editing. LR: Writing – review & editing. LP-E: Writing – review & editing, Conceptualization, Investigation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The project has been selected and funded as part of the Hospital Clinical Research Program (PHRC 13-0039) by the French Ministry of Health.

Acknowledgments

This work is a tribute to the memory of Gerard Chaouat, without whom it would never have been the light of day. We express our sincere gratitude to the patients who generously

consented to participate in this research, making this study possible. We extend our appreciation to Firas Turki, Cynthia Simon, Agathe Rousseau, Tiphaine Isnard, Mathilde Victoria, and Jennifer Uddin, whose dedicated support throughout their assistantships was instrumental in overseeing the patients at every stage of the trial. We acknowledge the patience and contributions of Carine Paré and Kim Mollet for monitoring data and safety during the research. Thanks also to Katia Vezmar for carrying out the first part of the analysis of the sample (RNA extraction and reverse transcription). Finally, our profound thanks are extended to Professor René Frydman, who laid the foundation for this research two decades ago. His ongoing curiosity has been an invaluable contribution to the study's development and completion. I extend my sincere gratitude to Professor Dijalli Hannane, our esteemed dean of UVSQ, for granting me the autonomy to pursue this research.

Conflict of interest

NL and MP created MatriceLAB Innove through a valorisation contract with the University Université Paris Saclay, UVSQ – UFR Simone Veil – Santé and hold a patent covering the endometrial immune assessment test and associated recommendations PCT/EP2013/065355.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Ombelet W. WHO fact sheet on infertility gives hope to millions of infertile couples worldwide. *Facts Views Vis Obgyn*. (2020) 12:249–51.

2. European IVF Monitoring Consortium (EIM), for the European Society of Human Reproduction and Embryology (ESHRE), Wyns C, De Geyter C, Calhaz-Jorge C, Kupka MS, Motrenko T, Smeenk J, et al. ART in Europe 2018: results generated from European registries by ESHRE. *Hum Reprod Open*. (2022) 2022: hoac022. doi: 10.1093/hropen/hoac022

3. Cui W. Mother or nothing: the agony of infertility. *Bull World Health Organ*. (2010) 88:881–2. doi: 10.2471/BLT.10.011210

4. Aghaeepour N, Ganio EA, McIlwain D, Tsai AS, Tingle M, Van Gassen S, et al. An immune clock of human pregnancy. *Sci Immunol*. (2017) 2(15):eaan2946. doi: 10.1126/sciimmunol.aan2946

5. Deglincerti A, Croft GF, Pietila LN, Zernicka-Goetz M, Siggia ED, Brivanlou AH. Self-organization of the *in vitro* attached human embryo. *Nature*. (2016) 533:251–4. doi: 10.1038/nature17948

6. Kanter JR, Mani S, Gordon SM, Mainigi M. Uterine natural killer cell biology and role in early pregnancy establishment and outcomes. *F S Rev*. (2021) 2:265–86. doi: 10.1016/j.xfnr.2021.06.002

7. Saito S. Role of immune cells in the establishment of implantation and maintenance of pregnancy and immunomodulatory therapies for patients with repeated implantation failure and recurrent pregnancy loss. *Reprod Med Biol*. (2024) 23:e12600. doi: 10.1002/rmb2.12600

8. Genest G, Banjar S, Almasri W, Beauchamp C, Benoit J, Buckett W, et al. Immunomodulation for unexplained recurrent implantation failure: where are we now? *Reproduction*. (2023) 165(2):R39–60. doi: 10.1530/REP-22-0150

9. Robertson SA, Moldenhauer LM, Green ES, Care AS, Hull ML. Immune determinants of endometrial receptivity: a biological perspective. *Fertil Steril*. (2022) 117(6):1107–20. doi: 10.1016/j.fertnstert.2022.04.023

10. Cheloufi M, Kazhalawi A, Pinton A, Rahmati M, Chevrier L, Prat-Ellenberg L, et al. The endometrial immune profiling may positively affect the management of recurrent pregnancy loss. *Front Immunol*. (2021) 12:656701. doi: 10.3389/fimmu.2021.656701

11. Ledee N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The uterine immune profile may help women with repeated unexplained embryo implantation failure after *in vitro* fertilization. *Am J Reprod Immunol*. (2016) 75:388–401. doi: 10.1111/aji.2016.75.issue-3

12. Ledee N, Petitbarat M, Prat-Ellenberg L, Dray G, Cassuto GN, Chevrier L, et al. Endometrial immune profiling: A method to design personalized care in assisted reproductive medicine. *Front Immunol*. (2020) 11:1032. doi: 10.3389/fimmu.2020.01032

13. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med*. (2007) 25:445–53. doi: 10.1055/s-2007-991042

14. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. *Clin Exp Reprod Med*. (2011) 38:119–25. doi: 10.5653/cerm.2011.38.3.119

15. Zhang J, Dunk C, Croy AB, Lye SJ. To serve and to protect: the role of decidual innate immune cells on human pregnancy. *Cell Tissue Res*. (2016) 363:249–65. doi: 10.1007/s00441-015-2315-4

16. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. *J Reprod Immunol*. (2017) 124:44–53. doi: 10.1016/j.jri.2017.10.045

17. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today*. (1993) 14:353–6. doi: 10.1016/0167-5699(93)90235-D

18. Chaouat G. The Th1/Th2 paradigm: still important in pregnancy? *Semin Immunopathol*. (2007) 29:95–113. doi: 10.1007/s10815-007-9142-9

19. Manaster I, Mandelboim O. The unique properties of uterine NK cells. *Am J Reprod Immunol*. (2010) 63:434–44. doi: 10.1111/j.1600-0897.2009.00794.x

20. Lob S, Ochmann B, Ma Z, Vilsmaier T, Kuhn C, Schmoeckel E, et al. The role of Interleukin-18 in recurrent early pregnancy loss. *J Reprod Immunol*. (2021) 148:103432. doi: 10.1016/j.jri.2021.103432

21. Tokmadzic VS, Tsuji Y, Bogovic T, Laskarin G, Cupurdija K, Strbo N, et al. IL-18 is present at the maternal-fetal interface and enhances cytotoxic activity of decidual lymphocytes. *Am J Reprod Immunol*. (2002) 48:191–200. doi: 10.1034/j.1600-0897.2002.01132.x

22. Yoshino O, Osuga Y, Koga K, Tsutsumi O, Yano T, Fujii T, et al. Evidence for the expression of interleukin (IL)-18, IL-18 receptor and IL-18 binding protein in the human endometrium. *Mol Hum Reprod*. (2001) 7:649–54. doi: 10.1093/molehr/7.7.649

23. Ashkar AA, Black GP, Wei Q, He H, Liang L, Head JR, et al. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. *J Immunol*. (2003) 171:2937–44. doi: 10.4049/jimmunol.171.6.2937

24. Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. *J Immunol*. (2008) 181:1869–76. doi: 10.4049/jimmunol.181.3.1869

25. Verma S, Hiby SE, Loke YW, King A. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. *Biol Reprod*. (2000) 62:959–68. doi: 10.1095/biolreprod62.4.959

26. Lei MZ, Qin LJ, Zhao DD, Wang AH, Zhao XJ, Jin YP, et al. Tumor necrosis factor-like weak inducer of apoptosis regulates the phenotype and cytotoxic activity of goat uterine natural killer cells. *J Anim Sci*. (2015) 93:589–97. doi: 10.2527/jas.2014-7942

27. Qi X, Lei M, Qin L, Xie M, Zhao D, Wang J. Endogenous TWEAK is critical for regulating the function of mouse uterine natural killer cells in an immunological model of pregnancy loss. *Immunology*. (2016) 148:70–82. doi: 10.1111/imm.2016.148.issue-1

28. Ledee N, Petitbarat M, Prat-Ellenberg L, Dray G, Vaucoret V, Kazhalawi A, et al. The next frontier in ART: harnessing the uterine immune profile for improved performance. *Int J Mol Sci*. (2023) 24(14):11322. doi: 10.3390/ijms241411322

29. Acosta AA, Elberger L, Borghi M, Calamera JC, Chemes H, Doncel GF, et al. Endometrial dating and determination of the window of implantation in healthy fertile women. *Fertil Steril*. (2000) 73:788–98. doi: 10.1016/S0015-0282(99)00605-6

30. Petitbarat M, Rahmati M, Serazin V, Dubanchet S, Morvan C, Wainer R, et al. TWEAK appears as a modulator of endometrial IL-18 related cytotoxic activity of uterine natural killers. *PloS One*. (2011) 6:e14497. doi: 10.1371/journal.pone.0014497

31. Almog B, Shalom-Paz E, Dufort D, Tulandi T. Promoting implantation by local injury to the endometrium. *Fertil Steril*. (2010) 94:2026–9. doi: 10.1016/j.fertnstert.2009.12.075

32. Barash A, Dekel N, Fieldust S, Segal I, Schechtman E, Granot I. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing *in vitro* fertilization. *Fertil Steril*. (2003) 79:1317–22. doi: 10.1016/S0015-0282(03)00345-5

33. Gnainsky Y, Granot I, Aldo PB, Barash A, Or Y, Schechtman E, et al. Local injury of the endometrium induces an inflammatory response that promotes successful implantation. *Fertil Steril*. (2010) 94:2030–6. doi: 10.1016/j.fertnstert.2010.02.022

34. Narvekar SA, Gupta N, Shetty N, Kottur A, Srinivas M, Rao KA. Does local endometrial injury in the nontransfer cycle improve the IVF-ET outcome in the subsequent cycle in patients with previous unsuccessful IVF? A randomized controlled pilot study. *J Hum Reprod Sci*. (2010) 3:15–9. doi: 10.4103/0974-1208.63116

35. Berndt S, Blacher S, Perrier d'Hauterive S, Thiry M, Tsampalas M, Cruz A, et al. Chorionic gonadotropin stimulation of angiogenesis and pericyte recruitment. *J Clin Endocrinol Metab*. (2009) 94:4567–74. doi: 10.1210/jc.2009-0443

36. Kane N, Kelly R, Saunders PT, Critchley HO. Proliferation of uterine natural killer cells is induced by human chorionic gonadotropin and mediated via the mannose receptor. *Endocrinology*. (2009) 150:2882–8. doi: 10.1210/en.2008-1309

37. Robertson SA, Sharkey DJ. Seminal fluid and fertility in women. *Fertil Steril*. (2016) 106:511–9. doi: 10.1016/j.fertnstert.2016.07.1101

38. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *J Immunol*. (2012) 188:2445–54. doi: 10.4049/jimmunol.1102736

39. Zhang J, Wang C, Zhang H, Zhou Y. Sequential cleavage and blastocyst embryo transfer and IVF outcomes: a systematic review. *Reprod Biol Endocrinol*. (2021) 19:142. doi: 10.1186/s12958-021-00824-y

40. Eddy JL, Krukowski K, Janusek L, Mathews HL. Glucocorticoids regulate natural killer cell function epigenetically. *Cell Immunol*. (2014) J290(1):120–30. doi: 10.1016/j.cellimm.2014.05.013

41. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. *Ann N Y Acad Sci*. (2004) 1024:138–46. doi: 10.1196/annals.1321.010

42. Ledee N, Prat-Ellenberg L, Petitbarat M, Chevrier L, Simon C, Irani EE, et al. Impact of prednisone in patients with repeated embryo implantation failures: Beneficial or deleterious? *J Reprod Immunol*. (2018) 127:11–5. doi: 10.1016/j.jri.2018.03.003

43. Ledee N, Vasseur C, Petitbarat M, Chevrier L, Vezmar K, Dray G, et al. Intralipid (R) may represent a new hope for patients with reproductive failures and simultaneously an over-immune endometrial activation. *J Reprod Immunol*. (2018) 130:18–22. doi: 10.1016/j.jri.2018.09.050

44. Raghupathy R, Szekeres-Bartho J. Progesterone: A unique hormone with immunomodulatory roles in pregnancy. *Int J Mol Sci*. (2022) 23(3):1333. doi: 10.3390/ijms23031333

45. Szekeres-Bartho J. Progesterone-mediated immunomodulation in pregnancy: its relevance to leukocyte immunotherapy of recurrent miscarriage. *Immunotherapy*. (2009) 1:873–82. doi: 10.2217/imt.09.54

46. Tsampalas M, Gridelet V, Berndt S, Foidart JM, Geenen V, Perrier d'Hauterive S. Human chorionic gonadotropin: a hormone with immunological and angiogenic properties. *J Reprod Immunol*. (2010) 85:93–8. doi: 10.1016/j.jri.2009.11.008

47. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril*. (2000) 73:1155–8. doi: 10.1016/S0015-0282(00)00518-5

48. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, et al. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod*. (1999) 14:2345–9. doi: 10.1093/humrep/14.9.2345

49. Boyer P, Boyer M. Non invasive evaluation of the embryo: morphology of preimplantation embryos. *Gynecol Obstet Fertil*. (2009) 37:908–16. doi: 10.1016/j.gyobfe.2009.09.018

50. ESHRE Working Group on Recurrent Implantation Failure, Cimadomo D, de Los Santos MJ, Griesinger G, Lainas G, Le Clef N, et al. ESHRE good practice recommendations on recurrent implantation failure. *Hum Reprod Open*. (2023) 2023:hoad023. doi: 10.1093/hropen/hoad023

51. Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, et al. Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PloS One*. (2010) 5:e10258. doi: 10.1371/journal.pone.0010258

52. Macklon NS. The true incidence of recurrent implantation failure. *Curr Opin Obstet Gynecol*. (2022) 34:147–50. doi: 10.1097/GCO.0000000000000781

53. Achilli C, Duran-Retamal M, Saab W, Serhal P, Seshadri S. The role of immunotherapy in *in vitro* fertilization and recurrent pregnancy loss: a systematic review and meta-analysis. *Fertil Steril*. (2018) 110:1089–100. doi: 10.1016/j.fertnstert.2018.07.004

54. Boomsma CM, Keay SD, Macklon NS. Peri-implantation glucocorticoid administration for assisted reproductive technology cycles. *Cochrane Database Syst Rev*. (2012) 6:CD005996. doi: 10.1002/14651858.CD005996.pub3

55. Sun Y, Cui L, Lu Y, Tan J, Dong X, Ni T, et al. Prednisone vs placebo and live birth in patients with recurrent implantation failure undergoing *in vitro* fertilization: A randomized clinical trial. *JAMA*. (2023) 329:1460–8. doi: 10.1001/jama.2023.5302

56. Rahmati M, Ledee N. Targeted endometrial scratching: an example of endometrial diagnosis usage in reproductive medicine. *Front Immunol*. (2020) 11:589677. doi: 10.3389/fimmu.2020.589677

57. Francis LW, Yao SN, Powell LC, Griffiths S, Berquand A, Piasecki T, et al. Highly glycosylated MUC1 mediates high affinity L-selectin binding at the human endometrial surface. *J Nanobiotechnology*. (2021) 19:50. doi: 10.1186/s12951-021-00793-9

58. Jarkovska K, Martinkova J, Liskova L, Halada P, Moos J, Rezabek K, et al. Proteome mining of human follicular fluid reveals a crucial role of complement cascade and key biological pathways in women undergoing *in vitro* fertilization. *J Proteome Res*. (2010) 9:1289–301. doi: 10.1021/pr900802u

59. Radwan P, Tarnowska A, Piekarska K, Wisniewski A, Krasinski R, Radwan M, et al. The impact of soluble HLA-G in IVF/ICSI embryo culture medium on implantation success. *Front Immunol*. (2022) 13:982518. doi: 10.3389/fimmu.2022.982518

60. Leese HJ, Brison DR, Sturmey RG. The Quiet Embryo Hypothesis: 20 years on. *Front Physiol*. (2022) 13:899485. doi: 10.3389/fphys.2022.899485

61. Leese HJ, Guerif F, Allgar V, Brison DR, Lundin K, Sturmey RG. Biological optimization, the Goldilocks principle, and how much is lagom in the preimplantation embryo. *Mol Reprod Dev*. (2016) 83:748–54. doi: 10.1002/mrd.22684

62. Favaro RR, Murrieta-Coxca JM, Gutierrez-Samudio RN, Morales-Prieto DM, Markert UR. Immunomodulatory properties of extracellular vesicles in the dialogue between placental and immune cells. *Am J Reprod Immunol*. (2021) 85:e13383. doi: 10.1111/aji.13383

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 28 April 2025
DOI 10.3389/fimmu.2025.1516473

Check for updates

OPEN ACCESS

EDITED BY
Maria Laura Zenclussen,
National Scientific and Technical Research Council (CONICET), Argentina

REVIEWED BY
Ann-Kristin Östlund Farrants,
Stockholm University, Sweden
Marta Tesone,
University of Buenos Aires, Argentina

*CORRESPONDENCE
Guizhi Ma
sandy0539@126.com
Xi Wu
wuxi533@163.com

†These authors have contributed equally to this work and share first authorship

RECEIVED 24 October 2024
ACCEPTED 07 April 2025
PUBLISHED 28 April 2025

CITATION
Liu L, Ge D, Lin Y, Han Z, Zhao H, Cao L, Wu X and Ma G (2025) Epigenetic regulation in oogenesis and fetal development: insights into m6A modifications. *Front. Immunol.* 16:1516473. doi: 10.3389/fimmu.2025.1516473

COPYRIGHT
© 2025 Liu, Ge, Lin, Han, Zhao, Cao, Wu and Ma. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Epigenetic regulation in oogenesis and fetal development: insights into m6A modifications

Lusheng Liu[1,2†], Danxia Ge[3†], Yumeng Lin[4†], Zhongyu Han[1†], Heng Zhao[5], Liqin Cao[6], Xi Wu[6*] and Guizhi Ma[1*]

[1]Department of Acupuncture and Moxibustion, Shanghai Traditional Chinese Medicine (TCM)-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [2]Shanghai Clinical Medical College of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [3]Department of Critical Care Medicine, Traditional Chinese Medicine Hospital of, Ningbo, Zhejiang, China, [4]Nanjing Tongren Hospital, School of Medicine, Southeast University, Nanjing, China, [5]Shanghai TCM-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [6]Department of Gynecology, Shanghai TCM-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

The unique physiological structure of women has led to a variety of diseases that have attracted the attention of many people in recent years. Disturbances in the reproductive system microenvironment lead to the progression of various female tumours and pregnancy disorders. Numerous studies have shown that epigenetic modifications crucially influence both oogenesis and foetal development. m6A, a modification at the mRNA level, consists of three parts, namely, writers, erasers, and readers, which are involved in several biological functions, such as the nucleation and stabilisation of mRNAs, thereby regulating the development of reproductive system diseases. In this manuscript, we delineate the constituents of m6A, their biological roles, and advancements in understanding m6A within the maternal–foetal immunological context. In addition, we summarise the mechanism of m6A in gynaecological diseases and provide a new perspective for targeting m6A to delay the progression of reproductive system diseases in clinical practice.

KEYWORDS

RNA modification, epigenetic modification, female reproductive diseases, reproductive system neoplasms, treatment

1 Introduction

In recent years, the incidence of female reproductive system diseases has steadily increased, and these diseases have become a common problem worldwide, seriously affecting women's quality of life (1). Common gynaecological diseases include benign diseases, such as endometriosis, premature ovarian failure, and adenomyosis; diseases during pregnancy, such as preeclampsia, recurrent miscarriage, and gestational diabetes;

and malignant tumours, such as cervical cancer (CC), ovarian cancer (OC), and endometrial cancer (EC). Therefore, there is an urgent need to understand the pathogenesis of female reproductive system diseases and find specific biomarkers, so as to improve the quality of life of women.

The N6-methyladenosine (m6A) modification, a form of internal chemical alteration within RNA, was first identified in the 1970s. This process involves the methylation of the nitrogen atom at the 6th position of the adenine (A) base within RNA, catalysed by methyltransferases. This modification is prevalent in both messenger RNA (mRNA) and non-coding RNA (ncRNAs) (2). Through writers, erasers, and readers, m6A is implicated in diverse mechanisms and metabolic processes, including pre-mRNA splicing, export, and translation; the regulation of translation efficiency; mRNA stability; the prevention or delay of mRNA degradation; and the processing of noncoding RNAs (3–5). M6A is associated with female reproductive system function, and some abnormal m6A modifications have also been observed to be involved in the generation, metastasis, and drug resistance of cancer cells (6). Further studies revealed that m6A methylation affects mainly carcinogenesis and malignant tumour progression by regulating gene expression in cancer (7, 8). Thus, the potential association of aberrant m6A with cancer was documented.

This article reviews the latest research progress on the biological role of m6A in gynaecological tumours and pregnancy-related diseases and discusses the mechanism of m6A regulatory proteins in the proliferation, invasion and metastasis of gynaecological tumours. This article also discusses the research progress and future directions of m6A modification in the maternal–foetal immune environment and the diagnosis and prognostic evaluation of gynaecological diseases, with the aim of revealing the potential role of RNA methylation in female reproductive system diseases to gain a deeper understanding of these diseases.

2 Mechanisms and regulation of m6A RNA methylation

2.1 Writers

The m6A writer is a methyltransferase with dynamic, reversible properties during the formation process and many proteins function as writers, including methyltransferase-like3(METTL3), methyltransferase-like5 (METTL5), methyltransferase-like 14 (METTL14), methyltransferase-like 16 (METTL16), Wilms tumour type 1 associated protein (WTAP), Vir-like m6A methyltransferase-associated protein (VIRMA), RNA-binding motif 15/15B (RBM15/15B, zinc finger CCCH-containing type 13 (ZC3H13, KIAA0853), and methyltransferase-like 16 (METTL16) (9–11) (Figure 1).

METTL3 was the first methyltransferase shown to be involved in m6A methylation in 1997, and its subunits play critical roles in the catalytic process to facilitate the transfer of adenine from S-adenosylmethionine (SAM) moieties to RNA (12). Until 2017, there were relevant studies on the role of METTL3 as an m6A methyltransferase in human cancer. It has been shown that METTL3 acts as an oncogene in AML and its activity depends on interaction with the CAAT-box binding protein CEBPZ, which promotes METTL3 localisation to chromatin and induces m6A modification on specific mRNA transcripts such as SP1 and SP2 (13).

The oncogene role of METTL3 in AML has been well-established and its overexpression and oncogenic activity have subsequently been revealed in a variety of cancer types, including hepatocellular carcinoma, gastric cancer, and non-small cell lung cancer (14–17). In gastric cancer, elevated expression levels of METTL3 were demonstrated to be an independent poor prognostic indicator (18). In bladder cancer, METTL3 overexpression has been shown to be associated with unfavourable prognostic outcomes, and this overexpression promotes methylation of specific mRNA molecules (19). In the female reproductive system, overexpression of METTL3 is thought to have a role in promoting oocyte maturation. In the genital vesicle stage of oocytes, the loss of function of Mettl3 severely inhibits meiotic maturation of oocytes, and its inactivation may lead to abnormal oocyte development, affect the stability of ovulation and meiosis, and then cause reproductive system diseases such as infertility (20).

METTL14 and METTL3 are molecular homologues that form stable heterodimeric complexes, and METTL14 can promote RNA recognition and binding by stabilising the conformation of METTL3, providing structural support, and enhancing its catalytic activity (21–25). METTL14 was initially found to be overexpressed in acute myeloid leukaemia and plays a key oncogenic role in the initiation and development of the disease (26). METTL14 has also been shown to be downregulated and exhibit tumour suppressive effects in some cancers. In patients with endometrial cancer, METTL14 levels are decreased in tumour samples, promoting the proliferation of endometrial cancer cells (27).

It has been shown that in hepatocellular carcinoma, down-regulation of METTL14 and m6A expression is significantly associated with the high metastatic ability of tumours and the prognosis of patients (28). In breast cancer, the expression levels of METTL14 and ALKBH5 regulate each other and together promote tumour development by regulating m6A modification levels in specific transcripts associated with epithelial-mesenchymal transition (EMT) and angiogenesis (29).

WTAP is a ubiquitously expressed nuclear protein whose transcription is regulated by WT-1 and interacts with the METTL3/METTL14 complex to affect m6A RNA methylation, thereby regulating gene transcription and RNA splicing. WTAP regulates transcription and translation of niche factors by placing m6A markers directly or indirectly on transcripts encoding niche factors (30). In acute myeloid leukaemia, WTAP has been shown to be up-regulated at its expression level and plays an important role in the proliferation and differentiation of tumours (31). In a zebrafish embryo model, a decrease in WTAP or METTL3 expression levels results in abnormal tissue differentiation and increased rates of apoptosis (9). WTAP has been identified as a prototypical biomarker of OC development and spread (32, 33). Liu et al. found that WTAP expression was higher in EC tissues than in surrounding normal tissues (34).

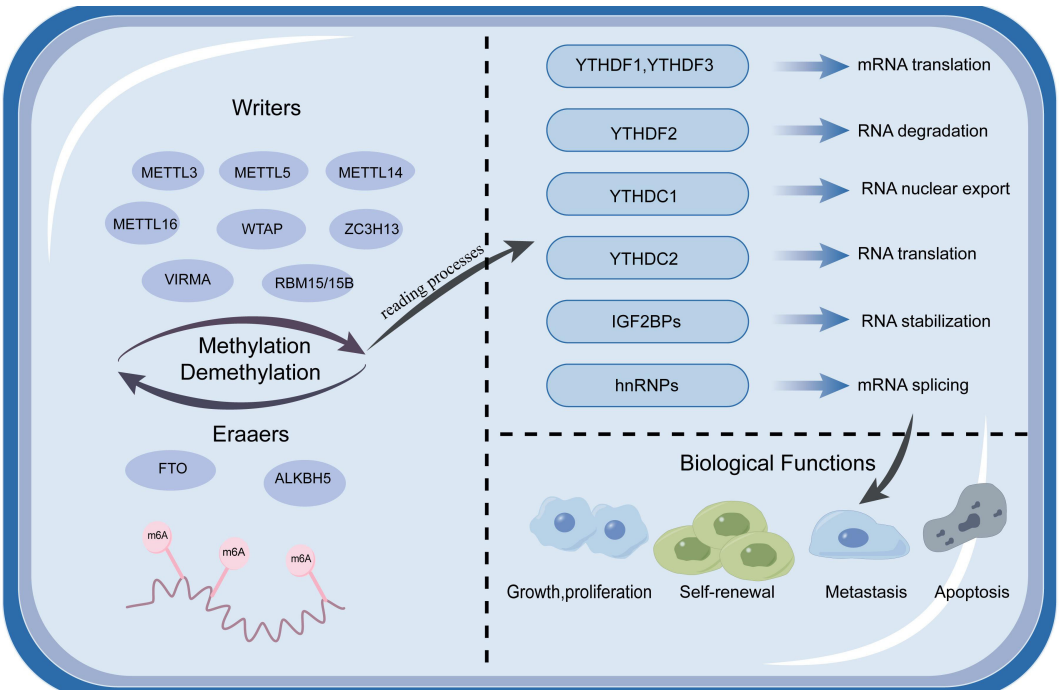


FIGURE 1
Shows the regulatory mechanism of m6A modification, which consists of three major parts and elaborates its working principle. N6-methyladenosine (m6A) is methylated by a methyltransferase complex containing METTL3, METTL14, WTAP, and KIAA1429, and these proteins called "writers" are responsible for adding the m6A modification; while "erasers" FTO and ALKBH5 are responsible for reversibly removing m6A. The reader proteins YTHDC1/2, YTHDF1/2/3, and IGF2BP1/2/3 are responsible for performing the biological functions of m6A, including translation, decay, splicing, and post-transcriptional regulation of RNA.

As an RNA-binding protein, RBM15 and RBM15B promote the recognition and assembly of mRNA molecules containing specific sequences by METTL3 and WTAP through their RNA-binding domains, which is critical for the precise regulation of m6A methylation. HAKAI protein plays a bridge role in this process, which mediates the binding of mRNA to methylation complexes and participates in the regulation of mRNA degradation. In particular, HAKAI ensures the stability of the sequence characteristics and functional status of mRNAs after m6A methylation, which has a crucial role for the accurate transmission of genetic information and the function of mRNAs in subsequent cellular processes, such as protein translation.

KIAA1429 can bind to WTAP, mediate the formation of the METTL3/METTL14/WTAP complex, preferentially bind to the 3'UTR and participate in alternative polyadenylation (APA) while preventing target RNA coding through selective methylation (10, 35).

METTL16 is a catalytically active m6A methyltransferase that regulates the homeostasis of the SAM content via the splicing of the U6 snRNP (36). METTL16 binds directly to MAT2A mRNA precursors, which have a conserved hairpin structure in the 3 'untranslated region (UTR) and affect alternative splicing of MAT2A and homeostasis of SAM content in cells. In addition, in mice, METTL16 also regulates SAM synthase expression, which in turn impacts early embryonic development (37, 38). Previous studies have shown that METTL16 expression levels are upregulated in multiple cancer types and are associated with poor prognosis, including gastric cancer, hepatocellular carcinoma, pancreatic cancer, and acute myeloid leukaemia (39–43).

2.2 Erasers

The m6A eraser is a demethylase that removes m6A methylation from the targeted RNAs, unlike other m6A proteins, and only the AlkB homologue 5 (ALKBH5) protein and fat mass and obesity-associated (FTO) protein have been identified as erasers (44–46).

FTO, as the first m6A demethylase identified, was examined and assigned to the AlkB family. It has the ability to remove m6A as well as n6, 2′ -O-dimethyladenosine (5′cap m6A$_m$) from the mRNA interior, thereby affecting m6A modification (47, 48). In addition, FTO localises predominantly in the nucleus and regulates about 10% of m6A modifications in all cell lines (49, 50). Recently, the discovery of the demethylase FTO revealed its reversible control of the kinetics of the m6A methylation process under physiological and pathological conditions (47, 48).

In CC, the interaction of FTO with E2F1 and MYC significantly decreased their translation efficiency. When E2F1 or MYC expression levels are elevated, they are able to complement the loss-of-function of FTO and have negative effects on cell proliferation and migration, suggesting that these two genes may play a mutually regulated role in CC cells (51). Wang and colleagues have suggested that FTO influences the destiny of lncRNA, thereby

advancing the progression of CC, and have shown that decreasing m6A levels stabilises HOXC13-AS in CC cellular contexts (52).

ALKBH5 is another m6A demethylase, and although FTO belongs to the same ALKB family, the two "erasers" function differently because of their differential expression in different tissues (53, 54). ALKBH5 can not only reverse m6A methylation but also affect mRNA export and RNA metabolism. ALKBH5 is classified as a nonheme oxygenase that is typically found in the nucleus and is generally expressed at low levels in the cytoplasm.

ALKBH5 expression is significantly higher in OC tissues than in normal ovary, yet it is comparatively lower in OC cell lines than in cultured normal ovarian cells (55). Under hypoxia, the expression of ALKBH5 in breast cancer cells was up-regulated by hypoxia-inducible factor, which resulted in a decrease in the m6A modification level of NANOG mRNA, which in turn increased the NANOG protein level and promoted the enrichment of breast cancer stem cells (56). In addition, it has been found that high ALKBH5 expression in glioblastoma stem cells is associated with poor clinical outcome in glioblastoma patients (57).

2.3 Readers

The m6A "reader" proteins are composed of a group of YTH domain-containing proteins, which include YTHDF1, YTHDF2, and YTHDF3, as well as YTHDC1 and YTHDC2. Additionally, this category encompasses IGF2BP, a protein that binds the mRNAs of insulin-like growth factors, and the HNRNP family, which are involved in the processing and regulation of RNA within the cell nucleus (58, 59). With increasing m6A "reader" research, these proteins have been shown to potentially change the fate of modified mRNAs.

For example, after YTHDF1 attaches to the m6A-modified site, it brings in the EIF3A complex, which is a transcription initiation factor, to stimulate the commencement of translation. This process facilitates the aggregation of ribosomes into polyribosomes, thereby enhancing the efficiency of protein synthesis (60). The YTH domain family proteins YTHDF1 and YTHDF3 accelerate the translation of mRNAs by recruiting translation initiation factors, whereas YTHDF2 synergises with YTHDF3 and YTHDF1 to reduce mRNA stability, strengthen the decay and demethylation of their RNAs, and induces the degradation of m6A-modified target mRNAs (61, 62). YTHDF2, as the first m6A reading protein to be discovered, has generally been demonstrated to play an oncogene role in a variety of cancers. Li et al. showed a close correlation between increased YTHDF2 protein levels and OC tissue in the clinic (63). In prostate cancer, YTHDF2 expression was increased in cancer tissues compared with adjacent normal tissues (64).

YTHDC1 binds competitively to SRSF3 and SRSF10 to recognise methylated mRNAs and regulate the alternative splicing of mRNAs (65–67). Furthermore, YTHDC1 plays a pivotal role in the nuclear export of mRNA, facilitating its transfer to the cytoplasmic receptor NXF1, which is crucial for mRNA transport out of the nucleus (68). YTHDC2 binds to m6A, promotes the efficient translation of target genes, mediates RNA decay, and decreases mRNA stability. The m6A reader YTHDC2 is essential for accelerating translation via m6A (69).

HNRNPS family proteins include HNRNPC, HNRNPG, and HNRNPA2B1 (70). HNRNPA2B1 interacts with RNA and regulates the alternative splicing of genes modified by m6A. The secondary structure of m6A-modified mRNA changes, regulating gene expression and enabling it to bind HNRNPC (71). HNRNPA2B1 was identified as an m6A reading protein thanks to its ability to promote alternative splicing of exons with METTL3. Meanwhile, the binding of HNRNPA2B1 to DGCR8 protein provides it with the function to process pre-miRNAs (58).

The IGF2BP family, consisting of IGF2BP1, IGF2BP2, and IGF2BP3, is known to increase translation and engage in the regulation of cellular metabolism. These proteins recognise m6A modification sites on target mRNAs, thereby stabilising these mRNAs and modulating their translational efficiency (72, 73). Increased expression of IGF2BP family proteins has already been observed in previous cancer-related studies (74). It can enhance the stability and translation efficiency of specific target mRNA transcripts in cervical cancer cells by recognising and combining m6A modifications, thereby promoting proliferation, colony formation, migration, and invasion of tumour cells (59).

2.4 m6A in mRNA nucleation

Following a complex series of processing, mRNA molecules that complete maturation in the nucleus are transported into the cytoplasm for protein synthesis. Throughout this process, the m6A modification is crucial for the regulation of mRNA translation, as it generates specific spatial hindrances that influence the translational machinery. The mRNA molecule that has been modified by m6A is identified by the nuclear protein YTHDC1. Through interactions with splicing factors and the nuclear export adapter protein SRSF3, YTHDC1 ensures the efficient handover of the mRNA to the nuclear export receptor NXF1 (66). YTHDC1, in complex with SRSF3, mediates RNA-NXF1 binding to increase nuclear mRNA export (66). In addition, FMRP, a reader of m6A-modified RNA, plays an integral role in exportin 1 (XPO1)-mediated nuclear export (75, 76).

2.5 m6A in mRNA translation

The m6A modification increases translation efficiency through interactions with translation-related proteins. m6A-related translational regulation plays an essential role in a variety of cancers, as well as some normal physiological processes. METTL3 can recognise m6A located in the 5 'UTR and 3' UTR, thereby accelerating translation (77). In addition, METTL3 interacts with eIF3, which further interacts with proteins associated with the cap structure of mRNAs to form mRNA loops.

METTL3 also affects ribosome biogenesis by regulating PES1 expression (78). Rui Su et al. reported that METTL16 can directly interact with eIF3a, eIF3b, and rRNA to promote translation and

accelerate the assembly of translation initiation complexes in the cytoplasm (40). Interactions between YTHDFs and the translation machinery have been shown to increase translation efficiency. However, the precise mechanism by which YTHDF2 functions remains to be fully elucidated (79, 80). YTHDF1 facilitates the translation and elongation of the Snail mRNA by engaging with eEF2 and connects with ribosomes via cap-dependent initiation, interacting with eIF4G and eIF3. The effect of YTHDF1 is not limited to synergy with other translation factors, which can also increase eIF3C expression in an m6A-dependent manner (81).

YTHDF3 augments cap-independent translation during breast cancer brain metastasis by promoting the interaction of eIF3a with the m6A residue located in the 5' UTR of the YTHDF3 mRNA (80). Although the eIF4 complex is required for the standard translation initiation process, YTHDF1 can initiate translation independently of the eIF4 complex by interacting with eIF3A and 3B (82). YTHDF3 works in concert with YTHDF1 to increase translation by interacting with the 40S ribosomal subunit (61). YTHDC2, a protein with RNA helicase activity, can improve the translation efficiency, but at the same time, it also leads to a decrease in mRNA abundance (83, 84).

2.6 m6A and mRNA stability

The m6A protein machinery is crucial for maintaining mRNA stability, regulating RNA metabolism, and sustaining the equilibrium of gene expression. Transcripts containing m6A-induced RNA decay via an m6A reader. The m6A modification exerts context-specific effects on mRNA stability, thereby regulating gene expression and contributing to cellular homeostasis (11, 85). Additional experimental findings indicate that the m6A modification exerts a dual influence on mRNA stability (86–88).

In particular, after YTHDF2 binds to m6A-modified mRNA, its degradation can proceed through at least two different pathways. On the one hand, when the binding domain of the temperature-sensitive protein HRSP12 and the cleavage domain for the endoribonuclease RNase P/MRP are located before and after the YTHDF2 binding domain, HRSP12 acts as an adaptor to link YTHDF2 with RNase P or MRP, thus promoting rapid degradation of RNA bound to YTHDF2 through the endoribonuclease route. On the other hand, YTHDF2 is capable of directly engaging the CCR4/NOT complex, which is associated with exosomes and processing bodies (P-bodies), to induce deadenylation, thereby initiating the breakdown of mRNAs that carry m6A modifications (89, 90). FTO can inhibit YTHDF2-mediated m6A-dependent RNA decay, thereby increasing MYC mRNA stability (91).

The YTHDF1 protein induces degradation by binding to the 3'-UTR m6A modification site of the MAT2A mRNA. YTHDF3 acts synergistically with YTHDF2 to induce the degradation of mRNA (61). Unlike YTHDF2, IGF2BP1/2/3 can increase mRNA stability by binding to RNA stabilisers. In addition, the FMRP and PRRC2A proteins enhance mRNA stabilisation in a manner dependent on the m6A modification by recognising m6A-modified mRNAs (86, 87). The intricacies of the mechanism regulating the m6A protein machinery have not been fully elucidated, necessitating future research to delve deeper into its precise mechanisms and identify additional RNA-binding proteins that may interact with this system.

3 m6A RNA methylation in female reproductive physiology

RNA methylation, especially m6A modification, plays a crucial role in oocyte maturation and early embryonic development, and plays an important regulatory role in gametogenesis and embryonic development. Oocyte maturation refers to the resumption of meiosis in oocytes in dominant follicles a few hours before ovulation and continued development from diplotene in the first meiotic prophase to metaphase in the second meiotic prophase (92) (Figure 2).

During oocyte maturation, it is often accompanied by a series of processes such as RNA storage, translation and degradation to maintain the homeostasis of relative changes in transcript dose in the transcriptome, so the precise regulation of intracellular RNA post-transcriptional levels appears to be particularly important during oocyte maturation, which has a very important role for egg quality, fertilisation process and early embryonic development (93). For example, Qi et al. showed that hypermethylated mRNAs were mainly enriched in progesterone-mediated oocyte maturation and cell cycle pathways, implying that m6A modification is involved in RNA translational regulation during oocyte maturation (94). Ivanova et al. further showed that YTHDF2 precisely regulates m6A modification-mediated mRNA degradation during oocyte maturation (95). In addition, the importance of METTL3 in oocyte maturation was highlighted by the study of Xia et al. by the arrest of oocyte maturation development in METTL3 mutants (96).

Embryonic development refers to the process from zygote development to embryo detachment from the oolemma. In early embryonic development, m6A modification similarly plays a critical role. Wu et al. revealed the important and unique significance of m6A modification during MZT through egg and embryo samples deficient in m6A modification (97). In addition, m6A modification in combination with miRNAs regulates the process of embryonic development, and a study by Hao et al. (98). MiR-670 was shown to play a key role in embryonic development by regulating IGF2BP1 expression through m6A modification. In summary, m6A modification plays an integral role in oocyte maturation and early embryonic development by precisely regulating RNA metabolism and stability, and its dynamic changes are essential for maintaining normal reproductive and developmental processes.

3.1 Germ cell development

Many studies have underscored the pivotal function of the m6A modification in governing cellular activities within the female reproductive system, including proliferation, differentiation,

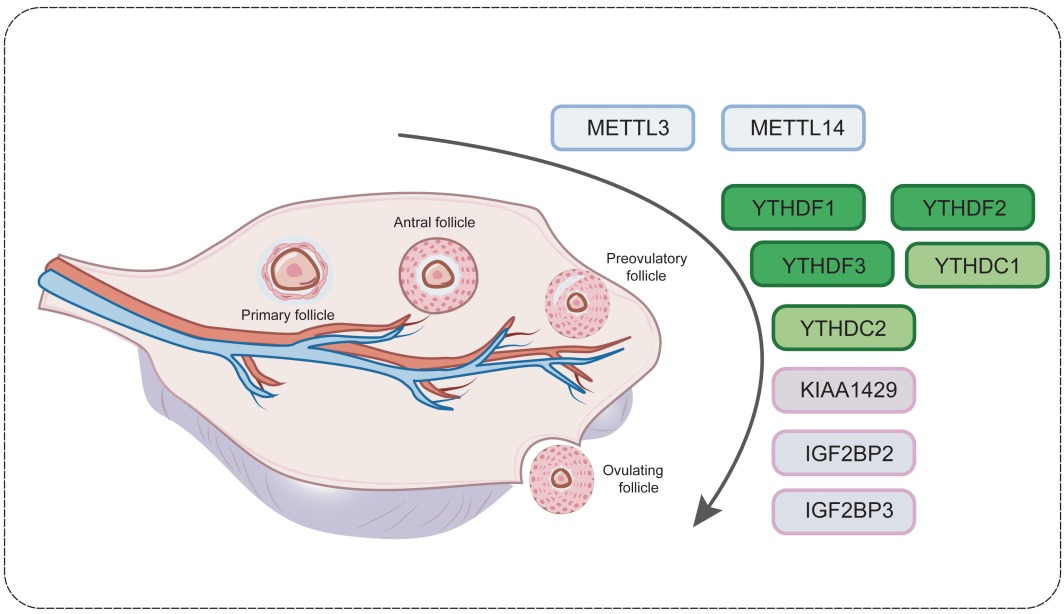


FIGURE 2
RNA methylation plays a pivotal role in early follicle development.

metabolic processes, and the cell cycle. Recent findings highlight the importance of the m6A modification during egg development. This conclusion is rooted in the observed significant differential clustering of m6A-modified genes in key signalling pathways involved in steroid synthesis, granulosa cell growth, and follicular maturation (99).

During the stages of egg maturation and late embryonic development, the RNA methylation process is involved in the fine regulation of RNA translation (94). Because transcriptional activity is not active during the initial stages of embryonic development, tight control of the translation process is essential for oocyte maturation (100). If the methylation level of maternal RNA is too high, it may interfere with the normal translation mechanisms and reduce the synthesis of proteins required for embryonic development and oocytes. In addition, recent findings support the role of m6A-related proteins in regulating ovulation processes, particularly METTL3, 14, YTHDC1/2, YTHDF1/2/3, and KIAA1429.

The absence of METTL3 function can lead to the blockade of the gamete maturation process, which in turn affects the reproductive capacity of individuals. This effect may be associated with a reduction in m6A modification levels, which may interfere with the normal expression of key genes involved in sex hormone synthesis and gonadotropin signalling pathways (101). When METTL3 activity is inhibited in oocytes, abnormal follicles form, which in turn affect the ovulatory process. In addition, the ability of METTL3 to regulate oocyte meiotic stability is enhanced by an m6A modification-dependent mechanism through cooperation with ITSN2, a protein that plays a restorative role during oocyte meiosis. In the presence of decreased METTL3 expression, females are unable to produce mature MII oocytes, and GV oocytes are smaller than normal in number and size, suggesting that METTL3 acts as an m6A methyltransferase to promote oocyte development by maintaining the stability of maternal mRNAs during GV (20). Although a reduction in METTL3 does not affect meiotic resumption, oocytes lacking METTL3 are defective in the formation of the first polar body and spindle.

L-Ascorbic acid treatment significantly reduces METTL14 mRNA levels in porcine oocytes, which may enhance the meiotic maturation and developmental competence of these cells (102). These findings suggest that METTL14 may play a critical role in oocytes during ovulation.

Several studies have shown that YTH domain-containing proteins such as YTHDC1/2 and YTHDF1/2/3 are essential for the ovulatory process (103–107). The oocyte maturation process is significantly affected by the YTHDC1 protein, and the loss of YTHDC1 is lethal to the embryo. Specifically, deletion of the m6A-modified reader YTHDC1 results in extensive changes in the polyadenylation pattern of the 3'-UTR, which affects microRNAs as well as protein-binding sites that are quite abundant in the 3'-UTR. When YTHDC1 function is inhibited after birth, female mice suffer a loss of fertility and are unable to form secondary and antral follicles. Notably, YTHDC1 deficiency also results in defective RNA metabolism and particle accumulation in oocytes, and these changes significantly affect oocyte maturation and ovulation processes (103).

Deletion of the YTHDC2 gene did not affect mouse survival but caused male mice to lose fertility and female mice to have a reduced ovarian size (106). YTHDC2 increases the efficiency of the translation process, stabilises target proteins by recognising m6A modifications, and may be involved in regulating self-renewal mechanisms in female germline stem cells. In the testes of male mice, YTHDC2 helps mitigate reproductive toxicity and cell cycle arrest (108).

Diminishing the expression of the YTHDF1 gene has been shown to curtail the self-renewal capacity of female germline stem cells in mice. Compared with cells derived from Sando's inbred

mouse embryos resistant to thioguanine and valine, these female mouse germline stem cells presented significant differences in global m6A levels mediated by METTL3, ALKBH5, YTHDF1/2, and YTHDC1/2 (109). In contrast to cells originating from Sando's inbred mouse embryos, which are resistant to thioguanine and valine, notable disparities in the overall m6A levels were observed among these female mouse germline stem cells, particularly with respect to the expression of METTL3, ALKBH5, YTHDF1/2, and YTHDC1/2 (81). The loss of YTHDF2, on the other hand, blocks the degradation of m6A-modified mRNAs, which in turn affects oocyte quality (110). When YTHDF2 and YTHDF3 are mutated at the same time, they impair the normal development of the female gonad (111). These findings support earlier findings that the m6A modification and its associated proteins and mechanisms remain consistent among regulators of gametogenesis (112).

KIAA1429, which is recognised as a novel enzyme associated with m6A methylation, plays a critical role in oogenesis. Studies have indicated that in the absence of KIAA1429, oocytes exhibit disrupted regulation of apoptosis and proliferation in granulosa cells during the early stages of folliculogenesis. Additionally, abnormalities in chromatin structure and RNA metabolism are observed, suggesting that KIAA1429 is essential for maintaining the normal developmental trajectory of oocytes (113). Several other m6A recognition proteins, such as IGF2BP2 and IGF2BP3, are essential for DNA repair and the stability of maternal mRNAs during meiosis during the maturation stage of eggs (63). While preserving mRNA stability, m6A may also be involved in oocyte maturation through various metabolic pathways. Currently, the significant role of the m6A modification in oogenesis is widely acknowledged; however, its potential as a therapeutic target for treating ovulatory disorders requires further in-depth investigation.

3.2 Embryo development

Many studies have shown that m6A-related proteins, including METTL3, METTL5, IGF2BP2, and IGF2BP3, are also involved in embryonic development. The absence of METTL3 has been noted to lead to increased levels of naïve pluripotency transcripts within mouse embryonic stem cells, an alteration that impedes the initiation of cell differentiation and their subsequent developmental progression (85). Similarly, studies have revealed the methylation of 18S rRNA by METTL5. When METTL5 was knocked down, mouse embryonic stem cells (mESCs) lost their pluripotency profile.

In vitro, although the loss of IGF2BP2 did not affect the process of egg formation, general decreases in transcriptional and translational activities were observed at the two-cell stage of embryonic development. IGF2BP2 significantly contributes to the advancement of embryonic development to the blastocyst phase and increases the overall quality of embryos through the modulation of IGF2 expression (114).

The IGF2BP3 protein has a strong affinity for maternal mRNA, preserving its stability by preventing its degradation during the initial phases of embryogenesis. In the absence of IGF2BP3, the degradation rate of maternal RNA is accelerated. IGF2BP3 deficiency does not negatively affect oocyte development, and these cells are still able to develop normally. Notably, cell division is impaired in cells with IGF2BP3 mutations, which may have an impact on other aspects of early embryonic development (115).

3.3 Foetal growth

In addition to its influence on female germ cell development and embryogenesis, the m6A demethylase FTO plays a significant role in foetal growth. Studies have shown that FTO activity may affect the process of foetal development by regulating the expression of genes associated with foetal growth through specific molecular mechanisms (116). In newborns with low birth weights, m6A modification levels in the 5' untranslated region (UTR) are higher, whereas m6A modification levels near the stop codon region are relatively lower than those in normal weight children of the same age (117, 118).

In particular, the expression of the FTO protein and the levels of the m6A modification on mRNA were significantly increased in the placentas of pigs with low birth weights under conditions of maternal obesity. Furthermore, in these low-birth-weight foetuses, the expression patterns of key genes involved in lipid metabolism and angiogenesis differed from those in normal foetuses. Researchers have hypothesised that elevated m6A levels might be involved in the regulation of the expression of key genes within placentas associated with low birth weight (119).

3.4 m6A functions in the innate immune response

Innate immunity constitutes a fundamental barrier against pathogen invasion, and a series of innate defensive cells, such as mononuclear phagocytes (MFs), natural killer (NK) cells, and dendritic cells (DCs), play key roles at the maternal–foetal interface. These cells have the ability to rapidly recognise invasive microorganisms and heterologous RNAs, which in turn trigger defence mechanisms against nonself pathogens (120, 121).

m6A modifications and their associated proteins are pivotal mediators of innate immune responses. They modulate the innate immune system by controlling mechanisms underlying cellular recognition and responses to external pathogens, unmodified tRNAs, exogenous RNAs, and aberrant endogenous RNAs. Specific knockout of the METTL3 gene in dendritic cells blocks the maturation of these cells when they encounter lipopolysaccharide (LPS), impairing their phenotype and normal function (122). In the innate immune system, macrophages constitute another indispensable cell population, and an analysis of RNA-binding proteins using CRISPR screening techniques identified preferential targets of m6A methylases that control LPS-induced macrophage activation. METTL3-deficient macrophages produced small amounts of TNF-α upon LPS stimulation. However, the suppression of METTL3 in macrophages notably amplified the production of proinflammatory cytokines such as TNF-α, IL-6, and nitric oxide (NO) (123).

The specific depletion of the m6A methylase METTL14 in macrophages disrupts the differentiation of $CD8^+$ T cells, which in turn weakens the ability of these cells to clear tumours (124). Natural killer (NK) cells are crucial for the defence against tumours and viral infections. As an m6A-binding protein, YTHDF2 expression is significantly increased when NK cells are activated by cytokines, tumour cells, or viruses. Elevated levels of YTHDF2 are implicated in modulating the tumour suppressor functions of NK cells and are linked to the final maturation stages of NK cells. This maturation is vital for governing the migration and function of NK cells, thereby influencing their capacity to exert antitumour and antiviral effects within the organism (125). However, the impacts of the m6A modification on the development and functions of macrophages and NK cells are not yet fully understood and require further research and scrutiny.

3.5 Maternal–foetal immune microenvironment

At the maternal–foetal interface, the equilibrium of the immune microenvironment is orchestrated by the dynamic interplay between placental and maternal cells, a balance that is subject to changes across the continuum of pregnancy. Placental trophoblasts serve as innate immune cells, engaging in communication with maternal cells to ensure that the embryo develops in an optimal immune environment conducive to its growth and well-being. Indeed, any dysfunction in placental cells or maternal decidual cells can precipitate pregnancy complications, with early pregnancy loss, including recurrent miscarriage, being a notable example. RNA methylation is crucial for modulating transcription across a spectrum of cellular processes. Many studies have underscored the pivotal function of m6A and its regulatory components throughout the process of embryo implantation. They play substantial roles in governing the behaviour of innate and adaptive immune cells, tuning immune responses, and shaping local and systemic immune contexts, which may subsequently affect the immune environment at the maternal–foetal junction. However, the role of the m6A modification at the maternal–foetal interface remains insufficiently explored. In this review, we delve into the critical functions of m6A during early embryonic development and assess current studies on the regulatory effects of m6A on immune cells and the tumour immune background. The m6A modification is believed to have the potential to promote embryonic development, bolster placentation, and aid in shaping the immune microenvironment at the maternal–foetal interface. A thorough comprehension of these key regulatory mechanisms may clarify the path for innovative therapeutic strategies that leverage RNA methylation, particularly during the early stages of pregnancy.

4 m6A modifications in female reproductive system neoplasms

The m6A RNA modification is central to the regulation of gene expression and cellular function, with evidence showing that the dysregulation of m6A is associated with the pathogenesis of a range of diseases, including gynaecological disorders (Figure 3).

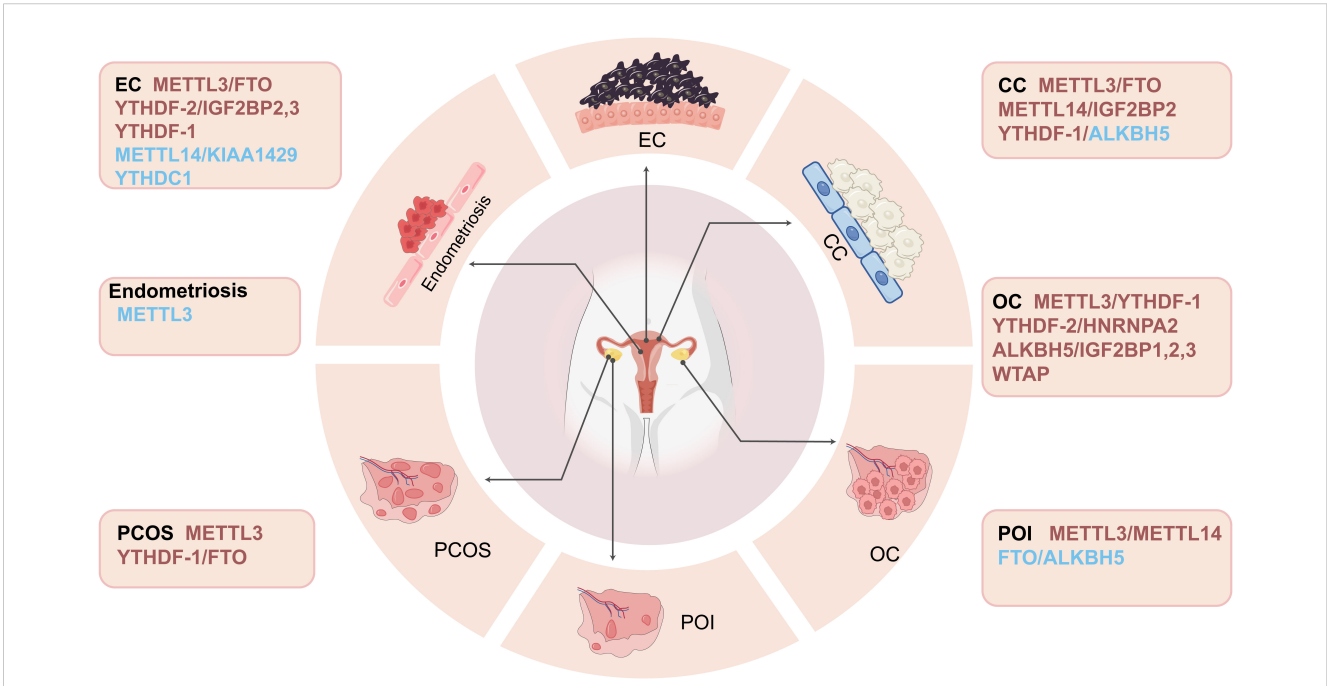


FIGURE 3
Illustrates the important role of m6A regulators in female reproductive disorders. These factors play a crucial role in the pathological changes of the female reproductive system, and they affect the pathogenesis of female reproductive disorders by regulating different molecular pathways and their corresponding targets (in which up-regulated targets are marked in red and down-regulated targets are marked in blue).

In tumour cells, the transcriptional process of epithelial cells promotes carcinogenesis by regulating the expression levels of m6A modification-related enzymes, including "writers", "readers", and "erasers". Specifically, up- or downregulation of the expression levels of these enzymes can affect the dynamic balance of m6A modifications, which in turn may drive tumour development (126–128). The m6A RNA modification is central to the pathogenesis of various cancers, with dysregulation of its modification patterns and underlying mechanisms contributing to the progression, growth, and spread of tumours by impacting the functional pathways of both coding and noncoding RNAs. Notably, specific m6A-modifying enzymes have been found to exert m6A-independent effects on certain malignancies, providing novel insights for cancer diagnostics and therapeutics.

Emerging research indicates that the m6A modification of mRNAs is intricately involved in reproductive processes, including oocyte maturation and the functionality of granulosa cells, through the regulation of RNA splicing, nuclear export, RNA degradation, and translation (129, 130). In addition, ROS-mediated oxidative stress leads to granulosa cell apoptosis and promotes follicular dysplasia, which can alter the m6A modification; however, how m6A participates in oxidative stress-induced ovarian cell apoptosis remains unclear (131, 132).

4.1 Preeclampsia

Placental trophoblasts play a key role in EMT in the initial stages of preeclampsia, a disease of placental origin. Upon the interference of multiple factors, the EMT process of trophoblasts may be abnormal, and this abnormality may lead to reduced invasion of trophoblasts into the uterine spiral artery, which in turn becomes a cause of preeclampsia (133). Numerous studies have revealed that trophoblasts in the placenta of patients with preeclampsia exhibit significantly increased levels of m6A methylation, METTL3, and HNRNPC1/C2 (134).

These chemical modifications have been shown to influence diverse biological attributes of placental trophoblasts. Some research teams have observed significant increases in m6A methylation and METTL3 expression levels in placental tissue from preeclampsia patients. Reducing METTL3 activity has been shown to significantly reduce m6A methylation levels in placental trophoblasts from individuals with preeclampsia, which in turn affects the expression of heterogeneous ribonucleoprotein complex members C1/C2 (hnRNPC1/C2); in addition, this process involves the maturation of miRNA 497-5p/145-5p (135). In the placenta of preeclampsia patients, the protein expression level of METTL14 has been shown to increase (136).

An in-depth exploration of the mechanism of action of METTL14 revealed that it was able to upregulate the expression of the FOXO3a gene through an m6A-dependent pathway, a process that not only promoted the autophagy and death of trophoblasts but also inhibited their proliferation and invasion (137). Compared with those in healthy placental tissue, no consensus is available on the changes in ALKBH5 and FTO levels in preeclamptic placentas. Some studies have suggested that the expression levels of these two proteins do not differ significantly in preeclamptic placentas, implying that their involvement in this condition might not be directly linked to their expression levels. Instead, it could be related to alternative regulatory mechanisms or functional alterations (134).

However, others have reported a significant increase in the expression level of ALKBH5 in placentas from preeclampsia patients, as well as in cells subjected to hypoxia or reoxidation (138). At the mechanistic level, the expression of the RNA demethylase ALKBH5 is notably elevated in placental tissues affected by preeclampsia. This increase in ALKBH5 expression decreases the stability of the PPARG mRNA, thereby disrupting its normal translation. Consequently, this process affects the demethylation of ALCAM by the histone demethylase KDM3B, causing a decrease in ALCAM expression. This chain of molecular events is implicated in the pathogenesis of preeclampsia (138).

Recent explorations of the pathomechanism of preeclampsia have focused mostly on the IGF2BP protein family, which is expressed at low levels in preeclamptic placental samples (139). IGF2BP3 contributes to increasing circRNA PAPPA2 stability by recognising and binding m6A modifications. In addition, the MNSFβ protein can directly interact with IGF2BP2 and enhance the invasion and migration of trophoblast cells (e.g., JEG-3 and HTR8/SVneo) (139).

A sequencing analysis of m6A-modified RNA in PE placentas revealed that circRNA m6A methylation levels were increased in preeclamptic placentas compared with normal placentas. Recent studies have shown that the YTHDC1 protein maintains the normal function of trophoblasts by accelerating the degradation of m6A-modified circMPP1 (140). Recent studies have shown that YTHDF3 expression levels increase in trophoblast cells under hypoxic conditions and that its inhibition of IGF1R gene expression by the m1A modification leads to decreased trophoblast invasion (141). Because hypoxia is involved in the pathogenesis of preeclampsia, YTHDF3 may be involved in the pathogenesis of preeclampsia.

4.2 Abortion

Spontaneous abortion represents one of the most prevalent complications encountered in pregnancy. Controlling the invasion of trophoblasts and reducing their atypical growth and division can reduce the impact on the health of the placenta *in utero*. Thus, trophoblasts are essential for the formation of the placenta. During pregnancy, dysfunction of trophoblast molecules may trigger serious pregnancy problems (142, 143). When human trophoblasts are exposed to BPDE, METTL14 expression levels are increased compared with those before exposure, which was also observed in the placental villous tissue of women with recurrent miscarriages.

Increased ALKBH5 levels in the placental villous tissue of those with recurrent miscarriages may explain the noted decrease in global m6A modification levels. ALKBH5 may affect trophoblast invasiveness by reducing CYR61 mRNA stability through an m6A-dependent mechanism (144). The expression levels of FTO, an RNA demethylase, are decreased in women who experienced a

spontaneous abortion. These findings suggest that the abnormal accumulation of m6A at the maternal–foetal interface may be a factor contributing to spontaneous abortion (145).

The m6A modification may increase IGFBP3 transcript levels, which in turn may increase MMP2 activity while decreasing the expression of its specific inhibitor tissue inhibitor TIMP-1. Eventually, spontaneous abortion occurs (146).

4.3 Endometriosis

Endometriosis, an oestrogen-independent condition, is characterised by the ectopic occurrence of endometrial-like tissues outside the uterine cavity. Patients typically present with intense pelvic pain and infertility, significantly impairing their daily life and productivity (147–149). Many studies have shown that the pathogenesis of endometriosis may be related to m6A, and some of these regulators, including METTL3, YTHDF2, YTHDF3, HNRNPC, HNRNPA2B1, and FTO, are significantly dysregulated in the ectopic endometrium (150).

Histopathological assessments of endometriosis patients have indicated a notably lower m6A modification level in endometrial tissues than in healthy control tissues, a finding that may be correlated with the decreased expression of METTL3. This finding suggests that decreased METTL3 expression enhances the migration and invasiveness of human endometrial stromal cells (HESCs) by inhibiting the DGCR8-mediated transformation of pri-miRNA126 to mature miRNA126, which in turn accelerates endometriosis development. On the other hand, METTL3 overexpression has the opposite effects, which may provide ideas for further studies of endometriosis (151).

HNRNPA2B1 and HNRNPC are involved in the regulation of immune responses, and their abnormal expression is closely related to the pathogenesis of endometriosis. Therefore, they can be used as biomarkers of endometriosis to assist with diagnosis and treatment (150). Although research on the role of the mechanisms of m6A-related proteins in endometriosis is still limited, the existing studies have highlighted their substantial effects on this condition. These insights are anticipated to yield potential biomarkers and therapeutic targets, paving the way for the improved clinical management of endometriosis.

4.4 Adenomyosis

Adenomyosis is a relatively common uterine disease of the female reproductive system that is characterised by the excessive proliferation of abnormally accumulated epithelial cells and stromal fibroblasts in the endometrium, causing hypertrophy and hyperplasia of adjacent smooth muscle cells (152–154). Currently, the pathophysiological mechanisms underlying adenomyosis are not fully understood, and effective biomarkers and treatment options are lacking.

Zhai et al. reported that the m6A protein machinery is involved in the development of adenomyosis. Notably, regulators of m6A RNA methylation, including METTL3, ZC3H13, and FTO, are expressed at reduced levels in the myometrium of individuals with adenomyosis, consequently affecting global m6A levels (155). A correlation between the significant upregulation of IGF1 and DDT gene expression levels and a reduction in METTL3 expression levels has already been validated. A number of additional potential target genes, such as cadherin 3 (CDH3), placenta-specific protein 8 (PLAC8), and sodium channel β-subunit 4 (SCN4B), have been shown to significantly contribute to processes such as cell adhesion, muscle contraction, and immune responses within the myometrium of patients with adenomyosis. Previous studies have indicated that these genes participate in the regulation of epithelial cell proliferation and migration.

Therefore, the pathogenesis of adenomyosis may be related to the cellular processes associated with these genes (155, 156). These studies provide new perspectives and possible therapeutic directions for the diagnosis and treatment of adenomyosis. However, these experimental results have not been validated in animal models or in clinical trials. Therefore, future studies need to further explore its mechanism of action and discover its diagnostic markers and therapeutic targets.

4.5 Polycystic ovary syndrome

PCOS is a highly complex disease with an uncertain aetiology in the metabolic and endocrine fields. Patients often show clinical associations with obesity, insulin resistance (IR), and cardiovascular disease (157, 158). In addition, obesity not only exacerbates metabolic disorders but also adversely impacts oocyte quality and may interfere with endometrial receptivity, factors that collectively influence fertility in women of reproductive age (159, 160). Recent studies have revealed elevated levels of the m6A modification in luteinised granulosa cells (GCs) from patients with PCOS. The loss of the m6A modification of the FOXO3 mRNA was also observed in luteinised granulosa cells from PCOS patients. By selectively knocking down m6A methyltransferases or demethylases, researchers have observed a change in FOXO3 expression in luteinised GCs from controls but not in those from PCOS patients. This series of findings suggests the possible inhibition of m6A-mediated transcriptional regulation of the FOXO3 gene in luteinised GCs from PCOS patients. FOXO3 is a key target of METTL3, which modifies the FOXO3 3'-UTR-untranslated region, increasing its stability and activating its autophagy-related pathway through a YTHDF1-dependent mechanism (161). Interestingly, autophagy is prevalent in ovarian tissue from patients with polycystic ovary syndrome (PCOS). In luteinised granulosa cells from PCOS patients, FTO facilitates m6A demethylation by downregulating the m6A modification of the FLOT2 mRNA, a mechanism that correlates with insulin resistance and a phenotype characterised by an elevated proliferation rate and diminished apoptosis in these cells (162). The above studies may reveal the potential pathogenesis of and future treatment ideas for PCOS, and more studies on PCOS are needed.

4.6 Primary ovarian insufficiency

Primary ovarian insufficiency (POI), also known as premature ovarian failure, is a clinical condition characterised by persistent amenorrhea, elevated follicle-stimulating hormone (FSH) levels, and reduced oestrogen levels in women under the age of 40. The exact pathomechanism of this condition has not been fully elucidated, but chemotherapeutic agents, particularly alkylating agents such as cyclophosphamide (CTX), may lead to impaired ovarian function by affecting m6A RNA methylation levels (163–165).

Specifically, CTX has been reported to increase m6A methylation levels and the expression of the methyltransferases METTL3 and METTL14 in a concentration- and time-dependent manner while decreasing the expression levels of the demethylase FTO and the effector protein YTHDC1 in human granulosa cells and mouse models (166). In a previous study, an increase in m6A levels and a decrease in FTO expression were observed in POI patients. In addition, the incidence of apoptosis increases, whereas the proliferation decreases in granulosa cells in which FTO expression is knocked down (132).

Increased levels of the m6A modification of mRNAs were observed in male mice deficient in the ALKBH5 gene. These mice presented a notable decrease in the sperm count and abnormal sperm morphology, along with altered sperm motility. In addition, mice lacking ALKBH5 may undergo apoptosis during the metaphase and coarse stages of spermatocytes, which is accompanied by disturbances in spermatogenesis. Given that ALKBH5 is expressed in the reproductive tissues of both males and females, it is inferred to play a significant role in germ cell development (167). Dysregulated levels of m6A modifiers could impact oocyte maturation by influencing cell division processes during meiosis.

4.7 Endometrial cancer

EC, which is prevalent in developed countries, typically arises from the endometrial epithelium and affects perimenopausal and postmenopausal women. Its diagnosis and treatment often necessitate invasive evaluations and surgical procedures (168, 169). The standard therapeutic approaches for endometrial cancer (EC) include surgical intervention, radiation therapy, hormone treatment, and targeted therapy (170). The prognosis is usually optimistic if the disease is diagnosed early and treated with surgery (171). However, patients with advanced or poorly differentiated EC face a high risk of recurrence and a poor prognosis even after multimodal treatment because of its invasive metastatic properties (172). Thus, exploring and investigating biomarkers and their molecular mechanisms in EC are essential to improve patient outcomes.

In EC, abnormal expression or dysfunction of m6A regulatory proteins may lead to the abnormal regulation of tumour-related genes, which leads to the development and progression of EC. In a 2018 study, mutations in METTL14 or decreased expression of METTL3 resulted in decreased m6A methylation levels in endometrial tumours, and this decrease promoted the proliferation and tumour formation of EC cells by activating the AKT signalling pathway and its regulators PHLPP2 and mTORC2 (27). Another study in the same year revealed that specific knockdown of the m6A methylase METTL14 in macrophages impaired the antitumour activity of CD8+ T cells, a phenomenon that was also observed in colorectal cancer, providing a new perspective for studies of the immune microenvironment in EC (173).

IGF2BP1 increases PEG10 mRNA expression by recognising the m6A modification site in the PEG10 mRNA, thereby promoting the proliferation of EC cells (174). In addition, the expression level of PADI2 is increased in EC and increases the expression of IGF2BP1, which in turn binds to the m6A locus in the 3' untranslated region of the SOX2 mRNA and increases its stability, leading to the malignant progression of EC (175, 176).

IGF2BP1 expression is increased in EC, and this high level of expression is strongly associated with a poor patient prognosis (177). IGF2BP1 is also involved in arginine guanidinium methylation in EC, and dysregulation of IGF2BP1 expression mediated by the PADI2/MEK1/ERK signalling pathway leads to the upregulation of SOX2 expression and subsequently promotes the malignant phenotype of EC cells (176). High expression levels of IGF2BP1 are also strongly associated with a poor prognosis for EC patients. These findings reveal multiple roles for IGF2BP1 in EC development, including promoting cell proliferation, regulating the tumour cell cycle, and promoting tumour progression.

In EC, a decrease in the level of m6A-modified RNA leads to a significant decrease in the expression level of KIAA1429 (176). The expression of the KIAA1429 gene is closely associated with nuclear metabolism. However, in liver cancer, KIAA1429 is known to promote liver tumour progression by regulating the posttranscriptional modification of GATA binding protein 3 (GATA3), a highly conserved essential transcription factor that is widely expressed in a variety of tissues, in an N6-methyladenosine-dependent manner (178). The specific mechanism of action of KIAA1429 in EC remains to be further elucidated.

In EC, ALKBH5 expression is notably elevated under hypoxic conditions, facilitating SOX2 transcription via mRNA demethylation. This process sustains the stem-like features and tumourigenic potential of endometrial cancer stem cells (ECSCs), thereby driving the aggressive progression of EC (179). Consistent observations from studies in 2020 have indicated that ALKBH5 levels are elevated in EC tissues. By diminishing the m6A methylation of IGF1R, ALKBH5 increases the stability of the IGF1R mRNA, stimulates IGF1R translation, and activates the IGF1R signalling cascade, consequently fuelling the proliferation and invasive capabilities of EC cells (180, 181).

In EC tissues, a marked increase in the expression of FTO has been observed. Increased levels of FTO can facilitate the demethylation of the m6A mark in the 3' untranslated region (3' UTR) of the HOXB13 mRNA, a modification that prevents YTHDF2 from recognising the m6A site. This demethylation leads to the activation of the WNT signalling pathway and subsequent regulation of downstream protein expression, ultimately promoting tumour metastasis and invasion (182, 183).

In EC, the suppression of YTHDC1 is associated with increased proliferation and invasion of cancer cells (184). However, YTHDF2 exerts the opposite effect and is capable of curbing the proliferation and invasive behaviour of EC cell lines. Specifically, the m6A reader YTHDF2 can identify and bind to the methylation site on the target transcript insulin receptor substrate 1 (IRS1), a mechanism that promotes the degradation of the IRS1 mRNA, thereby suppressing IRS1 expression and consequently impeding the IRS1/AKT signalling pathway to ultimately curb the tumourigenic potential of EC (185).

WTAP, which is recognised as an essential m6A methyltransferase, facilitates the addition of m6A modifications to mRNAs, particularly within the 3' untranslated region (3'-UTR) of transcripts such as CAV-1. A decrease in CAV-1 expression triggers the activation of the NF-κB signalling cascade, thereby significantly contributing to the progression of EC (186). Based on the aforementioned research, a distinct correlation exists between m6A regulators and their integral roles in both the clinical treatment and diagnosis of various diseases.

4.8 Cervical cancer

Cervical cancer (CC) is indeed one of the most prevalent gynaecological tumours globally, with a well-established link to persistent human papillomavirus (HPV) infection, which is identified as a significant risk factor for its development. In summary, the TME significantly influences the initiation, progression, and outcomes of CC (187). Although the widespread use of HPV vaccines has reduced the incidence of CC to some extent, because the early symptoms of CC are not obvious, early detection and treatment are more difficult in clinical practice, with a five-year survival rate of less than 20% and a poor prognosis for patients with CC recurrence (188, 189). Hence, understanding the molecular mechanisms of CC is crucial for identifying novel therapeutic targets and enhancing clinical treatment approaches.

The m6A modification is instrumental in the progression of CC, with METTL3, in particular, shown to increase the proliferation and invasiveness of CC cells (190). Wang et al. observed that METTL3 levels are substantially elevated in CC tissues and cell lines, closely correlating with lymph node metastasis and unfavourable patient outcomes (191). In terms of the molecular mechanisms, METTL3 can target the 3'-UTR of the hexokinase 2 (HK2) mRNA and increase the stability of HK2 by recruiting YTHDF1, thereby promoting the Warburg effect in CC (191).

Furthermore, METTL3 facilitates the m6A modification of pyruvate dehydrogenase kinase 4 (PDK4), increases PDK4 mRNA translation, stimulates cancer cell glycolysis, and subsequently promotes the growth and progression of CC (192). Recent research has revealed an innovative pathway through which METTL3 enhances the invasiveness of CC by suppressing miR-193b activity, thereby upregulating "CCND1" expression and accelerating CC progression and severity (193). An earlier study revealed that elevated METTL3 levels in CC tissues were positively correlated with the presence of $CD33^+$ cells within the tumour and with the clinical outcomes of patients (194). Elevated levels of METTL3 in CC tissues markedly increase the expression and stability of FOXD2-AS1, thereby further promoting the malignancy of CC (195).

METTL14 stimulates the expression of the tumour development and progression-associated protein seven in absentia homologue 2 (Siah2). The suppression of Siah2 hinders the proliferation and cytotoxic capabilities of T cells, as it sustains the expression of PD-L1 on tumour cells. Moreover, an examination of samples from patients subjected to anti-PD-1 immunotherapy indicated that tumours exhibiting reduced Siah2 levels responded more positively to the treatment (196).

FTO is highly expressed in human CC tissues and is closely associated with disease progression and patient prognosis (51). At the molecular level, FTO engages directly with the E2F1 and Myc mRNAs. The suppression of FTO markedly impacts the translation of these key oncogenes, subsequently curbing the proliferation and migration of CC cells (51).

In addition, the expression of GAS5-AS1 was decreased in CC tissues, and GAS5-AS1 combined with ALKBH5 and reduced the m6A modification of GAS5, which increased its stability, inhibited the proliferation and metastasis of CC cells, and affected the prognosis of patients (197). However, different investigators have different views on the relationship between FTO and CC, and some studies have revealed how HOXC13-AS promotes the proliferation, invasion, and EMT of CC cells by increasing the level of FZD6 and activating the Wnt/β-catenin signalling pathway (198).

Zhou et al. discovered that FTO modulates the expression of β-catenin by reducing m6A levels in the β-catenin mRNA, which in turn increases the chemoresistance of cancer cells both *in vivo* and *in vitro* (199). These findings reveal the multiple roles of FTO in CC development and highlight its potential as a novel therapeutic strategy and a critical target for prognostic evaluations.

YTHDF1 promotes translation of the RANBP2 protein through an m6A-dependent mechanism but does not alter the expression level of the RANBP2 mRNA, which in turn enhances the growth and invasion of CC cells. It is significantly correlated with unfavourable outcomes of CC (197).

Research indicates that the expression of the circRNA circARHGAP12 is increased in CC tissues, where it stabilises the FOXM1 mRNA through its interaction with IGF2BP2 in an m6A-dependent manner, contributing to the malignancy of CC (200). A separate study revealed that the long noncoding RNA KCNMB2-AS1 is markedly overexpressed in CC and is correlated with an unfavourable prognosis for patients. IGF2BP3 can identify and bind to the m6A-modified site on KCNMB2-AS1, increasing its stability and thus facilitating the progression of CC (201).

In addition, the IGF2BP3 and YTHDF1/eEF-2 complexes increase PDK4 mRNA stability through the m6A modification system, a process that promotes the glycolytic process of cancer cells and accelerates CC development (192).

4.9 Ovarian cancer

OC is a common cause of death in women with gynaecologic cancer, and its five-year survival rate is between approximately 30%

and 40% (202, 203). Because early symptoms are not significant, many patients are already in an advanced metastatic state at the time of diagnosis, and the tumour has also spread to adjacent tissues (204).

The presence of metastatic tumours is a major cause of high mortality in OC patients. Although most patients' symptoms can be significantly relieved by appropriate treatment at an early stage, approximately 70% of patients with advanced disease experience relapse (205). The cure rate and survival rate for patients with advanced OC have not improved significantly in recent years. This lack of improvement is partly attributed to the absence of effective early diagnostic screening and detection methods, coupled with high rates of tumour metastasis and recurrence (206). Consequently, a thorough investigation into the high-risk factors and the precise molecular mechanisms underlying the progression of OC is crucial for facilitating early intervention and treatment, thereby increasing patient survival rates.

Proteins associated with the m6A modification are instrumental in the initiation and progression of OC (207). Recent studies have shown that METTL3 plays a key role in the TME, and its increased expression in OC tissues is significantly correlated with the clinical characteristics of tumours. Luo et al. reported that the m6A modification can affect antigen presentation processes in the immune system and plays a critical role in cell infiltration in the TME of OC (208).

Hua and colleagues showed that stable overexpression of METTL3 under *in vitro* experimental conditions significantly enhanced the ability of nude mouse cells to proliferate, form tumours, migrate, invade, and form tumours (207). In contrast, in cell lines in which METTL3 expression was downregulated by a short hairpin RNA (shRNA), its function in cancer was effectively inhibited. Further mechanistic analysis revealed that METTL3 increases AXL mRNA expression, a process that accelerates the EMT, which in turn promotes the proliferation, tumour formation, migration, and invasion of OC cells.

The proportion of apoptotic cells increases when METTL3 expression is suppressed in OC cells, a phenomenon that may stem from the activation of mitochondria-mediated apoptotic pathways. A study by Liang et al. revealed that METTL3 may also play an active role in OC progression by increasing the phosphorylation of AKT and the expression level of Cyclin D1. In addition, METTL3 expression was significantly increased in OC tissues compared with other tumour tissues.

However, the expression levels of METTL3 and m6A are significantly increased in epithelial OC, and the degree of malignancy is high (108). Therefore, METTL3 may be used as a specific indicator of epithelial OC in the future.

YTH domain family proteins, through the chemical modification of m6A, can affect the development of OC at different levels. YTHDF1 and hnRNPA2 promote the development and metastasis of OC by increasing the expression levels of EIF3C and Lin28B, respectively, and are associated with a poor prognosis (81, 209).

The binding of the YTHDF1 protein to m6A-modified TRIM29 promotes the translation of TRIM29 in cisplatin-resistant OC cells, enhances the cancer stem cell characteristics in cisplatin-resistant OC cells, and then promotes the development of malignant tumours. These findings suggest that it may become a potential new target for OC treatment (109).

YTHDF2 increases proliferation and migration and inhibits apoptosis in epithelial OC cells through the YTHDF2/m6A regulatory axis while downregulating global mRNA m6A levels (63). YTHDF2 is a new substrate of the FBW7 enzyme, and the expression level of FBW7 is decreased in OC tissues. This decrease is closely related to a poor prognosis and a decrease in the m6A modification level. Further studies revealed that FBW7 triggers the proteasomal degradation of YTHDF2, thereby counteracting its tumour-promoting effect (210).

Recent studies on the tumour suppressor protein IFFO1 have revealed how METTL3 and the YTHDF2 axis regulate the stability of the IFFO1 mRNA through an m6A-dependent mechanism, which in turn inhibits the invasive ability of OC cells and reduces their drug resistance (211). When METTL14 is overexpressed, it effectively inhibits granulosa cell proliferation by inactivating PI3K/AKT/mTOR signalling. WTAP can regulate the proliferation and invasion of high-grade serous OC (HGSOC) cells by activating the AKT signalling pathway (212). Reducing the expression levels of METTL14 and WTAP has limited effects on the tumour development and behaviour of endometrioid epithelial OC cells cultured *in vitro* (108).

WTAP might stimulate the growth and mobility of high-grade serous ovarian cancer (HGSOC) cells and impede their apoptosis by triggering the MAPK and AKT signalling pathways. Furthermore, the long noncoding RNA UBA6-AS1 is able to recruit the RBM15 protein, which in turn increases the m6A methylation of the UBA6 mRNA, leading to the inhibition of UBA6 expression. This process is associated with the increased proliferation, migration, and invasion of ovarian cancer (OC) cells (213).

Zhang et al. discovered that FTO expression was elevated in OC tissues, where it increased OC cell viability and autophagy by stimulating AKT phosphorylation, thereby inhibiting apoptosis in cancer cells (214). Unlike previous studies in which FTO was used to promote tumourigenesis, a study focused on OC revealed that FTO expression levels are decreased in this type of cancer and inhibit tumourigenesis *in vivo* by affecting cAMP signalling pathways and blocking the self-renewal process of OC stem cells (215).

In epithelial ovarian cancer, ALKBH5 expression is notably elevated, and its suppression reduces the activity of the EGFR/PI3K/AKT signalling pathway (216). This process leads to an increase in autophagy signalling and the suppression of OC cell proliferation and invasion. Concurrently, research has indicated that ALKBH5 is more abundantly expressed in OC tissues, with reduced expression in OC cell lines, mirroring the expression pattern of TLR4. The activation of the m6A modification by TLR4 bolstered the NF-κB pathway, consequently increasing ALKBH5 expression levels (217).

Elevated expression levels of ALKBH5 in OC promote tumour cell proliferation and migration and inhibit the autophagy process by increasing BCL-2 mRNA stability, which in turn activates the EGFR-PIK3CA-AKT-mTOR signalling pathway (218). ALKBH5

overexpression is associated with OC with lymph node metastasis and may promote lymphangiogenesis and the lymph node metastasis of ovarian tumours by affecting the m6A modification and the ITGB1/FAK signalling pathway (219). ALKBH5 forms a regulatory loop with HOXA10 that can activate the JAK2/STAT3 signalling pathway by regulating the m6A demethylation of JAK2, a process that causes epithelial OC (EOC) to become resistant to cisplatin drugs (217).

IGF2BP1 increases SRF expression in an m6A-dependent manner by preventing SRF mRNA degradation by miRNAs and sustaining PDLIM7 and FOXK1 levels, thereby promoting tumour cell proliferation and invasion in OC (220). In high-grade serous OC (HGSOC), which presents mesenchymal features, the expression level of IGF2BP1 increases significantly, promoting the invasive growth of OC cells (221). In the same year, another study revealed that increased IGF2BP2 protein expression promoted the proliferation and invasion of OC cells by binding to circ0000745 and increasing its stability (222). IGF2BP3 is an RNA-binding protein that is upregulated in ovarian clear cell carcinoma (OCCC) and promotes cancer cell proliferation and tumour formation (216).

In summary, METTL3/14, YTHDF1/2, WTAP, FTO, ALKBH5, and IGF2BP1/2/3 play roles in promoting or inhibiting OC, and these factors are expected to be tools for the diagnosis and treatment of OC. However, many types of OC, including high-grade serous OC, endometrioid OC, and clear cell OC, have been identified. Whether the function of m6A is consistent in multiple OCs requires further investigation.

5 Conclusions and perspectives

Despite significant advancements in the field, considerable challenges remain in thoroughly understanding the role of m6A RNA methylation in the context of infertility and various reproductive diseases. This complex epigenetic modification is known to play a crucial role in regulating gene expression, but its specific mechanisms and implications in reproductive health issues have not yet been fully elucidated. Researchers continue to encounter difficulties in deciphering the intricate interplay between m6A modifications and the molecular pathways that contribute to fertility and the development of reproductive disorders. The heterogeneity of patient populations, the complexity of the female reproductive system, and the dynamic nature of RNA methylation patterns all contribute to ongoing challenges in this area of study.

First, the role of m6A modification in gynaecological tumours is currently based primarily on bioinformatics analyses and animal model research, with relatively few clinical trials. With the identification of proteins associated with the m6A modification, our understanding of the underlying mechanisms and signalling pathways has significantly increased. Despite the recognition that the m6A modification can either stimulate or suppress the progression of gynaecological cancers, a comprehensive grasp of the intricate processes driving oncogenesis is still lacking. Accordingly, more clinical trials are needed to elucidate these mechanisms, facilitating the development of more potent therapeutic strategies in the future.

Second, some m6A regulators may be associated with gynaecological diseases that have yet to be explored. The intricate operational mechanisms of m6A-related proteins within a range of prevalent gynaecological disorders are not yet fully understood, highlighting the need for additional investigations into how m6A influences multiple pathways that govern gene expression. Therefore, more in-depth studies are necessary to identify more m6A regulators associated with gynaecological diseases.

Third, reports on m6A-specific modification sites are currently limited, which limits their potential for clinical application. Although m6A RNA methylation is thought to influence other molecules, our understanding of its molecular mechanisms and cellular effects on gynaecologic reproductive system diseases is not comprehensive. Furthermore, the irregular expression patterns of m6A-modified regulatory molecules in gynaecological malignancies remain unclear. Enhancing the sensitivity and specificity of pertinent biomarkers is essential for advancing the clinical application of m6A regulators in diagnosis and therapeutics.

Fourth, research indicates that modulating m6A levels and the protein machinery could target specific reproductive diseases; however, clinical validation regarding safety, efficacy, and potential adverse effects, particularly on a large scale, is still pending. Currently, the m6A modification remains challenging despite advances in gynaecologic oncology research.

Fifth, no consensus is available on whether m6A modifications differ in their impacts on different tumour subtypes. More in-depth studies are needed to obtain answers for this question. The regulatory mechanisms associated with the m6A modification have been exhaustively explored in many types and different pathological stages of gynaecological malignancies.

In conclusion, the study of the m6A modification has emerged as a focal point of scientific inquiry, garnering significant attention for its potential implications across various fields of research. It is instrumental throughout the metabolic journey of mRNA, from its synthesis in the nucleus to its translation and ultimate degradation within the cytoplasm, thereby determining the ultimate fate of the mRNA. Relative to other relevant reviews, this manuscript provides an exhaustive examination of the relationship between the m6A modification and the physiological and pathological aspects of the female reproductive system. It encompasses a wide range of conditions, from germ cell genesis and embryonic development to benign and malignant diseases affecting female reproductive organs. The hope is that the m6A modification will become a pivotal factor in diagnosing and treating gynaecological conditions in the future.

Author contributions

LL: Writing – original draft. DG: Writing – original draft. YL: Writing – original draft. ZH: Writing – original draft. HZ: Writing – original draft. LC: Writing – original draft. XW: Writing – original draft. GM: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. The work was supported by the 2024 Hongkou District Health Commission's Traditional Chinese Medicine Research Project (HKZYY-2024-07).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. Chatgpt.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Dwivedi SKD, Rao G, Dey A, Mukherjee P, Wren JD, Bhattacharya R. Small non-coding-rna in gynecological Malignancies. *Cancers (Basel)*. (2021) 13:1085. doi: 10.3390/cancers13051085

2. Schäfer KP. Rna synthesis and processing reactions in a subcellular system from mouse L cells. *Hoppe Seylers Z Physiol Chem*. (1982) 363:33–43. doi: 10.1515/bchm2.1982.363.1.33

3. Roignant JY, Soller M. M(6)a in mrna: an ancient mechanism for fine-tuning gene expression. *Trends Genet*. (2017) 33:380–90. doi: 10.1016/j.tig.2017.04.003

4. Lipshitz HD, Claycomb JM, Smibert CA. Post-transcriptional regulation of gene expression. *Methods*. (2017) 126:1–2. doi: 10.1016/j.ymeth.2017.08.007

5. Gilbert WV, Bell TA, Schaening C. Messenger rna modifications form distribution and function. *SIGNALS IN RNA*. (2016) 352:1408–12. doi: 10.1126/science.aad8711

6. Sun X, Lu J, Li H, Huang B. The role of M(6)a on female reproduction and fertility: from gonad development to ovarian aging. *Front Cell Dev Biol*. (2022) 10:884295. doi: 10.3389/fcell.2022.884295

7. Huang W, Chen TQ, Fang K, Zeng ZC, Ye H, Chen YQ. N6-methyladenosine methyltransferases: functions, regulation, and clinical potential. *J Hematol Oncol*. (2021) 14:117. doi: 10.1186/s13045-021-01129-8

8. Ma S, Chen C, Ji X, Liu J, Zhou Q, Wang G, et al. The interplay between M6a rna methylation and noncoding rna in cancer. *J Hematol Oncol*. (2019) 12:121. doi: 10.1186/s13045-019-0805-7

9. Ping X-L, Sun B-F, Wang L, Xiao W, Yang X, Wang W-J, et al. Mammalian wtap is a regulatory subunit of the rna N6-methyladenosine methyltransferase. *Cell Res*. (2014) 24:177–89. doi: 10.1038/cr.2014.3

10. Yue Y, Liu J, Cui X, Cao J, Luo G, Zhang Z, et al. Virma mediates preferential M(6)a mrna methylation in 3'utr and near stop codon and associates with alternative polyadenylation. *Cell Discov*. (2018) 4:10. doi: 10.1038/s41421-018-0019-0

11. Schwartz S, Mumbach MR, Jovanovic M, Wang T, Maciag K, Bushkin GG, et al. Perturbation of M6a writers reveals two distinct classes of mrna methylation at internal and 5' Sites. *Cell Rep*. (2014) 8:284–96. doi: 10.1016/j.celrep.2014.05.048

12. Huang E, Chen L. Rna N(6)-methyladenosine modification in female reproductive biology and pathophysiology. *Cell Commun Signal*. (2023) 21:53. doi: 10.1186/s12964-023-01078-4

13. Barbieri I, Tzelepis K, Pandolfini L, Shi J, Millán-Zambrano G, Robson SC, et al. Promoter-bound mettl3 maintains myeloid leukaemia by M(6)a-dependent translation control. *Nature*. (2017) 552:126–31. doi: 10.1038/nature24678

14. Vu LP, Pickering BF, Cheng Y, Zaccara S, Nguyen D, Minuesa G, et al. The N(6)-methyladenosine (M(6)a)-forming enzyme mettl3 controls myeloid differentiation of normal hematopoietic and leukemia cells. *Nat Med*. (2017) 23:1369–76. doi: 10.1038/nm.4416

15. Chen M, Wei L, Law CT, Tsang FH, Shen J, Cheng CL, et al. Rna N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through ythdf2-dependent posttranscriptional silencing of socs2. *Hepatology*. (2018) 67:2254–70. doi: 10.1002/hep.29683

16. He H, Wu W, Sun Z, Chai L. Mir-4429 prevented gastric cancer progression through targeting mettl3 to inhibit M(6)a-caused stabilization of sec62. *Biochem Biophys Res Commun*. (2019) 517:581–7. doi: 10.1016/j.bbrc.2019.07.058

17. Choe J, Lin S, Zhang W, Liu Q, Wang L, Ramirez-Moya J, et al. Mrna circularization by mettl3-eif3h enhances translation and promotes oncogenesis. *Nature*. (2018) 561:556–60. doi: 10.1038/s41586-018-0538-8

18. Yue B, Song C, Yang L, Cui R, Cheng X, Zhang Z, et al. Mettl3-mediated N6-methyladenosine modification is critical for epithelial-mesenchymal transition and metastasis of gastric cancer. *Mol Cancer*. (2019) 18:142. doi: 10.1186/s12943-019-1065-4

19. Han J, Wang JZ, Yang X, Yu H, Zhou R, Lu HC, et al. Mettl3 promote tumor proliferation of bladder cancer by accelerating pri-mir221/222 maturation in M6a-dependent manner. *Mol Cancer*. (2019) 18:110. doi: 10.1186/s12943-019-1036-9

20. Mu H, Zhang T, Yang Y, Zhang D, Gao J, Li J, et al. Mettl3-mediated mrna N(6)-methyladenosine is required for oocyte and follicle development in mice. *Cell Death Dis*. (2021) 12:989. doi: 10.1038/s41419-021-04272-9

21. Pan F, Lin XR, Hao LP, Chu XY, Wan HJ, Wang R. The role of rna methyltransferase mettl3 in hepatocellular carcinoma: results and perspectives. *Front Cell Dev Biol*. (2021) 9:674919. doi: 10.3389/fcell.2021.674919

22. Wang T, Kong S, Tao M, Ju S. The potential role of rna N6-methyladenosine in cancer progression. *Mol Cancer*. (2020) 19:88. doi: 10.1186/s12943-020-01204-7

23. Chen XY, Zhang J, Zhu JS. The role of M(6)a rna methylation in human cancer. *Mol Cancer*. (2019) 18:103. doi: 10.1186/s12943-019-1033-z

24. Sledz P, Jinek M. Structural insights into the molecular mechanism of the M(6)a writer complex. *Elife*. (2016) 5:e18434. doi: 10.7554/eLife.18434

25. Wang P, Doxtader KA, Nam Y. Structural basis for cooperative function of mettl3 and mettl14 methyltransferases. *Mol Cell*. (2016) 63:306–17. doi: 10.1016/j.molcel.2016.05.041

26. Weng H, Huang H, Wu H, Qin X, Zhao BS, Dong L, et al. Mettl14 inhibits hematopoietic stem/progenitor differentiation and promotes leukemogenesis via mrna M(6)a modification. *Cell Stem Cell*. (2018) 22:191–205.e9. doi: 10.1016/j.stem.2017.11.016

27. Liu J, Eckert MA, Harada BT, Liu SM, Lu Z, Yu K, et al. M(6)a mrna methylation regulates akt activity to promote the proliferation and tumorigenicity of endometrial cancer. *Nat Cell Biol*. (2018) 20:1074–83. doi: 10.1038/s41556-018-0174-4

28. Ma JZ, Yang F, Zhou CC, Liu F, Yuan JH, Wang F, et al. Mettl14 suppresses the metastatic potential of hepatocellular carcinoma by modulating N(6)-methyladenosine-dependent primary microrna processing. *Hepatology*. (2017) 65:529–43. doi: 10.1002/hep.28885

29. Panneerdoss S, Eedunuri VK, Yadav P, Timilsina S, Rajamanickam S, Viswanadhapalli S, et al. Cross-talk among writers, readers, and erasers of M(6)a regulates cancer growth and progression. *Sci Adv*. (2018) 4:eaar8263. doi: 10.1126/sciadv.aar8263

30. Jia GX, Lin Z, Yan RG, Wang GW, Zhang XN, Li C, et al. Wtap function in sertoli cells is essential for sustaining the spermatogonial stem cell niche. *Stem Cell Rep*. (2020) 15:968–82. doi: 10.1016/j.stemcr.2020.09.001

31. Bansal H, Yihua Q, Iyer SP, Ganapathy S, Proia DA, Penalva LO, et al. Wtap is a novel oncogenic protein in acute myeloid leukemia. *Leukemia*. (2014) 28:1171–4. doi: 10.1038/leu.2014.16

32. Wu LS, Qian JY, Wang M, Yang H. Identifying the role of wilms tumor 1 associated protein in cancer prediction using integrative genomic analyses. *Mol Med Rep*. (2016) 14:2823–31. doi: 10.3892/mmr.2016.5528

33. Barbolina MV, Adley BP, Shea LD, Stack MS. Wilms tumor gene protein 1 is associated with ovarian cancer metastasis and modulates cell invasion. *Cancer*. (2008) 112:1632–41. doi: 10.1002/cncr.23341

34. Li Q, Wang C, Dong W, Su Y, Ma Z. Wtap facilitates progression of endometrial cancer via cav-1/nf-Kb axis. *Cell Biol Int*. (2021) 45:1269–77. doi: 10.1002/cbin.11570

35. Li Y, Ge YZ, Xu L, Xu Z, Dou Q, Jia R. The potential roles of rna N6-methyladenosine in urological tumors. *Front Cell Dev Biol*. (2020) 8:579919. doi: 10.3389/fcell.2020.579919

36. Ruszkowska A. Mettl16, methyltransferase-like protein 16: current insights into structure and function. *Int J Mol Sci*. (2021) 22:2176. doi: 10.3390/ijms22042176

37. Satterwhite ER, Mansfield KD. Rna methyltransferase mettl16: targets and function. *Wiley Interdiscip Rev RNA*. (2022) 13:e1681. doi: 10.1002/wrna.1681

38. Mendel M, Chen KM, Homolka D, Gos P, Pandey RR, McCarthy AA, et al. Methylation of structured rna by the M(6)a writer mettl16 is essential for mouse embryonic development. *Mol Cell*. (2018) 71:986–1000.e11. doi: 10.1016/j.molcel.2018.08.004

39. Zeng X, Zhao F, Cui G, Zhang Y, Deshpande RA, Chen Y, et al. Mettl16 antagonizes mre11-mediated DNA end resection and confers synthetic lethality to parp inhibition in pancreatic ductal adenocarcinoma. *Nat Cancer*. (2022) 3:1088–104. doi: 10.1038/s43018-022-00429-3

40. Su R, Dong L, Li Y, Gao M, He PC, Liu W, et al. Mettl16 exerts an M(6)a-independent function to facilitate translation and tumorigenesis. *Nat Cell Biol*. (2022) 24:205–16. doi: 10.1038/s41556-021-00835-2

41. Wang XK, Zhang YW, Wang CM, Li B, Zhang TZ, Zhou WJ, et al. Mettl16 promotes cell proliferation by up-regulating cyclin D1 expression in gastric cancer. *J Cell Mol Med*. (2021) 25:6602–17. doi: 10.1111/jcmm.16664

42. Dai YZ, Liu YD, Li J, Chen MT, Huang M, Wang F, et al. Mettl16 promotes hepatocellular carcinoma progression through downregulating rab11b-as1 in an M(6)a-dependent manner. *Cell Mol Biol Lett*. (2022) 27:41. doi: 10.1186/s11658-022-00342-8

43. Han L, Dong L, Leung K, Zhao Z, Li Y, Gao L, et al. Mettl16 drives leukemogenesis and leukemia stem cell self-renewal by reprogramming bcaa metabolism. *Cell Stem Cell*. (2023) 30:52–68.e13. doi: 10.1016/j.stem.2022.12.006

44. Fedeles BI, Singh V, Delaney JC, Li D, Essigmann JM. The alkb family of fe(Ii)/alpha-ketoglutarate-dependent dioxygenases: repairing nucleic acid alkylation damage and beyond. *J Biol Chem*. (2015) 290:20734–42. doi: 10.1074/jbc.R115.656462

45. Yang Z, Yu GL, Zhu X, Peng TH, Lv YC. Critical roles of fto-mediated mrna M6a demethylation in regulating adipogenesis and lipid metabolism: implications in lipid metabolic disorders. *Genes Dis*. (2022) 9:51–61. doi: 10.1016/j.gendis.2021.01.005

46. Zarza-Rebollo JA, Molina E, Rivera M. The role of the fto gene in the relationship between depression and obesity. A systematic review. *Neurosci Biobehav Rev*. (2021) 127:630–7. doi: 10.1016/j.neubiorev.2021.05.013

47. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, et al. N6-methyladenosine in nuclear rna is a major substrate of the obesity-associated fto. *Nat Chem Biol*. (2011) 7:885–7. doi: 10.1038/nchembio.687

48. Linder B, Grozhik AV, Olarerin-George AO, Meydan C, Mason CE, Jaffrey SR. Single-nucleotide-resolution mapping of M6a and M6am throughout the transcriptome. *Nat Methods*. (2015) 12:767–72. doi: 10.1038/nmeth.3453

49. Wei J, Liu F, Lu Z, Fei Q, Ai Y, He PC, et al. Differential M(6)a, M(6)a(M), and M(1)a demethylation mediated by fto in the cell nucleus and cytoplasm. *Mol Cell*. (2018) 71:973–85.e5. doi: 10.1016/j.molcel.2018.08.011

50. Zhang X, Wei LH, Wang Y, Xiao Y, Liu J, Zhang W, et al. Structural insights into fto's catalytic mechanism for the demethylation of multiple rna substrates. *Proc Natl Acad Sci U S A*. (2019) 116:2919–24. doi: 10.1073/pnas.1820574116

51. Zou D, Dong L, Li C, Yin Z, Rao S, Zhou Q. The M(6)a eraser fto facilitates proliferation and migration of human cervical cancer cells. *Cancer Cell Int*. (2019) 19:321. doi: 10.1186/s12935-019-1045-1

52. Wang T, Li W, Ye B, Zhang S, Lei X, Zhang D. Fto-stabilized lncrna hoxc13-as epigenetically upregulated fzd6 and activated wnt/B-catenin signaling to drive cervical cancer proliferation, invasion, and emt. *J buon*. (2021) 26:1279–91.

53. Qu J, Hou Y, Chen Q, Chen J, Li Y, Zhang E, et al. Rna demethylase alkbh5 promotes tumorigenesis in multiple myeloma via traf1-mediated activation of nf-kappab and mapk signaling pathways. *Oncogene*. (2022) 41:400–13. doi: 10.1038/s41388-021-02095-8

54. He Y, Yue H, Cheng Y, Ding Z, Xu Z, Lv C, et al. Alkbh5-mediated M(6)a demethylation of kcnk15-as1 inhibits pancreatic cancer progression via regulating kcnk15 and pten/akt signaling. *Cell Death Dis*. (2021) 12:1121. doi: 10.1038/s41419-021-04401-4

55. Jiang Y, Wan Y, Gong M, Zhou S, Qiu J, Cheng W. Rna demethylase alkbh5 promotes ovarian carcinogenesis in a simulated tumour microenvironment through stimulating nf-Kb pathway. *J Cell Mol Med*. (2020) 24:6137–48. doi: 10.1111/jcmm.15228

56. Zhang C, Samanta D, Lu H, Bullen JW, Zhang H, Chen I, et al. Hypoxia induces the breast cancer stem cell phenotype by hif-dependent and alkbh5-mediated M^6a-demethylation of nanog mrna. *Proc Natl Acad Sci U S A*. (2016) 113:E2047–56. doi: 10.1073/pnas.1602883113

57. Zhang S, Zhao BS, Zhou A, Lin K, Zheng S, Lu Z, et al. M(6)a demethylase alkbh5 maintains tumorigenicity of glioblastoma stem-like cells by sustaining foxm1 expression and cell proliferation program. *Cancer Cell*. (2017) 31:591–606.e6. doi: 10.1016/j.ccell.2017.02.013

58. Alarcon CR, Goodarzi H, Lee H, Liu X, Tavazoie S, Tavazoie SF. Hnrnpa2b1 is a mediator of M(6)a-dependent nuclear rna processing events. *Cell*. (2015) 162:1299–308. doi: 10.1016/j.cell.2015.08.011

59. Huang H, Weng H, Sun W, Qin X, Shi H, Wu H, et al. Recognition of rna N(6)-methyladenosine by igf2bp proteins enhances mrna stability and translation. *Nat Cell Biol*. (2018) 20:285–95. doi: 10.1038/s41556-018-0045-z

60. Chen Z, Zhong X, Xia M, Zhong J. The roles and mechanisms of the M6a reader protein ythdf1 in tumor biology and human diseases. *Mol Ther Nucleic Acids*. (2021) 26:1270–9. doi: 10.1016/j.omtn.2021.10.023

61. Shi H, Wang X, Lu Z, Zhao BS, Ma H, Hsu PJ, et al. Ythdf3 facilitates translation and decay of N(6)-methyladenosine-modified rna. *Cell Res*. (2017) 27:315–28. doi: 10.1038/cr.2017.15

62. Li A, Chen YS, Ping XL, Yang X, Xiao W, Yang Y, et al. Cytoplasmic M(6)a reader ythdf3 promotes mrna translation. *Cell Res*. (2017) 27:444–7. doi: 10.1038/cr.2017.10

63. Li J, Wu L, Pei M, Zhang Y. Ythdf2, a protein repressed by mir-145, regulates proliferation, apoptosis, and migration in ovarian cancer cells. *J Ovarian Res*. (2020) 13:111. doi: 10.1186/s13048-020-00717-5

64. Li J, Meng S, Xu M, Wang S, He L, Xu X, et al. Downregulation of N(6)-methyladenosine binding ythdf2 protein mediated by mir-493-3p suppresses prostate cancer by elevating N(6)-methyladenosine levels. *Oncotarget*. (2018) 9:3752–64. doi: 10.18632/oncotarget.23365

65. Li Y, Lu T, Wang J, Zhuo Z, Miao L, Yang Z, et al. Ythdc1 gene polymorphisms and neuroblastoma susceptibility in chinese children. *Aging (Albany NY)*. (2021) 13:25426–39. doi: 10.18632/aging.203760

66. Roundtree IA, Luo GZ, Zhang Z, Wang X, Zhou T, Cui Y, et al. Ythdc1 mediates nuclear export of N(6)-Methyladenosine methylated mrnas. *Elife*. (2017), 6. doi: 10.7554/eLife.31311

67. Widagdo J, Anggono V, Wong JJ. The multifaceted effects of ythdc1-mediated nuclear M(6)a recognition. *Trends Genet*. (2022) 38:325–32. doi: 10.1016/j.tig.2021.11.005

68. Liu R, Kasowitz SD, Homolka D, Leu NA, Shaked JT, Ruthel G, et al. Ythdc2 is essential for pachytene progression and prevents aberrant microtubule-driven telomere clustering in male meiosis. *Cell Rep*. (2021) 37:110110. doi: 10.1016/j.celrep.2021.110110

69. Hsu PJ, Zhu Y, Ma H, Guo Y, Shi X, Liu Y, et al. Ythdc2 is an N(6)-methyladenosine binding protein that regulates mammalian spermatogenesis. *Cell Res*. (2017) 27:1115–27. doi: 10.1038/cr.2017.99

70. Zhou KI, Shi H, Lyu R, Wylder AC, Matuszek Z, Pan JN, et al. Regulation of co-transcriptional pre-mrna splicing by M(6)a through the low-complexity protein hnrnpg. *Mol Cell*. (2019) 76:70–81 e9. doi: 10.1016/j.molcel.2019.07.005

71. Jiang F, Tang X, Tang C, Hua Z, Ke M, Wang C, et al. Hnrnpa2b1 promotes multiple myeloma progression by increasing akt3 expression via M6a-dependent stabilization of ilf3 mrna. *J Hematol Oncol*. (2021) 14:54. doi: 10.1186/s13045-021-01066-6

72. Korn SM, Ulshofer CJ, Schneider T, Schlundt A. Structures and target rna preferences of the rna-binding protein family of igf2bps: an overview. *Structure*. (2021) 29:787–803. doi: 10.1016/j.str.2021.05.001

73. Nielsen J, Lykke-Andersen J, Wewer UM. A family of insulin-like growth factor ii mrna-binding proteins represses translation in late development. *Mol Cell Biol*. (1999) 19:1262–70. doi: 10.1128/MCB.19.2.1262

74. Degrauwe N, Suvà ML, Janiszewska M, Riggi N, Stamenkovic I. Imps: an rna-binding protein family that provides a link between stem cell maintenance in normal development and cancer. *Genes Dev*. (2016) 30:2459–74. doi: 10.1101/gad.287540.116

75. Edens BM, Vissers C, Su J, Arumugam S, Xu Z, Shi H, et al. Fmrp modulates neural differentiation through M(6)a-dependent mrna nuclear export. *Cell Rep*. (2019) 28:845–54 e5. doi: 10.1016/j.celrep.2019.06.072

76. Hsu PJ, Shi H, Zhu AC, Lu Z, Miller N, Edens BM, et al. The rna-binding protein fmrp facilitates the nuclear export of N(6)-methyladenosine-containing mrnas. *J Biol Chem*. (2019) 294:19889–95. doi: 10.1074/jbc.AC119.010078

77. Bourgeois CF, Mortreux F, Auboeuf D. The multiple functions of rna helicases as drivers and regulators of gene expression. *Nat Rev Mol Cell Biol*. (2016) 17:426–38. doi: 10.1038/nrm.2016.50

78. Ianniello Z, Sorci M, Ceci Ginistrelli L, Iaiza A, Marchioni M, Tito C, et al. New insight into the catalytic -dependent and -independent roles of mettl3 in sustaining aberrant translation in chronic myeloid leukemia. *Cell Death Dis*. (2021) 12:870. doi: 10.1038/s41419-021-04169-7

79. Mao Y, Dong L, Liu XM, Guo J, Ma H, Shen B, et al. M(6)a in mrna coding regions promotes translation via the rna helicase-containing ythdc2. *Nat Commun*. (2019) 10:5332. doi: 10.1038/s41467-019-13317-9

80. Chang G, Shi L, Ye Y, Shi H, Zeng L, Tiwary S, et al. Ythdf3 induces the translation of M(6)a-enriched gene transcripts to promote breast cancer brain metastasis. *Cancer Cell*. (2020) 38:857–71 e7. doi: 10.1016/j.ccell.2020.10.004

81. Liu T, Wei Q, Jin J, Luo Q, Liu Y, Yang Y, et al. The M6a reader ythdf1 promotes ovarian cancer progression via augmenting eif3c translation. *Nucleic Acids Res*. (2020) 48:3816–31. doi: 10.1093/nar/gkaa048

82. Orouji E, Peitsch WK, Orouji A, Houben R, Utikal J. Oncogenic role of an epigenetic reader of M(6)a rna modification: ythdf1 in merkel cell carcinoma. *Cancers (Basel)*. (2020) 12:202. doi: 10.3390/cancers12010202

83. Kretschmer J, Hackert P, Sloan KE, Bohnsack MT. The M(6)a reader protein ythdc2 interacts with the small ribosomal subunit and the 5 '-3 ' Exoribonuclease xrn1. *RNA*. (2018) 24:1339–50. doi: 10.1261/rna.064238.117

84. Wojtas MN, Pandey RR, Mendel M, Homolka D, Sachidanandam R, Pillai RS. Regulation of M(6)a transcripts by the 3'–>5' Rna helicase ythdc2 is essential for a successful meiotic program in the mammalian germline. *Mol Cell*. (2017) 68:374–87 e12. doi: 10.1016/j.molcel.2017.09.021

85. Geula S, Moshitch-Moshkovitz S, Mansour AA, Kol N, Hershkovitz V, Peer E, et al. M6a mrna methylation facilitates resolution of naive pluripotency toward differentiation. *Science*. (2015) 347:1002–6. doi: 10.1126/science.1261417

86. Edupuganti RR, Geiger S, Lindeboom RGH, Shi H, Hsu PJ, Lu Z, et al. N(6)-methyladenosine (M(6)a) recruits and repels proteins to regulate mrna homeostasis. *Nat Struct Mol Biol*. (2017) 24:870–8. doi: 10.1038/nsmb.3462

87. Zhang F, Kang Y, Wang M, Li Y, Xu T, Yang W, et al. Fragile X mental retardation protein modulates the stability of its M6a-marked messenger rna targets. *Hum Mol Genet*. (2018) 27:3936–50. doi: 10.1093/hmg/ddy292

88. Huang H, Weng H, Sun W, Qin X, Shi H, Wu H, et al. Publisher correction: recognition of rna N(6)-methyladenosine by igf2bp proteins enhances mrna stability and translation. *Nat Cell Biol*. (2020) 22:1288. doi: 10.1038/s41556-020-00580-y

89. Luo Y, Na Z, Slavoff SA. P-bodies: Composition, Properties, and Functions. *Biochemistry*. (2018) 57:2424–31. doi: 10.1021/acs.biochem.7b01162

90. Zheng D, Ezzeddine N, Chen CY, Zhu W, He X, Shyu AB. Deadenylation is prerequisite for P-body formation and mrna decay in mammalian cells. *J Cell Biol*. (2008) 182:89–101. doi: 10.1083/jcb.200801196

91. Su R, Dong L, Li C, Nachtergaele S, Wunderlich M, Qing Y, et al. R-2hg exhibits anti-tumor activity by targeting fto/M(6)a/myc/cebpa signaling. *Cell*. (2018) 172:90–105 e23. doi: 10.1016/j.cell.2017.11.031

92. Moor RM, Dai Y, Lee C, Fulka J Jr. Oocyte maturation and embryonic failure. *Hum Reprod Update*. (1998) 4:223–36. doi: 10.1093/humupd/4.3.223

93. Gindi N, Grossman H, Bar-Joseph H, Miller I, Nemerovsky L, Hadas R, et al. Fyn and argonaute 2 participate in maternal-mrna degradation during mouse oocyte maturation. *Cell Cycle*. (2022) 21:792–804. doi: 10.1080/15384101.2022.2031427

94. Qi ST, Ma JY, Wang ZB, Guo L, Hou Y, Sun QY. N6-methyladenosine sequencing highlights the involvement of mrna methylation in oocyte meiotic maturation and embryo development by regulating translation in xenopus laevis. *J Biol Chem*. (2016) 291:23020–6. doi: 10.1074/jbc.M116.748889

95. Ivanova I, Much C, Di Giacomo M, Azzi C, Morgan M, Moreira PN, et al. The rna M(6)a reader ythdf2 is essential for the post-transcriptional regulation of the maternal transcriptome and oocyte competence. *Mol Cell*. (2017) 67:1059–67.e4. doi: 10.1016/j.molcel.2017.08.003

96. Xia H, Zhong C, Wu X, Chen J, Tao B, Xia X, et al. Mettl3 mutation disrupts gamete maturation and reduces fertility in zebrafish. *Genetics*. (2018) 208:729–43. doi: 10.1534/genetics.117.300574

97. Wu Y, Xu X, Qi M, Chen C, Li M, Yan R, et al. N(6)-methyladenosine regulates maternal rna maintenance in oocytes and timely rna decay during mouse maternal-to-zygotic transition. *Nat Cell Biol*. (2022) 24:917–27. doi: 10.1038/s41556-022-00915-x

98. Hao J, Hu H, Jiang Z, Yu X, Li C, Chen L, et al. Microrna-670 modulates igf2bp1 expression to regulate rna methylation in parthenogenetic mouse embryonic development. *Sci Rep*. (2020) 10:4782. doi: 10.1038/s41598-020-61816-3

99. Cao Z, Zhang D, Wang Y, Tong X, Avalos LFC, Khan IM, et al. Identification and functional annotation of M6a methylation modification in granulosa cells during antral follicle development in pigs. *Anim Reprod Sci*. (2020) 219:106510. doi: 10.1016/j.anireprosci.2020.106510

100. Li L, Zheng P, Dean J. Maternal control of early mouse development. *Development*. (2010) 137:859–70. doi: 10.1242/dev.039487

101. Lubzens E, Young G, Bobe J, Cerda J. Oogenesis in teleosts: how eggs are formed. *Gen Comp Endocrinol*. (2010) 165:367–89. doi: 10.1016/j.ygcen.2009.05.022

102. Yu XX, Liu YH, Liu XM, Wang PC, Liu S, Miao JK, et al. Ascorbic acid induces global epigenetic reprogramming to promote meiotic maturation and developmental competence of porcine oocytes. *Sci Rep*. (2018) 8:6132. doi: 10.1038/s41598-018-24395-y

103. Kasowitz SD, Ma J, Anderson SJ, Leu NA, Xu Y, Gregory BD, et al. Nuclear M6a reader ythdc1 regulates alternative polyadenylation and splicing during mouse oocyte development. *PloS Genet*. (2018) 14:e1007412. doi: 10.1371/journal.pgen.1007412

104. Bailey AS, Batista PJ, Gold RS, Chen YG, de Rooij DG, Chang HY, et al. The conserved rna helicase ythdc2 regulates the transition from proliferation to differentiation in the germline. *Elife*. (2017) 6:e26116. doi: 10.7554/eLife.26116

105. Zeng M, Dai X, Liang Z, Sun R, Huang S, Luo L, et al. Critical roles of mrna M(6)a modification and ythdc2 expression for meiotic initiation and progression in female germ cells. *Gene*. (2020) 753:144810. doi: 10.1016/j.gene.2020.144810

106. Zhao X, Tian GG, Fang Q, Pei X, Wang Z, Wu J. Comparison of rna M(6)a and DNA methylation profiles between mouse female germline stem cells and sto cells. *Mol Ther Nucleic Acids*. (2021) 23:431–9. doi: 10.1016/j.omtn.2020.11.020

107. Kontur C, Jeong M, Cifuentes D, Giraldez AJ. Ythdf M(6)a readers function redundantly during zebrafish development. *Cell Rep*. (2020) 33:108598. doi: 10.1016/j.celrep.2020.108598

108. Ma Z, Li Q, Liu P, Dong W, Zuo Y. Mettl3 regulates M6a in endometrioid epithelial ovarian cancer independently of mettl14 and wtap. *Cell Biol Int*. (2020) 44:2524–31. doi: 10.1002/cbin.11459

109. Hao L, Wang JM, Liu BQ, Yan J, Li C, Jiang JY, et al. M6a-ythdf1-mediated trim29 upregulation facilitates the stem cell-like phenotype of cisplatin-resistant ovarian cancer cells. *Biochim Biophys Acta Mol Cell Res*. (2021) 1868:118878. doi: 10.1016/j.bbamcr.2020.118878

110. Pendleton KE, Chen B, Liu K, Hunter OV, Xie Y, Tu BP, et al. The U6 snrna M(6)a methyltransferase mettl16 regulates sam synthetase intron retention. *Cell*. (2017) 169:824–35 e14. doi: 10.1016/j.cell.2017.05.003

111. Zou S, Toh JD, Wong KH, Gao YG, Hong W, Woon EC. N(6)-methyladenosine: A conformational marker that regulates the substrate specificity of human demethylases fto and alkbh5. *Sci Rep*. (2016) 6:25677. doi: 10.1038/srep25677

112. Galganski L, Urbanek MO, Krzyzosiak WJ. Nuclear speckles: molecular organization, biological function and role in disease. *Nucleic Acids Res*. (2017) 45:10350–68. doi: 10.1093/nar/gkx759

113. Zhao X, Yang Y, Sun BF, Shi Y, Yang X, Xiao W, et al. Fto-dependent demethylation of N6-methyladenosine regulates mrna splicing and is required for adipogenesis. *Cell Res*. (2014) 24:1403–19. doi: 10.1038/cr.2014.151

114. Liu HB, Muhammad T, Guo Y, Li MJ, Sha QQ, Zhang CX, et al. Rna-binding protein igf2bp2/imp2 is a critical maternal activator in early zygotic genome activation. *Adv Sci (Weinh)*. (2019) 6:1900295. doi: 10.1002/advs.201900295

115. Ren F, Lin Q, Gong G, Du X, Dan H, Qin W, et al. Igf2bp3 maintains maternal rna stability and ensures early embryo development in zebrafish. *Commun Biol*. (2020) 3:94. doi: 10.1038/s42003-020-0827-2

116. Barton SJ, Mosquera M, Cleal JK, Fuller AS, Crozier SR, Cooper C, et al. Relation of fto gene variants to fetal growth trajectories: findings from the southampton women's survey. *Placenta*. (2016) 38:100–6. doi: 10.1016/j.placenta.2015.12.015

117. Taniguchi K, Kawai T, Kitawaki J, Tomikawa J, Nakabayashi K, Okamura K, et al. Epitranscriptomic profiling in human placenta: N6-methyladenosine modification at the 5'-untranslated region is related to fetal growth and preeclampsia. *FASEB J*. (2020) 34:494–512. doi: 10.1096/fj.201900619RR

118. Bassols J, Prats-Puig A, Vazquez-Ruiz M, Garcia-Gonzalez MM, Martinez-Pascual M, Avelli P, et al. Placental fto expression relates to fetal growth. *Int J Obes (Lond)*. (2010) 34:1365–70. doi: 10.1038/ijo.2010.62

119. Song T, Lu J, Deng Z, Xu T, Yang Y, Wei H, et al. Maternal obesity aggravates the abnormality of porcine placenta by increasing N(6)-methyladenosine. *Int J Obes (Lond)*. (2018) 42:1812–20. doi: 10.1038/s41366-018-0113-2

120. Mor G, Aldo P, Alvero AB. The unique immunological and microbial aspects of pregnancy. *Nat Rev Immunol*. (2017) 17:469–82. doi: 10.1038/nri.2017.64

121. Tang L, Wei X, Li T, Chen Y, Dai Z, Lu C, et al. Emerging perspectives of rna N(6)-methyladenosine (M(6)a) modification on immunity and autoimmune diseases. *Front Immunol*. (2021) 12:630358. doi: 10.3389/fimmu.2021.630358

122. Wang H, Hu X, Huang M, Liu J, Gu Y, Ma L, et al. Mettl3-mediated mrna M(6)a methylation promotes dendritic cell activation. *Nat Commun*. (2019) 10:1898. doi: 10.1038/s41467-019-09903-6

123. Tong J, Wang X, Liu Y, Ren X, Wang A, Chen Z, et al. Pooled crispr screening identifies M(6)a as a positive regulator of macrophage activation. *Sci Adv*. (2021) 7: eabd4742. doi: 10.1126/sciadv.abd4742

124. Dong L, Chen C, Zhang Y, Guo P, Wang Z, Li J, et al. The loss of rna N(6)-adenosine methyltransferase mettl14 in tumor-associated macrophages promotes cd8 (+) T cell dysfunction and tumor growth. *Cancer Cell*. (2021) 39:945–57 e10. doi: 10.1016/j.ccell.2021.04.016

125. Ma S, Yan J, Barr T, Zhang J, Chen Z, Wang LS, et al. The rna M6a reader ythdf2 controls nk cell antitumor and antiviral immunity. *J Exp Med*. (2021) 218: e20210279. doi: 10.1084/jem.20210279

126. Huang H, Weng H, Chen J. M(6)a modification in coding and non-coding rnas: roles and therapeutic implications in cancer. *Cancer Cell*. (2020) 37:270–88. doi: 10.1016/j.ccell.2020.02.004

127. Deng X, Su R, Weng H, Huang H, Li Z, Chen J. Rna N(6)-methyladenosine modification in cancers: current status and perspectives. *Cell Res*. (2018) 28:507–17. doi: 10.1038/s41422-018-0034-6

128. Dong S, Wu Y, Liu Y, Weng H, Huang H. N(6) -methyladenosine steers rna metabolism and regulation in cancer. *Cancer Commun (Lond)*. (2021) 41:538–59. doi: 10.1002/cac2.12161

129. Yang Y, Hsu PJ, Chen YS, Yang YG. Dynamic transcriptomic M(6)a decoration: writers, erasers, readers and functions in rna metabolism. *Cell Res*. (2018) 28:616–24. doi: 10.1038/s41422-018-0040-8

130. Shi H, Wei J, He C. Where, when, and how: context-dependent functions of rna methylation writers, readers, and erasers. *Mol Cell*. (2019) 74:640–50. doi: 10.1016/j.molcel.2019.04.025

131. Li Q, Li X, Tang H, Jiang B, Dou Y, Gorospe M, et al. Nsun2-mediated M5c methylation and mettl3/mettl14-mediated M6a methylation cooperatively enhance P21 translation. *J Cell Biochem*. (2017) 118:2587–98. doi: 10.1002/jcb.25957

132. Ding C, Zou Q, Ding J, Ling M, Wang W, Li H, et al. Increased N6-methyladenosine causes infertility is associated with fto expression. *J Cell Physiol*. (2018) 233:7055–66. doi: 10.1002/jcp.26507

133. Tang Z, Ju Y, Dai X, Ni N, Liu Y, Zhang D, et al. Ho-1-mediated ferroptosis as a target for protection against retinal pigment epithelium degeneration. *Redox Biol*. (2021) 43:101971. doi: 10.1016/j.redox.2021.101971

134. Gu Y, Chu X, Morgan JA, Lewis DF, Wang Y. Upregulation of mettl3 expression and M6a rna methylation in placental trophoblasts in preeclampsia. *Placenta*. (2021) 103:43–9. doi: 10.1016/j.placenta.2020.10.016

135. Li R, Qiu X, He M, Qiao J, He J, Zhong M. Retracted article: mettl3-mediated mature mir-497-5p/195-5p inhibits trophoblast migration and invasion by targeting wwp1 in preeclampsia. *Cell Cycle*. (2022) 21:iii–xviii. doi: 10.1080/15384101.2021.1982527

136. Zhang Y, Yang H, Long Y, Zhang Y, Chen R, Shi J, et al. Circrna N6-methyladenosine methylation in preeclampsia and the potential role of N6-methyladenosine-modified circpappa2 in trophoblast invasion. *Sci Rep*. (2021) 11:24357. doi: 10.1038/s41598-021-03662-5

137. Fan W, Zhou W, Yan Q, Peng Y, Wang H, Kong C, et al. Upregulation of mettl14 contributes to trophoblast dysfunction by elevating foxo3a expression in an M (6)a-dependent manner. *Placenta*. (2022) 124:18–27. doi: 10.1016/j.placenta.2022.05.008

138. Guo Y, Song W, Yang Y. Inhibition of alkbh5-mediated M(6) a modification of pparg mrna alleviates H/R-induced oxidative stress and apoptosis in placenta trophoblast. *Environ Toxicol*. (2022) 37:910–24. doi: 10.1002/tox.23454

139. Yang Q, Ma Y, Liu Y, Shao X, Jia W, Yu X, et al. Mnsfbeta regulates placental development by conjugating igf2bp2 to enhance trophoblast cell invasiveness. *Cell Prolif*. (2021) 54:e13145. doi: 10.1111/cpr.13145

140. Wang D, Guan H, Xia Y. Ythdc1 maintains trophoblasts function by promoting degradation of M6a-modified circmpp1. *Biochem Pharmacol*. (2023) 210:115456. doi: 10.1016/j.bcp.2023.115456

141. Zheng Q, Gan H, Yang F, Yao Y, Hao F, Hong L, et al. Cytoplasmic M(1)a reader ythdf3 inhibits trophoblast invasion by downregulation of M(1)a-methylated igf1r. *Cell Discov*. (2020) 6:12. doi: 10.1038/s41421-020-0144-4

142. Qin X, Chen Y, Chen S, Liu X, Zeng W, Tian F, et al. Plasmacytoma variant translocation 1 (Pvt1) regulates trophoblast viability, proliferation, and migration and is downregulated in spontaneous abortion. *Am J Reprod Immunol*. (2019) 81:e13048. doi: 10.1111/aji.13048

143. Huppertz B. Traditional and new routes of trophoblast invasion and their implications for pregnancy diseases. *Int J Mol Sci*. (2019) 21:289. doi: 10.3390/ijms21010289

144. Li XC, Jin F, Wang BY, Yin XJ, Hong W, Tian FJ. The M6a demethylase alkbh5 controls trophoblast invasion at the maternal-fetal interface by regulating the stability of cyr61 mrna. *Theranostics*. (2019) 9:3853–65. doi: 10.7150/thno.31868

145. Zhang J, Qiu Q, Wang H, Chen C, Luo D. Trim46 contributes to high glucose-induced ferroptosis and cell growth inhibition in human retinal capillary endothelial cells by facilitating gpx4 ubiquitination. *Exp Cell Res*. (2021) 407:112800. doi: 10.1016/j.yexcr.2021.112800

146. She J, Tan K, Liu J, Cao S, Li Z, Peng Y, et al. The alteration of M(6)a modification at the transcriptome-wide level in human villi during spontaneous abortion in the first trimester. *Front Genet*. (2022) 13:861853. doi: 10.3389/fgene.2022.861853

147. Chapron C, Marcellin L, Borghese B, Santulli P. Rethinking mechanisms, diagnosis and management of endometriosis. *Nat Rev Endocrinol*. (2019) 15:666–82. doi: 10.1038/s41574-019-0245-z

148. Giudice LC, Kao LC. Endometriosis. *Lancet*. (2004) 364:1789–99. doi: 10.1016/S0140-6736(04)17403-5

149. Vercellini P, Vigano P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nat Rev Endocrinol*. (2014) 10:261–75. doi: 10.1038/nrendo.2013.255

150. Jiang L, Wu J, Wang S, Yang X, Yi M, Zhang X, et al. Exploring diagnostic M6a regulators in endometriosis. *Aging (Albany NY)*. (2020) 12:25916–38. doi: 10.18632/aging.202163

151. Li X, Xiong W, Long X, Dai X, Peng Y, Xu Y, et al. Inhibition of mettl3/M6a/mir126 promotes the migration and invasion of endometrial stromal cells in endometriosisdagger. *Biol Reprod*. (2021) 105:1221–33. doi: 10.1093/biolre/ioab152

152. Szubert M, Koziróg E, Olszak O, Krygier-Kurz K, Kazmierczak J, Wilczynski J. Adenomyosis and infertility—Review of medical and surgical approaches. *Int J Environ Res Public Health*. (2021) 18:1235. doi: 10.3390/ijerph18031235

153. Bulun SE, Yildiz S, Adli M, Wei J-J. Adenomyosis pathogenesis: insights from next-generation sequencing. *Hum Reprod Update*. (2021) 27:1086–97. doi: 10.1093/humupd/dmab017

154. Cunningham RK, Horrow MM, Smith RJ, Springer J. Adenomyosis: A sonographic diagnosis. *RadioGraphics*. (2018) 38:1576–89. doi: 10.1148/rg.2018180080

155. Zhai J, Li S, Sen S, Opoku-Anane J, Du Y, Chen ZJ, et al. M(6)a rna methylation regulators contribute to eutopic endometrium and myometrium dysfunction in adenomyosis. *Front Genet*. (2020) 11:716. doi: 10.3389/fgene.2020.00716

156. Wang Q, Wei Y, Zhang J. Combined knockdown of D-dopachrome tautomerase and migration inhibitory factor inhibits the proliferation, migration, and invasion in human cervical cancer. *Int J Gynecol Cancer*. (2017) 27:634–42. doi: 10.1097/IGC.0000000000000951

157. Jeanes YM, Reeves S. Metabolic consequences of obesity and insulin resistance in polycystic ovary syndrome: diagnostic and methodological challenges. *Nutr Res Rev*. (2017) 30:97–105. doi: 10.1017/S0954422416000287

158. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. *Nat Rev Endocrinol*. (2018) 14:270–84. doi: 10.1038/nrendo.2018.24

159. Schulte MM, Tsai JH, Moley KH. Obesity and pcos: the effect of metabolic derangements on endometrial receptivity at the time of implantation. *Reprod Sci*. (2015) 22:6–14. doi: 10.1177/1933719114561552

160. Snider AP, Wood JR. Obesity induces ovarian inflammation and reduces oocyte quality. *Reproduction*. (2019) 158:R79–R90. doi: 10.1530/rep

161. Lin Z, Niu Y, Wan A, Chen D, Liang H, Chen X, et al. Rna M(6) a methylation regulates sorafenib resistance in liver cancer through foxo3-mediated autophagy. *EMBO J*. (2020) 39:e103181. doi: 10.15252/embj.2019103181

162. Zhou L, Han X, Li W, Wang N, Yao L, Zhao Y, et al. N6-methyladenosine demethylase fto induces the dysfunctions of ovarian granulosa cells by upregulating flotillin 2. *Reprod Sci*. (2022) 29:1305–15. doi: 10.1007/s43032-021-00664-6

163. Morgan S, Anderson RA, Gourley C, Wallace WH, Spears N. How do chemotherapeutic agents damage the ovary? *Hum Reprod Update*. (2012) 18:525–35. doi: 10.1093/humupd/dms022

164. Brodie MJ, Mintzer S, Pack AM, Gidal BE, Vecht CJ, Schmidt D. Enzyme induction with antiepileptic drugs: cause for concern? *Epilepsia*. (2013) 54:11–27. doi: 10.1111/j.1528-1167.2012.03671.x

165. Blumenfeld Z. Preservation of fertility and ovarian function and minimalization of chemotherapy associated gonadotoxicity and premature. *Mol Cell Endocrinol*. (2002) 187:93–105. doi: 10.1016/S0303-7207(01)00712-2

166. Huang B, Ding C, Zou Q, Wang W, Li H. Cyclophosphamide regulates N6-methyladenosine and M6a rna enzyme levels in human granulosa cells and in ovaries of a premature ovarian aging mouse model. *Front Endocrinol (Lausanne)*. (2019) 10:415. doi: 10.3389/fendo.2019.00415

167. Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, et al. Alkbh5 is a mammalian rna demethylase that impacts rna metabolism and mouse fertility. *Mol Cell*. (2013) 49:18–29. doi: 10.1016/j.molcel.2012.10.015

168. Zhao J, Lu L. Interplay between rna methylation eraser fto and writer mettl3 in renal clear cell carcinoma patient survival. *Recent Pat Anticancer Drug Discov*. (2021) 16:363–76. doi: 10.2174/1574892816666210204125155

169. Colombo N, Creutzberg C, Amant F, Bosse T, Gonzalez-Martin A, Ledermann J, et al. Esmo-esgo-estro consensus conference on endometrial cancer: diagnosis, treatment and follow-up. *Ann Oncol*. (2016) 27:16–41. doi: 10.1093/annonc/mdv484

170. Kong A, Johnson N, Kitchener HC, Lawrie TA. Adjuvant radiotherapy for stage I endometrial cancer: an updated cochrane systematic review and meta-analysis. *J Natl Cancer Inst*. (2012) 104:1625–34. doi: 10.1093/jnci/djs374

171. McMeekin DS. Where is the future of endometrial cancer therapy? *Ann Oncol*. (2009) 20:1757–61. doi: 10.1093/annonc/mdp493

172. Montejo M, Werner TL, Gaffney D. Current challenges in clinical management of endometrial cancer. *Adv Drug Delivery Rev*. (2009) 61:883–9. doi: 10.1016/j.addr.2009.04.014

173. Gu J, Bi F. Significance of N6-methyladenosine rna methylation regulators in immune infiltrates of ovarian cancer. *Front Genet*. (2021) 12:671179. doi: 10.3389/fgene.2021.671179

174. Zhang L, Wan Y, Zhang Z, Jiang Y, Gu Z, Ma X, et al. Igf2bp1 overexpression stabilizes peg10 mrna in an M6a-dependent manner and promotes endometrial cancer progression. *Theranostics*. (2021) 11:1100–14. doi: 10.7150/thno.49345

175. Nie X, Tan J. N6-methyladenosine-related lncrnas is a potential marker for predicting prognosis and immunotherapy in ovarian cancer. *Hereditas*. (2022) 159:17. doi: 10.1186/s41065-022-00222-3

176. Xue T, Liu X, Zhang M, Liu S, Zou M, Li Y, et al. Padi2-catalyzed mek1 citrullination activates erk1/2 and promotes igf2bp1-mediated sox2 mrna stability in endometrial cancer. *Adv Sci (Weinh)*. (2021) 8:2002831. doi: 10.1002/advs.202002831

177. Bhan A, Soleimani M, Mandal SS. Long noncoding rna and cancer: A new paradigm. *Cancer Res*. (2017) 77:3965–81. doi: 10.1158/0008-5472.CAN-16-2634

178. Zhang C, Liu J, Guo H, Hong D, Ji J, Zhang Q, et al. M6a rna methylation regulators were associated with the Malignancy and prognosis of ovarian cancer. *Bioengineered*. (2021) 12:3159–76. doi: 10.1080/21655979.2021.1946305

179. Chen G, Liu B, Yin S, Li S, Guo Y, Wang M, et al. Hypoxia induces an endometrial cancer stem-like cell phenotype via hif-dependent demethylation of sox2 mrna. *Oncogenesis*. (2020) 9:81. doi: 10.1038/s41389-020-00265-z

180. Li Q, Ren CC, Chen YN, Yang L, Zhang F, Wang BJ, et al. A risk score model incorporating three M6a rna methylation regulators and a related network of mirnas-M6a regulators-M6a target genes to predict the prognosis of patients with ovarian cancer. *Front Cell Dev Biol*. (2021) 9:703969. doi: 10.3389/fcell.2021.703969

181. Pu X, Gu Z, Gu Z. Alkbh5 regulates igf1r expression to promote the proliferation and tumorigenicity of endometrial cancer. *J Cancer*. (2020) 11:5612–22. doi: 10.7150/jca.46097

182. Zhang L, Wan Y, Zhang Z, Jiang Y, Lang J, Cheng W, et al. Fto demethylates M6a modifications in hoxb13 mrna and promotes endometrial cancer metastasis by activating the wnt signalling pathway. *RNA Biol.* (2021) 18:1265–78. doi: 10.1080/15476286.2020.1841458

183. Fan L, Lin Y, Lei H, Shu G, He L, Yan Z, et al. A newly defined risk signature, consisting of three M(6)a rna methylation regulators, predicts the. *Aging (Albany NY).* (2020) 12:18453–75. doi: 10.2139/ssrn.3514606

184. Zhu W, Zhao L, Kong B, Liu Y, Zou X, Han T, et al. The methylation modification of M6a regulators contributes to the prognosis of ovarian cancer. *Ann Transl Med.* (2022) 10:59. doi: 10.21037/atm-21-6462

185. Jiao J, Jiang L, Luo Y. N6-methyladenosine-related rna signature predicting the prognosis of ovarian cancer. *Recent Pat Anticancer Drug Discov.* (2021) 16:407–16. doi: 10.2174/1574892816666210615164645

186. Wei Q, Yang D, Liu X, Zhao H, Yang Y, Xu J, et al. Exploration of the role of M (6) a rna methylation regulators in Malignant progression and clinical prognosis of ovarian cancer. *Front Genet.* (2021) 12:650554. doi: 10.3389/fgene.2021.650554

187. Liu Y, Li L, Li Y, Zhao X. Research progress on tumor-associated macrophages and inflammation in cervical cancer. *BioMed Res Int.* (2020) 2020:6842963. doi: 10.1155/2020/6842963

188. Ferrall L, Lin KY, Roden RBS, Hung CF, Wu TC. Cervical cancer immunotherapy: facts and hopes. *Clin Cancer Res.* (2021) 27:4953–73. doi: 10.1158/1078-0432.CCR-20-2833

189. Tewari KS, Monk BJ. New strategies in advanced cervical cancer: from angiogenesis blockade to immunotherapy. *Clin Cancer Res.* (2014) 20:5349–58. doi: 10.1158/1078-0432.Ccr-14-1099

190. Pan J, Xu L, Pan H. Development and validation of an M6a rna methylation regulator-based signature for prognostic prediction in cervical squamous cell carcinoma. *Front Oncol.* (2020) 10:1444. doi: 10.3389/fonc.2020.01444

191. Wang Q, Guo X, Li L, Gao Z, Su X, Ji M, et al. N(6)-methyladenosine mettl3 promotes cervical cancer tumorigenesis and warburg effect through ythdf1/hk2 modification. *Cell Death Dis.* (2020) 11:911. doi: 10.1038/s41419-020-03071-y

192. Li Z, Peng Y, Li J, Chen Z, Chen F, Tu J, et al. N(6)-methyladenosine regulates glycolysis of cancer cells through pdk4. *Nat Commun.* (2020) 11:2578. doi: 10.1038/s41467-020-16306-5

193. Huang C, Liang J, Lin S, Wang D, Xie Q, Lin Z, et al. N(6)-methyladenosine associated silencing of mir-193b promotes cervical cancer aggressiveness by targeting ccnd1. *Front Oncol.* (2021) 11:666597. doi: 10.3389/fonc.2021.666597

194. Ni HH, Zhang L, Huang H, Dai SQ, Li J. Connecting mettl3 and intratumoural cd33(+) mdscs in predicting clinical outcome in cervical cancer. *J Transl Med.* (2020) 18:393. doi: 10.1186/s12967-020-02553-z

195. Ji F, Lu Y, Chen S, Lin X, Yu Y, Zhu Y, et al. M(6)a methyltransferase mettl3-mediated lncrna foxd2-as1 promotes the tumorigenesis of cervical cancer. *Mol Ther Oncolytics.* (2021) 22:574–81. doi: 10.1016/j.omto.2021.07.004

196. Zheng H, Zheng WJ, Wang ZG, Tao YP, Huang ZP, Yang L, et al. Decreased expression of programmed death ligand-L1 by seven in absentia homolog 2 in cholangiocarcinoma enhances T-cell-mediated antitumor activity. *Front Immunol.* (2022) 13:845193. doi: 10.3389/fimmu.2022.845193

197. Wang H, Luo Q, Kang J, Wei Q, Yang Y, Yang D, et al. Ythdf1 Aggravates the Progression of Cervical Cancer through M(6)a-Mediated up-Regulation of Ranbp2. *Front Oncol.* (2021) 11:650383. doi: 10.3389/fonc.2021.650383

198. Wangm T, Ye B, Zhang S, Lei X, Zhang D. Fto-stabilized lncrna hoxc13-as epigenetically upregulated. *J BUON.* (2021) 26:1279–91.

199. Zhou S, Bai ZL, Xia D, Zhao ZJ, Zhao R, Wang YY, et al. Fto regulates the chemo-radiotherapy resistance of cervical squamous cell carcinoma (Cscc) by targeting beta-catenin through mrna demethylation. *Mol Carcinog.* (2018) 57:590–7. doi: 10.1002/mc.22782

200. Ji F, Lu Y, Chen S, Yu Y, Lin X, Zhu Y, et al. Igf2bp2-modified circular rna circarhgap12 promotes cervical cancer progression by interacting M(6)a/foxm1 manner. *Cell Death Discov.* (2021) 7:215. doi: 10.1038/s41420-021-00595-w

201. Zhang Y, Wang D, Wu D, Zhang D, Sun M. Long noncoding rna kcnmb2-as1 stabilized by N(6)-methyladenosine modification promotes cervical cancer growth through acting as a competing endogenous rna. *Cell Transplant.* (2020) 29:963689720964382. doi: 10.1177/0963689720964382

202. Govindarajan M, Wohlmuth C, Waas M, Bernardini MQ, Kislinger T. High-throughput approaches for precision medicine in high-grade serous ovarian cancer. *J Hematol Oncol.* (2020) 13:134. doi: 10.1186/s13045-020-00971-6

203. Arora T MS, Lekkala MR. *Ovarian Cancer*. Treasure Island (FL: StatPearls Publishing Copyright (2022).

204. Nameki R, Chang H, Reddy J, Corona RI, Lawrenson K. Transcription factors in epithelial ovarian cancer: histotype-specific drivers and novel therapeutic targets. *Pharmacol Ther.* (2021) 220:107722. doi: 10.1016/j.pharmthera.2020.107722

205. Baert T, Ferrero A, Sehouli J, O'Donnell DM, Gonzalez-Martin A, Joly F, et al. The systemic treatment of recurrent ovarian cancer revisited. *Ann Oncol.* (2021) 32:710–25. doi: 10.1016/j.annonc.2021.02.015

206. Wang Z, Meng F, Zhong Z. Emerging targeted drug delivery strategies toward ovarian cancer. *Advanced Drug Delivery Rev.* (2021) 178:113969. doi: 10.1016/j.addr.2021.113969

207. Hua W, Zhao Y, Jin X, Yu D, He J, Xie D, et al. Mettl3 promotes ovarian carcinoma growth and invasion through the regulation of axl translation and epithelial to mesenchymal transition. *Gynecol Oncol.* (2018) 151:356–65. doi: 10.1016/j.ygyno.2018.09.015

208. Luo Y, Sun X, Xiong J. Characterization of M6a regulator-mediated methylation modification patterns and tumor microenvironment infiltration in ovarian cancer. *Front Cell Dev Biol.* (2021) 9:794801. doi: 10.3389/fcell.2021.794801

209. Yang Y, Wei Q, Tang Y, Yuanyuan W, Luo Q, Zhao H, et al. Loss of hnrnpa2b1 inhibits Malignant capability and promotes apoptosis via down-regulating lin28b expression in ovarian cancer. *Cancer Lett.* (2020) 475:43–52. doi: 10.1016/j.canlet.2020.01.029

210. Xu F, Li J, Ni M, Cheng J, Zhao H, Wang S, et al. Fbw7 suppresses ovarian cancer development by targeting the N(6)-methyladenosine binding protein ythdf2. *Mol Cancer.* (2021) 20:45. doi: 10.1186/s12943-021-01340-8

211. Zhang Y, Qiu J-G, Jia X-Y, Ke Y, Zhang M-K, Stieg D, et al. Mettl3-mediated N6-methyladenosine modification and hdac5/yy1 promote iffo1 downregulation in tumor development and chemo-resistance. *Cancer Lett.* (2023) 553:215971. doi: 10.1016/j.canlet.2022.215971

212. Yu HL, Ma XD, Tong JF, Li JQ, Guan XJ, Yang JH. Wtap is a prognostic marker of high-grade serous ovarian cancer and regulates the progression of ovarian cancer cells. *Onco Targets Ther.* (2019) 12:6191–201. doi: 10.2147/OTT.S205730

213. Wang Y, Chen Z. Long noncoding rna uba6-as1 inhibits the Malignancy of ovarian cancer cells via suppressing the decay of uba6 mrna. *Bioengineered.* (2022) 13:178–89. doi: 10.1080/21655979.2021.2011640

214. Zhao L, Kong X, Zhong W, Wang Y, Li P. Fto accelerates ovarian cancer cell growth by promoting proliferation, inhibiting apoptosis, and activating autophagy. *Pathol Res Pract.* (2020) 216:153042. doi: 10.1016/j.prp.2020.153042

215. Huang H, Wang Y, Kandpal M, Zhao G, Cardenas H, Ji Y, et al. (6)-methyladenosine modifications inhibit ovarian cancer stem cell self-renewal by blocking camp signaling. *Cancer Res.* (2020) 80:3200–14. doi: 10.1158/0008-5472.CAN-19-4044

216. Jiang X, Liu B, Nie Z, Duan L, Xiong Q, Jin Z, et al. The role of M6a modification in the biological functions and diseases. *Signal Transduction Targeted Ther.* (2021) 6:74. doi: 10.1038/s41392-020-00450-x

217. Jiang Y, Wan Y, Gong M, Zhou S, Qiu J, Cheng W. Rna demethylase alkbh5 promotes ovarian carcinogenesis in a simulated tumour microenvironment through stimulating nf-kappab pathway. *J Cell Mol Med.* (2020) 24:6137–48. doi: 10.1111/jcmm.15228

218. Zhu H, Gan X, Jiang X, Diao S, Wu H, Hu J. Alkbh5 inhibited autophagy of epithelial ovarian cancer through mir-7 and bcl-2. *J Exp Clin Cancer Res.* (2019) 38:163. doi: 10.1186/s13046-019-1159-2

219. Sun R, Yuan L, Jiang Y, Wan Y, Ma X, Yang J, et al. Alkbh5 activates fak signaling through M6a demethylation in itgb1 mrna and enhances tumor-associated lymphangiogenesis and lymph node metastasis in ovarian cance. *Theranostics.* (2023) 13:833–48. doi: 10.7150/thno.77441

220. Muller S, Glass M, Singh AK, Haase J, Bley N, Fuchs T, et al. Igf2bp1 promotes srf-dependent transcription in cancer in a M6a- and mirna-dependent manner. *Nucleic Acids Res.* (2019) 47:375–90. doi: 10.1093/nar/gky1012

221. Bley N, Schott A, Muller S, Misiak D, Lederer M, Fuchs T, et al. Igf2bp1 is a targetable src/mapk-dependent driver of invasive growth in ovarian cancer. *RNA Biol.* (2021) 18:391–403. doi: 10.1080/15476286.2020.1812894

222. Wang S, Li Z, Zhu G, Hong L, Hu C, Wang K, et al. Rna-binding protein igf2bp2 enhances circ_0000745 abundancy and promotes aggressiveness and stemness of ovarian cancer cells via the microrna-3187-3p/erbb4/pi3k/akt axis. *J Ovarian Res.* (2021) 14:154. doi: 10.1186/s13048-021-00917-7

Frontiers in Immunology

Explores novel approaches and diagnoses to treat immune disorders.

The official journal of the International Union of Immunological Societies (IUIS) and the most cited in its field, leading the way for research across basic, translational and clinical immunology.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact