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Citochine nel liquido follicolare predittive del successo della gravidanza nelle tecniche di procreazione medicalmente assistita

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Al mio adorato babbo Rossano

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ABBREVIAZIONI

ESHRE= European Society of Human Reproduction and Embriology

FF= Fluido follicolare

FGF= Fibroblast growth factor

FIVET=Fertilizzazione in vitro con trasferimento di embrione

FSH= Follicle stimulating hormone

GIFT= Gamete intrafallopian transfer

GnRH= Ormone di rilascio ipotalamico delle gonadotropine

G-CSF= Granulocyte colony-stimulating factor

GM-CSF= Granulocyte macrophage colony-stimulating factor

HCG= Ormone umano della gonadotropina corionica

HLAG-G=Human leukocyte antigen -G

ICSI= Intracitoplasmatic sperm injection

IFN=Interferone

IP-10= Interferon gamma-induced protein 10

IL=Interleuchina

IVF=In vitro fertilization

LH=Luteinizing hormone

MALDI TOF MS= Matrix-assisted laser desorption ionization time of flight mass spectrometry

MCP-1= Monocyte chemoattractant protein-1

MESA= Microsurgical epididymal sperm aspiration

MIP-1α= Macrophage inflammatory proteins-1α

MIP-1β= Macrophage inflammatory proteins-1β

OHSS= Ovarian hyperstimulation syndrome

PDGF= Platelet-derived growth factor

PGS= Pre implantation genetic screening

PMA= Procreazione medicalmente assistita

PROST= Pronuclear stage transfer

RIF=Recurrent implantation failure

RANTES= Regulated on activation, normal T cell expressed and secreted

SET=Single embryo transfert

TET= Tubal embryo transfer

TESA= Testicular sperm aspiration

Th=T helper

TNF= Tumor necrosis factor

VEGF= Vascular endothelial growth factor

ZIFT=Zigote intrafallopian transfer

1. INTRODUZIONE

1.1 Procreazione Medicalmente Assistita

La procreazione medicalmente assistita (PMA) è definita come quell'insieme di tecniche cliniche e di laboratorio che permettono a coppie affette da sterilità o sub fertilità (sia maschile che femminile), di ovviare alla propria condizione patologica. Nel tempo la richiesta di questo tipo di approccio è andata in crescendo.

Il primo successo in ambito di procreazione medicalmente assistita (PMA) nel 1978 è stato realizzato da R.G. Edwards, P. Steptoe e J. Purdy e portò alla nascita di Louise Brown, la prima bambina nata grazie alla procreazione medicalmente assistita (Steptoe PC e Edwards RG, 1978). Da allora la comunità scientifica si è mossa nell'ottica di garantire, a sempre maggior coppie che sono alla ricerca di un figlio, il successo della PMA.

Dai vari registri internazionali si stima che vengono eseguiti al mondo più di un milione di cicli, e secondo il 16esimo report dell' European IVF-monitoring Consortium (EIM) sotto l'egida dell'European Society of Human Reproduction and Embriology (ESHRE) (Calhaz-Jorge C, 2012) relativo all'anno 2012, sono stati

effettuati in Europa il 4.9% di cicli in più rispetto al 2011, con un incremento a carico in particolar modo della tecnica ICSI (Jain T, 2007; Nyobe A, 2008).

Per quanto riguarda l'Italia secondo la relazione del Ministro della Salute al Parlamento sullo stato di attuazione della legge contenente norme in materia di Procreazione Medicalmente Assistita, relativa all'attività di centri italiani per la PMA nell'anno 2016, sono stati effettuati 97.656 cicli per 77.722 coppie trattate, con un 14% di successo inteso come percentuale di nati vivi.

Nel corso del tempo, con sempre maggior raccolta di dati e grazie all'avanzamento tecnologico sono state messe a punto sempre più strategie per aumentare l'efficacia dei trattamenti.

1.2 L'infertilità

L'organizzazione mondiale della sanità descrive l'infertilità come una patologia che viene definita come il mancato concepimento dopo 1-2 anni di rapporti sessuali non protetti. Secondo stime e secondo i vari registri nazionali, l'infertilità riguarda il 15% delle coppie. Le cause dell'infertilità sono sia di origine maschile che femminile e possono essere di diversa natura e di diversa severità.

1.2.1 L'infertilità femminile

L'infertilità femminile è responsabile del 35-40% dell'infertilità di coppia e può dipendere da svariate cause quali: fattori endocrini, fattori ovarici, fattori tubarici,

patologie uterine, età della paziente, malattie sistemiche o genetiche, o fattori riguardanti lo stile di vita.

I fattori ormonali possono incidere sulla fertilità della donna, infatti se non ci sono gli adeguati livelli ormonali, non può essere garantita la fisiologica crescita e selezione del follicolo e l'inadeguata produzione ormonale può alterare il normale ciclo fisiologico fino a quadri di anovulazione. A queste ipotesi è riconducibile quasi il 40% dei casi di sterilità femminile. L'anovulazione, in particolare, può essere determinata sia da cause "esterne" come un continuo stato di stress o una condotta di vita disordinata, sia da cause organiche come la presenza di adenomi, tumori dell'ipofisi o la sindrome dell'ovaio policistico, che colpisce il 18% della popolazione ed è causa di infertilità anovulatoria nel 70% delle donne affette (Brassard M, 2008).

I fattori ovarici sono principalmente rappresentati dal già citato ovaio policistico, dall'endometriosi, che colpisce il 10% delle donne in età riproduttiva e causa infertilità nel 30-50% dei casi di pazienti affette. Fortunatamente con la terapia chirurgica viene ripristinata la normale fisiologia e la prognosi riproduttiva migliora decisamente (Vercellini P, 2006).

I fattori tubarici rappresentano quelle anomalie a carico delle tube di Falloppio sia a livello morfologico che funzionale. Le tube infatti possono essere affette da disfunzionalità a causa di infezioni, come ad esempio la Clamydia, di endometriosi, di interventi chirurgici, o di malformazioni congenite.

Il fattore uterino è rappresentato da anomalie che compromettono la sua struttura. Le anomalie sono in maggioranza congenite come nel caso dell'utero unicorne, bicorne,

didelfo, settato, arcuato che si verificano nel 4% delle pazienti e sono associati a un aumentato rischio di aborto (Raga F, 1997).

Il fattore cervicale può essere responsabile dell'infertilità della donna sia per alterazioni a livello morfologico (come nel caso delle aderenze), oppure funzionale (variazione della densità del muco cervicale o la presenza di infiammazioni) che compromettono la risalita degli spermatozoi verso le tube di Falloppio.

Le malattie sistemiche che possono interferire con la fertilità di una donna possono essere malattie che riguardano l'alterazione dell'asse ipotalamo-ipofisi (Sindrome di Kallmann, iperprolattinemia, ipopituitarismo, Sindrome di Cushing) oppure diabete mellito, disordini della tiroide, malattie delle ghiandole surrenali, malattie epatiche o renali.

Anche lo stile di vita può incidere sulla fertilità. Fattori come il fumo, gli stupefacenti, l'alcool, ma anche l'anoressia o l'eccesso ponderale possono influire.

Infine il fattore età ovviamente è essenziale, dato che la fertilità della donna decresce comunque con l'aumento anagrafico, infatti dopo i 36 anni in media subisce un rapido declino.

Esiste un'altra causa di infertilità legata al trattamento chemioterapico e radioterapico di giovani pazienti oncologiche. Il danno sulla fertilità è determinato ovviamente dalla tipologia di farmaco, dalla durata del trattamento e dalle dosi siano esse di chemioterapia o radioterapia (Matthews ML ,2012).

1.2.2 Infertilità maschile

Il 60% dei casi di infertilità di coppia è rappresentata dal fattore maschile. Le anomalie degli spermatozoi che riducono la possibilità di concepimento risiedono sia a livello quantitativo come basso numero, sia a livello qualitativo (ridotta motilità, morfologia anomala, danneggiamento del DNA).

Le cause dell'infertilità maschile possono essere presenti sia a livello genetico, sia organico per un non corretto sviluppo dei testicoli, per infezioni uro-genitali, che per altre patologie come ad esempio il varicocele (dilatazione delle vene dei testicoli), le orchiti (infiammazioni e infezioni a danno dei testicoli), il criptorchidismo (mancata discesa, alla nascita, di uno o entrambi i testicoli nello scroto). Possono essere presenti poi dei fattori extra testicolari come ad esempio la azoospermia dovuta all'ostruzione dell'epididimo, dei dotti eiaculatori e di quelli deferenti. Le ostruzioni di queste strutture possono essere congenite, svilupparsi in seguito a infiammazioni di prostata e vescichette seminali, oppure essere causate da una malattia autoimmune.

Ovviamente in base alla natura e al grado di severità dell'infertilità viene scelta la tecnica più adeguata e meno invasiva per curare tale patologia e garantire alla coppia la possibilità di concepimento.

1.3 Le tecniche

Le tecniche di procreazione medicalmente assistita si suddividono in tecniche di primo (I) secondo (II) e terzo (III) livello. Il livello ovviamente viene scelto in base alle esigenze della coppia rispettando la minor invasività.

Le tecniche di primo livello prevedono una fecondazione *in vivo* e sono le inseminazioni. Le tecniche di secondo livello, che prevedono l'aspirazione chirurgica degli ovociti dalle ovaie sono la FIVET (Fecondazione In Vitro con Trasferimento di Embrione) e la ICSI (Iniezione IntraCitoplasmatica dello Sperma). Le tecniche di terzo livello, prevedono il trasferimento in tuba degli ovociti e degli spermatozoi (gamete intrafallopian transfer, GIFT), il trasferimento in tuba degli zigoti (zigote intrafallopian transfer, ZIFT), il trasferimento di embrioni allo stadio di due pronuclei (pronuclear stage transfer, PROST) e il trasferimento in tuba degli embrioni (tubal embryo transfer, TET).

1.3.1 Tecniche di I livello

Le tecniche di I livello costituiscono il primo approccio clinico in casi di infertilità idiopatica o di un fattore lieve di infertilità maschile. Il liquido seminale opportunamente preparato viene inserito all'interno della cavità uterina mediante l'utilizzo di un catetere che permette un passaggio indolore attraverso il canale cervicale. Lo sperma viene prima opportunamente trattato in modo tale da selezionare gli spermatozoi mobili e vitali inducendo la capacitazione.

I molteplici studi presenti in letteratura (Opsahl MS, 2001; Schachter M, 2001; Oliveness F, 2002; Sbracia M, 2005) hanno messo in evidenza che la possibilità di successo è aumentata se all'inseminazione viene preceduto un protocollo di stimolazione ovarica per l'ottenimento di più follicoli ovulatori.

1.3.2 Tecniche di II livello

I trattamenti di II livello prevedono una iniziale stimolazione ovarica della donna. In un ciclo naturale un solo follicolo matura e diventa "dominante". La stimolazione ovarica permette la maturazione di più follicoli in modo tale da avere a disposizione più ovociti per la successiva fecondazione. Questa iperproduzione di follicoli può essere ottenuta con varie sostanze a seconda dei diversi protocolli. I protocolli prevedono solitamente l'utilizzo di un farmaco antagonista del GnRH (ormone di rilascio ipotalamico delle gonadotropine) che inibisce la produzione endogena degli ormoni ipofisari che regolano l'attività ovulatoria. Il temporaneo blocco dell'attività ormonale ha lo scopo di evitare interferenze nel trattamento da parte degli ormoni endogeni e di inibire il picco di ormone luteinizzante (LH) per evitare che i follicoli maturi vadano incontro ad ovulazione spontanea prima del prelievo ovocitario.

Durante il periodo di stimolazione ovarica la paziente è tenuta sotto controllo tramite tecnica ecografica transvaginale e tramite prelievo di sangue venoso per il dosaggio plasmatico dell'estradiolo. Tali controlli vengono effettuati a giorni alterni in modo da monitorare se la risposta della paziente è adeguata e quindi se non ci siano un numero troppo basso di follicoli (vengono così a mancare i presupposti per un successo clinico) o troppo elevato (con il rischio per la paziente di sviluppare la sindrome da iperstimolazione ovarica severa (Ovarian HyperStimulation Syndrome, OHSS). Se la risposta della paziente è nel range corretto, quando i follicoli in maturazione raggiungono la giusta dimensione periovulatoria (cioè quando hanno un diametro $\geq$18 mm) e i livelli di estradiolo sono nel giusto range, viene somministrato l'ormone umano della gonadotropina corionica (hCG) in modo tale da stimolare i follicoli alla maturazione. Dopo 36 ore dalla stimolazione con hCG si procede con il reperimento degli ovociti della paziente.

Il pick up ovocitario viene effettuato per via vaginale con aghi molto sottili, sotto controllo ecografico in anestesia locale o generale. Vengono aspirati con una siringa da 10 ml, tutti i follicoli idonei presenti. Nell'aspirazione dell'ovocita viene aspirato anche il liquido follicolare, che rappresenta il liquido normalmente presente intorno all'ovocita. I campioni di aspirato dove l'ovocita non era presente vengono scartati mentre quelli con l'ovocita vengono valutati per l'aspetto. Il giorno del pick up viene definito come giorno "0".

Se il campione spermatico ha parametri nel corretto range per numero e motilità si può procedere con la fecondazione in vitro con trasferimento di embrione (FIVET). Gli ovociti prelevati vengono singolarmente posti in coltura in appositi supporti con un certo numero di spermatozoi, in una piccola quantità di terreno di coltura apposito. Lo sperma in genere viene ottenuto mediante eiaculazione o con tecniche chirurgiche di prelievo dalle vie seminali quali la TESA (testicular sperm aspiration), la MESA (microsurgical epididymal sperm aspiration) e crioconservato. Nel caso della FIVET gli ovociti con le cellule del cumulo ooforo vengono poste in coltura con gli spermatozoi.

Se il campione maschile presenta un numero basso di spermatozoi o se questi non hanno una buona motilità, non è idoneo per poter fecondare l'ovocita semplicemente tramite una cocoltura come nel caso della FIVET. La tecnica della iniezione intracitoplasmatica dello spermatozoo (ICSI), permettendo la microiniezione del singolo spermatozoo, ha rappresentato una grande conquista medico scientifica in quanto ha permesso di ottenere una fecondazione anche in presenza di sperma con parametri estremamente lontani dalla norma in termini di numero o di motilità degli spermatozoi. La ICSI prevede di iniettare un unico spermatozoo direttamente all'interno del citoplasma dell'ovocita. Nel caso della ICSI il cumulo ooforo deve

essere rimosso e questa tecnica può essere eseguita solo se l'ovocita ha espulso il primo globulo polare (ovocita in metafase II). Sotto guida microscopica, il singolo spermatozoo viene iniettato nel citoplasma dell'ovocita attraverso uno strumento chiamato micromanipolatore.

1.3.3 Fecondazione e trasferimento dell'embrione

Dopo 15-20 ore dalla cocoltura viene valutata l'avvenuta fecondazione. La presenza dello zigote viene discriminata attraverso l'analisi della presenza dei due pronuclei. L'ovocita fecondato espelle il secondo globulo polare (che può essere utilizzato per approfondire l'analisi cromosomica degli ovociti). Lo zigote viene quindi rimesso in coltura dopo un cambio di terreno fresco e controllato ogni 20-24 ore per monitorarne lo sviluppo. La percentuale di fecondazione si aggira intorno ad una media del 75% mentre lo sviluppo embrionale intorno all'80%. Dopo 24 ore la fecondazione, quindi al giorno "2" un normale sviluppo embrionale deve portare alla formazione di un embrione a 2-4 cellule. Il giorno successivo, indicato come giorno "3" l'embrione deve essere a 6-8 cellule, il giorno "4" a 16 cellule nello stadio definito Morula, al giorno "5" l'embrione definito blastocisti, deve essere formato da almeno 100 cellule. Questo rappresenta lo stadio dal quale inizia l'annidamento dell'embrione nell'utero materno, e fino a questo stadio attualmente l'embrione può essere coltivato al di fuori del corpo della donna in condizioni di laboratorio, senza avere alterazione delle sue caratteristiche morfologiche funzionali.

Circa il 15-20% degli embrioni si arrestano durante i primi tre giorni di sviluppo, se sono presenti embrioni vitali si può procedere al trasferimento. Il trasferimento può essere effettuato al giorno "3" o al giorno "5". La metodica di trasferimento è in

genere indolore e viene eseguita mediante l'utilizzo di un sottile catetere attraverso il canale cervicale.

1.3.4 Tecniche di III livello

Le tecniche di III livello sono rappresentate dal trasferimento nella tuba di Falloppio degli ovociti e degli spermatozoi (GIFT), il trasferimento nella tuba di Falloppio degli zigoti (ZIFT), il trasferimento di embrioni allo stadio di due pronuclei (PROST) e il trasferimento nella tuba di Falloppio degli embrioni (TET). La GIFT consiste nel trasferimento nella tuba di Falloppio, per via laparoscopica degli spermatozoi che sono stati ottenuti dopo capacitazione del liquido seminale e degli ovociti recuperati con il pick up ovocitario. Per quanto riguarda ZIFT, PROST e TET vengono, trasferiti in sede della tuba di Falloppio zigoti, embrioni allo stadio di due pronuclei ed embrioni rispettivamente. Il catetere viene fatto penetrare delicatamente all'interno della tuba di Falloppio e qui viene depositato il gamete o gli zigoti o gli embrioni. Le indicazioni della GIFT consistono essenzialmente nella sterilità idiopatica, ma anche nei casi di fattore peritoneale (endometriosi) e fattore maschile lieve. La GIFT e la TET possono essere impiegate anche nei casi di fattore maschile moderato (Ménézo YJ, 1996; Tournaye H, 1996).

1.4 Tasso di impianto

Nei cicli di fecondazione in vitro un passaggio cruciale è il trasferimento dell'embrione e il suo tasso di impianto nell'utero materno. Il tasso di impianto dipende dalla morfologia dell'embrione, dalla qualità del trattamento e dall'età della

paziente. In molti centri di PMA per sopperire al basso tasso di impianto vengono impiantati più embrioni. L'impianto di un numero maggiore di embrioni con il rischio, in caso di successo, di sviluppo di gravidanze multiple con rischi clinici per la salute della donna pone la comunità scientifica davanti all'obiettivo di attuare quella che viene definita la politica del trasferimento del singolo embrione o SET dall'acronimo Single Embryo Transer. Come descritto in letteratura (Fiddelers AA, 2006) la SET ha un importante impatto sulla riduzione della mortalità e morbosità materno fetale. Il maggior impedimento all'applicazione della politica della SET è legata proprio al tasso di impianto. Essendo infatti molto basso, si parla di una percentuale intorno al 15-20%, si preferisce trasferire almeno due embrioni dato che questo incrementa notevolmente il successo della gravidanza (Van Mortfoort AP, 2006). Attualmente a livello sia Europeo ed ancora di più negli Stati Uniti d'America vengono impiantati più embrioni e questo trend potrebbe subire un cambiamento se si riuscisse ad intervenire sul potenziale di impianto incrementandolo. Oltre a limitare il potenziale utilizzo della politica della SET, il basso tasso di impianto presente nella attuale pratica clinica delle fecondazioni in vitro, fa emergere il problema di quello che viene definito fallimento di impianto ricorrente (Recurrent Implantation Faiulure RIF). Il RIF può essere definito come la ripetuta mancanza di impianto dopo il trasferimento dell'embrione in un normale ciclo di IVF. Prima infatti dell'avvento delle tecniche di fecondazione *in vitro* i trattamenti avevano come parametro di monitoring l'avvenuta gravidanza o meno. Con l'avvento delle tecniche di PMA e la possibilità quindi di compartimentalizzare la procedura e quindi di conoscere se dopo il trasferimento c'è stato impianto, il RIF è divenuto un fenomeno clinicamente identificabile. Pur essendo vero ciò, definire quando c'è la presenza di RIF non è semplice. Definire il termine ricorrente è una sfida,

analogamente alla definizione di infertilità o di insuccesso di gravidanza . Nel 2005 la società europea di riproduzione umana ed embriologia ESHRE ha definito il mancato impianto ricorrente, come un numero maggiore di 3 embrioni di alta qualità trasferiti e non impiantati (Thornhill AR, 2005). Molti parametri entrano in gioco in questa definizione perché fattori come l'età, la qualità degli embrioni, il giorno in cui viene effettuato il trasferimento nonché il fatto che c'è un forte declino del tasso di gravidanza via via che il numero di cicli cresce (Shapiro BS, 2001), influiscono sul fallimento di impianto.

1.4.1 Potenziale di impianto dell'ovocita

Un utile approccio per ridurre il RIF e poter pronosticare l'applicazione della politica della SET è quello di selezionare a monte l'ovocita con il più elevato potenziale di impianto. Questo potrebbe far alzare il tasso di impianto, diminuendo il RIF e potrebbe portare all'applicazione della SET evitando il trasferimento di embrioni soprannumerari, con il rischio di gravidanze multiple.

Ad oggi la letteratura ha descritto molti metodi per aumentare il tasso di impianto dell'embrione come ad esempio il dosaggio dell'HLA-G solubile nel surnatante di embrioni. L'HLA-G è una molecola appartenente al sistema HLA non classico. Scarsamente polimorfico è una delle molecole HLA presente sul trofoblasto. La sua forma solubile (sHLA-G) viene prodotta dal trofoblasto stesso ed induce le cellule T helper presenti nel microambiente uterino a virare verso la sottopopolazione Th2 (producente IL-4) benefica per il successo della gravidanza (Piccinni MP, 2010; Lombardelli L, 2013). L'sHLA-G sembra essere insieme ad altri importanti fattori come il LIF e il M-CSF (Piccinni MP, 1998) una molecola importante per l'impianto.

Dato che l'sHLA-G viene prodotto dall'embrione alcuni gruppi di ricerca hanno investigato se il suo dosaggio potesse essere correlato con la qualità dell'embrione stesso. E' stato in effetti osservato come, nel terreno di coltura di embrioni di ottima qualità, fosse presente una quantità maggiore di sHLA-G, questo però solo nelle donne giovani che putroppo non sono il gruppo più numeroso che si approccia alla PMA (Fisch JD, 2007). Infatti il rapporto del Ministro della Salute in materia di procreazione medicalmente assistita, descrive proprio come siano in calo i cicli effettuati sulle donne giovani e in aumento quelli richiesti ed effettuati sulle donne al di sopra di 40 anni. Inoltre in letteratura sono presenti molti lavori contrastanti sull'effettiva presenza misurabile dell'sHLA-G nel surnatante di embrioni in coltura. Secondo uno studio (Sageshima N, 2007) infatti le diverse sensibilità dei test ELISA utilizzati per detectare l'sHLA-G e le diverse condizioni di coltura degli embrioni nei vari centri per la procreazione medicalmente assistita, portano a dati contrastanti sul possibile utilizzo del dosaggio dell'sHLA-G come metodo prognostico per individuare l'embrione di miglior qualità.

Altri approcci prevedono metodi più invasivi per effettuare analisi genetiche, tali metodologie prevedono la biopsia dell'embrione. Gli ultimi dati del Consorzio Europeo che monitora l'IVF indica che non ci sono stati significativi aumenti nel tasso di nascita negli ultimi anni a seguito dell'utilizzo di tale tecniche (Kupka MS, 2010). Recentemente lo screening genetico di preimpianto (PGS) è stato descritto non aver incrementato il tasso di nascita (Verpoest W, 2018) tanto che il metodo è stato messo in discussione data la sua invasività e i suoi costi.

Alcuni recenti studi descrivono approcci per garantire la presenza di un embrione di ottima qualità mediante l'analisi di proteine, metaboliti e RNA non codificanti

(Gardner DK, 2015; Rodgaard T, 2015) anche se ancora questo tipo di indagine è solamente all'inizio.

Alcuni gruppi hanno tentato di soddisfare il bisogno di identificare un metodo non invasivo per determinare la qualità dell'embrione, utilizzando la spettrometria ionica di massa per mettere in luce delle proteine uniche utilizzabili come biomarkers per la qualità dell'embrione (Cortezzi SS, 2011). La complessità della preparazione del campione, il suo processamento, il costo e le capacità tecniche richieste però rendono difficile il suo utilizzo come metodo prognostico.

E' stato analizzato il mezzo di coltura di una blastocisti con il MALDI TOF MS (matrix-assisted laser desorption ionization time of flight mass spectrometry) (Iles RK, 2019). Sembra che tramite questa tecnologia si possa identificare i campioni con un maggiore potenziale di impianto. Ma non ci sono dati abbastanza robusti, presenti in letteratura a sostegno di tale metodologia per la valutazione del potenziale di impianto dell'embrione.

Ad oggi nessuna delle metodologie sopra descritte è riuscita non solo a sostituire ma anche ad affiancare l'utilizzo del criterio morfologico, che rappresenta ancora il miglior metodo di screening prognostico della capacità di un embrione a impiantarsi, nonostante sia ovviamente un criterio aleatorio in quanto legato all'operatore.

1.5 Citochine nel Fluido follicolare

Ultimamente la ricerca si è mossa nell'ottica di individuare un biomarker che potesse indicare l'ovocita con il maggior potenziale di generazione di un embrione di alta qualità e che garantisse un elevato tasso di impianto. Un utile approccio sembra

essere quello della quantizzazione delle citochine nel fluido follicolare (FF). Le citochine, sono mediatori peptidici, prodotti principalmente dalle cellule del sistema immunitario ma non solo, con azione autrocrina e paracrina, capaci di regolare finemente molti processi cellulari. Esse intervengono anche a livello del sistema riproduttivo regolando la funzione ovarica (Tabibzadeh S, 1994), in particolare la crescita follicolare, l'ovulazione e la formazione del corpo luteo (Field SL, 2014), ma possono esercitare il loro effetto anche su embrioni, cellule immunitarie e non presenti nell'ovaio e nell'utero creando un microambiente uterino immunologicamente protetto e recettivo, influenzando quindi l'ovocita e l'embrione sia nel suo impianto che nel suo sviluppo. Il fluido follicolare (FF) è un trasudato sierico con contenuto proteico (McNatty KP, 1978; Gosden RG, 1988), che provvede al microambiente di crescita e sviluppo della cellula uovo. Il follicolo rappresenta una formazione dell'organo ovarico. L'unità funzionale dell'ovaio è costituita dall'ovocita e da uno strato di epitelio follicolare ovarico. Dopo la pubertà un numero di follicoli definiti primordiali sono indotti, sotto stimolazione ormonale a riprendere l'ovogenesi portando al fenomeno fisiologico del ciclo ovarico. Nella donna la maturazione del follicolo avviene in cadenza mensile. I follicoli ovarici cominciano a svilupparsi sotto l'influenza di una complessa rete ormonale e un follicolo, diventato dominante o antrale, rilascia l'ovocita sotto il controllo dell'LH. La rottura del follicolo e il successivo rilascio dell'ovocita riduce ovviamente la pressione del fluido follicolare e l'ovocita è espulso insieme al fluido follicolare che era presente all'interno del follicolo in una zona cava definita antro.

In letteratura sono presenti molti lavori che descrivono la presenza di molteplici citochine nel fluido follicolare. Ad esempio il Fattore di Necrosi Tumorale- α (TNF-α) gioca un ruolo nella follicologenesi, influenza la qualità ovocitaria e induce la

sintesi di progesterone nelle cellule dell'ovaio (Wang LJ, 1992; Lee KS, 2000; Bornstein SR, 2004). L'interferone-γ (IFN-γ) e l'Interleuchina(IL)-2 hanno un particolare effetto sul clivaggio dell'embrione (Ledee N, 2008), mentre l'IL-6 si è dimostrata un regolatore delle cellule del cumulo ooforo contribuendo inoltre ad aumentare la qualità dell'ovocita (Liu Z, 2009). Inoltre l'IL-6 insieme ad all'IL-8 regolano la produzione degli ormoni ovarici steroidei e il processo infiammatorio durante l'ovulazione (Van der Hoek KH, 1998; Busher U, 1999). Inoltre è stato dimostrato che l'IL-10, nota citochina antifiammatoria, prodotta dai linfociti T è stata detectata nel terreno di coltura dell'embrione umano pre-impianto (Ozornek MH, 1995), anche se il suo valore predittivo sulla qualità dell'embrione non è ancora chiaro (Vujisic S, 2004). Ulteriormente l'IL-10 e il VEGF aumentano l'angiogenesi del follicolo (Brannstrom M, 1998). Dunque le citochine presenti nel FF si sono rivelate dei buoni candidati per essere elette a marcatori prognostici della competenza ovocitaria. Molti studi in letteratura hanno messo in evidenza una relazione tra alcune citochine e il successo dell'impianto nel cicli di fecondazione in vitro e in particolare nell'utilizzo della tecnica ICSI (Hammadeh ME, 2002; Salmassi A, 2005). Il limite di questi studi risiede nel fatto che il dosaggio delle citochine è stato effettuato su un pool di follicoli, la cui tracciabilità era alquanto compromessa (Franchin R, 2007) e questo fattore rendeva impossibile la correlazione delle citochine presenti nel FF degli ovociti, con la qualità dell'ovocita stesso.

1.5.1 Granulocyte colony stimulating factor (G-CSF)

In uno studio antecedente il nostro laboratorio si è occupato del dosaggio nei fluidi follicolari, collezionati da follicoli individuali di donne che approcciavano alla

fecondazione *in vitro* con programma ICSI. Gli ovociti erano ottenuti mediante protocollo classico di iperstimolazione ovarica, conseguente fertilizzazione e trasferimento in utero. In ogni liquido follicolare aspirato (quindi con estrema tracciabilità) è stato possibile dosare 27 tra citochine e chemochine in modo simultaneo. I risultati dimostrano come si evince dall'area sotto la curva ROC (Fig.1) che i fluidi follicolari che contenevano più Granulocyte-Colony Stimulating Factor (G-CSF) correlavano con un maggiore tasso di impianto.

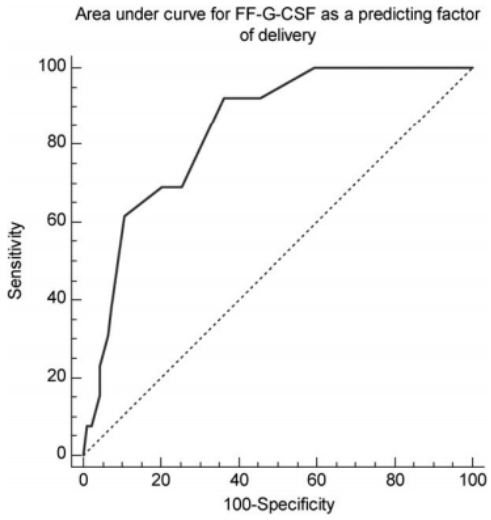


Fig.1 Curva ROC per la misurazione del G-CSF in fluidi follicolari individuali in pazienti con o senza impianto.

In dettaglio quando i livelli di G-CSF erano inferiori a 20 pg/ml la media del tasso di impianto era del 9%, mentre il tasso di nascita del 6%. Quando i livelli di G-CSF erano maggiori di 24 pg/ml la media del tasso di impianto raggiungeva il 44% e il tasso di gravidanza raggiungeva il medesimo livello (Ledee N, 2008). Sia il tasso di impianto che di nascita risultavano statisticamente significativi ($p<0.0001$) in quei FF dove il G-CSF era superiore a 24 pg\ml rispetto a quelli con G-CSF inferiore a 20 pg\ml. Nel caso in cui la concentrazione di G-CSF fosse all'interno di questo range la

media del tasso di impianto e di nascita (18% e 15,6% rispettivamente) erano significativamente più alte ($p<0.0005$ e $p<0.0001$) rispetto a quelli trovati quando i livelli i G-CSF erano inferiori a 20 pg/ml (Ledee N, 2008, 2010). Il G-CSF pur essendo presente negli embrioni con miglior morfologia in concentrazioni superiori a 20 pg/ml, non è risultata statisticamente significativa la differenza di livello di G-CSF tra gli embrioni con miglior morfologia rispetto a quelli con peggior morfologia, lasciando intuire che lo score morfologico e il G-CSF dosato nei FF fossero fattori predittivi dell'impianto indipendenti (Ledee N, 2011, 2013)

Successivamente in uno studio retrospettivo a cieco, sono stati analizzati i cicli naturali modificati. Anche qui ogni FF individuale è stato analizzato per la presenza di 27 citochine e chemochine ed è stata valutata per ogni molecola la sua potenzialità come biomarker. Andando a compiere un analisi di regressione, una combinazione di G-CSF e IL-15 si è rivelata ottimale per la definizione della competenza ovocitaria (Area curva ROC=0.85). In particolar modo alti livelli di G-CSF e bassi di IL-15 sembrava rappresentare un metodo assolutamente non invasivo, per individuare l'ovocita con il maggiore tasso di impianto (Ledee N, 2011). Il G-CSF sembra essere quindi un buon marcatore non invasivo per definire la competenza ovocitaria, parametro essenziale per tutta la procedura della procreazione medicalmente assistita.

Il G-CSF è stato storicamente descritto come il fattore di crescita che stimola la differenziazione, la sopravvivenza e la migrazione delle cellule ematopoietiche della linea neutrofilica ed inserito a pieno titolo nella famiglia dei colony-stimulating factor (Clark SC, 1987). E' una glicoproteina prodotta da svariati tipi cellulari. Nell'uomo per quanto riguarda il sistema riproduttivo è stato descritto essere prodotto dalle cellule della granulosa (Salmassi A, 2004), dalle cellule endometriali

(Giacomini G, 1995) e dalle cellule con origine deciduale (Duan JS, 1990) nonchè da vari tessuti fetali (Calhoun DA, 1999). Durante l'ovulazione le concentrazioni di G-CSF sono più elevate nei fluidi follicolari rispetto al siero (Salmassi A, 2005). Il recettore del G-CSF e la sua presenza è stata individuata nelle cellule della granulosa nel follicolo e nelle cellule luteali. In letteratura sono presenti considerevoli studi che analizzano il ruolo del G-CSF nell'impianto. Il G-CSF sembra alzarsi nella sua concentrazione, nel momento dell'impianto se analizziamo i cicli naturali (Yanagi K, 2002) e dopo tentativi di IVF\ICSI andati a buon fine (Salmassi A, 2005).

In base quindi ai lavori presenti in letteratura e ai lavori antecedenti del nostro gruppo sembra proprio che il G-CSF possa essere un ottimo candidato come biomarker di competenza ovocitaria, in quanto più elevato nei FF appartenenti agli ovociti che hanno generato embrioni con il più elevato tasso di impianto, e quindi essere un marcatore della qualità dell'embrione. Tali studi però erano di tipo retrospettivo ed eseguiti in presenza di diversi stimoli ovarici, fattore che ampliava la variabilità dello studio e rendeva difficile l'eventuale messa a punto del dosaggio del G-CSF come biomarker della qualità dell'embrione.

2. SCOPO DELLA TESI

La prima gravidanza nel campo della procreazione *in vitro* nel 1978 fu ottenuta nell'ambito di un ciclo naturale, con la coltura di un unico embrione e il suo conseguente trasferimento nella cavità uterina (Steptoe PC e Edwards RG, 1978). Fin dall'inizio degli anni '80 si osserva come l'efficacia dei trattamenti cresceva in maniera esponenziale se nel ciclo di fecondazione *in vitro* veniva inserito l'utilizzo di farmaci induttori dell'ovulazione, in grado di portare alla maturazione di più follicoli contemporaneamente e conseguentemente al prelievo di più ovociti. Questa pratica prevedeva il trasferimento di più embrioni all'interno dell'utero della donna. La prima gravidanza ottenuta con farmaci per la stimolazione ovarica, risale al 1980 e da allora l'induzione e la crescita follicolare multipla è divenuta una tappa fondamentale per i cicli di PMA. Da allora nella normale pratica clinica di PMA è divenuta prassi l'iperstimolazione ovarica con gonadotropine esogene per ottenere un numero maggiore di follicoli e nel corso del tempo nuovi farmaci sono stati messi a punto per garantire tale produzione. Nonostante ciò molte pazienti non sono in grado di produrre un adeguata crescita di più follicoli: circa il 15% presenta infatti risposte ovariche di scarsa entità che ovviamente possono compromettere il trattamento in corso. Inoltre il prelievo di più ovociti e più embrioni fertilizzati e trasferiti, ha aumentato l'incidenza di gravidanze plurigemellari. La gravidanza multipla in effetti è classificabile come una complicanza delle tecniche di procreazione medicalmente assistita, in quanto espone la futura madre e i nascituri a severi rischi per la salute fisica e mentale. Quindi sarebbe consigliabile nelle pazienti trasferire un solo embrione (Single Embryo Transfer), per ridurre l'incidenza ancora troppo elevata di gravidanze gemellari a maggior rischio di complicanze sia per la madre che per i feti.

In pazienti giovani si attua già questa politica cercando anche di utilizzare stimolazioni ovariche sempre meno aggressive. E' vero però che secondo il rapporto del Ministero della Sanità in materia di PMA l'età delle pazienti che decidono di sottoporsi alle tecniche di fecondazione *in vitro* sta aumentando. Alla luce di ciò l'applicazione della SET sembra essere un obiettivo molto ostico da raggiungere. Un utile approccio potrebbe essere quello di selezionare l'embrione o ancora meglio l'ovocita con il potenziale di impianto migliore, aumentando così la percentuale di successo delle tecniche, permettendo così l'utilizzo della SET e riducendo il rischio di gravidanze multiple.

Attualmente la scelta dell'embrione o degli embrioni da trasferire nella cavità uterina è basata su un criterio di tipo morfologico. La valutazione della qualità degli embrioni è basata sull'osservazione al microscopio al seguito della quale viene attribuito uno score morfologico ad ogni embrione. Vengono valutate complessivamente il numero di cellule (blastomeri), il grado di frammentazione e la presenza di multinucleazione (Nagy ZP, 2003). In base a tali caratteristiche viene assegnato un punteggio (score A-B-C) che esprime la qualità dell'embrione. L'embrione ottimale (score A) è considerato un embrione che è costituito da 4 blastomeri di uguale dimensione mononucleati e con frammentazione inferiore al 10% in seconda giornata, oppure 8 blastomeri mononucleati di uguale dimensione in terza giornata (Van Royen E, 1999). Successivamente, in quarta giornata, la classificazione embrionale si basa sulla compattazione cellulare, secondo cui la morula ottimale ha un grado di compattezza che riguarda l'intero volume dell'embrione. In quinta giornata post inseminazione l'embrione raggiunge lo stadio di blastocisti. La blastocisti con score più elevato, ha un alto grado di espansione

della sua cavità interna, detta blastocele, con una buona massa interna (Lemmen JG, 2008).

Ovviamente il criterio morfologico nonostante l'utilizzo del time-lapse, rimane sempre operatore dipendente. Questo introduce inevitabilmente una variabile imprescindibile di giudizio. La scoperta di tecniche non operatore dipendente, che sono in grado di identificare l'embrione con il maggiore potenziale di impianto risulta essere assolutamente indispensabile (Guerif F, 2007).

In letteratura sono presenti studi condotti sul fluido follicolare (FF) che è rappresentato dal fluido che circonda l'ovocita e che durante un ciclo di procreazione medicalmente assistita viene aspirato insieme all'ovocita e scartato. E' stato determinato che nel FF sono presenti delle molecole come ad esempio l'IL-6, l'IL-8 e il GM-CSF che potrebbero incidere sul successo di cicli di fecondazione *in vitro* ma non possono essere valutate per uno scopo prognostico (Hammadeh ME, 2002), o come il VEGF e inibina A e B (Ocal P, 2004), le gonadotropine e steroidi anch'essi non prognostici (Westergaard LG, 2004), la leptina (Asimakopolous B, 2005), il G-CSF (Salmassi A, 2005) o l'HLA-G solubile. Il più grande limite di tutti questi studi è rappresentato dall'impossibilità di legare una determinazione relativa ad un fluido follicolare con l'ovocita corrispondente dato che gli studi sono stati effettuati su pool di fluidi follicolari ottenuti da più follicoli aspirati dallo stesso ovaio.

Il nostro gruppo ha dimostrato che il G-CSF dosato nei fluidi follicolari individuali (aspirati separatamente) può essere correlato con il potenziale di impianto dell'embrione generato dall'ovocita corrispondente (Ledee N, 2008, 2010, 2011).

Gli studi eseguiti erano di tipo retrospettivo. La messa a punto dell'uso del G-CSF come biomarker prognostico della qualità dell'ovocita prevede uno studio di tipo

prospettico, con la selezione dell'ovocita di alta qualità di fertilizzare sulla base del contenuto in G-CSF dei FF individuali (corrispondenti ad ogni ovocita aspirato) e l'applicazione della SET che prevede il transfer di una singola blastocisti corrispondente al fluido follicolare con alti livelli di G-CSF.

I risultati riportati precedentemente dal nostro gruppo sono stati ottenuti con pazienti stimolati con i diversi protocolli di stimolazione ovarica, abbiamo dimostrato recentemente che il protocollo di stimolazione influenza il contenuto citochinico dei FF (pubblicazione in preparazione). Lo studio attuale sarà quindi eseguito usando un unico stimolo rappresentato da FSHr in unione a LHr.

A conferma che lo stimolo ovarico influenza il contenuto citochinico dei fluidi follicolari abbiamo dimostrato che la combinazione di G-CSF con IL-15 è un fattore predittivo del potenziale di impianto dell'embrione solo nei cicli naturali modificati. In questo studio determineremo se tale combinazione si riconferma come fattore predittivo del potenziale di impianto, anche nel caso di un ciclo di stimolazione ovarica con stimolo ovarico rappresentato da FSHr e LHr.

Infine per questo studio solo la blastocisti a 5 giorni, selezionata sulla base del G-CSF e dell'IL-15 nel fluido follicolare corrispondente e non l'embrione coltivato a due giorni sarà trasferita.

Nel presente studio di tipo prospettivo osservazionale randomizzato, saranno valutati il tasso di fertilizzazione e l'avvenuto o meno impianto di un'unica blastocisti in 2 gruppi di pazienti sottoposte a ICSI: gruppo 1, per il quale la scelta della blastocisti sarà eseguita in base alla scelta della miglior morfologia, e il "gruppo 2" per il quale la scelta sarà basata su maggiori quantità di G-CSF e minori quantità di IL-15 nel

fluido follicolare in confronto ai risultati di citochine ottenuti da tutti gli altri fluidi follicolari aspirati dall'ovaio della paziente.

Dopo il prelievo ovocitario, i fluidi follicolari individuali ottenuti dagli ovociti aspirati, saranno valutati mediante tecnologia multiplex baed-based assay per la presenza di 27 citochine e chemochine (Interleuchina(IL)-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 IL-13, IL-15, IL-17, Eotaxin, Fibroblast Growth Factor (FGF), G-CSF, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), IFN-G, Interferon gamma-induced protein 10 (IP-10), Monocyte Chemoattractant Protein 1 (MCP-1), Macrophage Inflammatory Proteins-1α (MIP-1α), Macrophage Inflammatory Proteins-1β (MIP-1β), Platelet-derived growth factor (PDGF), Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), Tumor necrosis factor-α (TNF-A), Vascular Endothelial Growth Factor (VEGF)). Sarà comunicato al centro di procreazione assistita (Demetra) il numero identificativo del fluido follicolare con una migliore combinazione di G-CSF e IL-15 e quindi l'ovocita con il probabile potenziale d'impianto migliore. Il centro di procreazione assistita provvederà, per il gruppo di studio, alla fertilizzazione dell'ovocita corrispondente a quel FF e al transfer della blastocisti ottenuta nella cavità uterina (Fig.2).

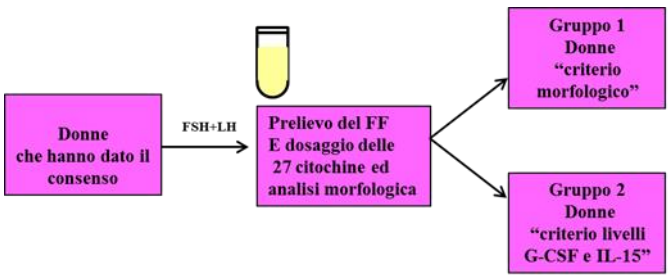


Fig.2 Protocollo dello studio prospettivo osservazionale

Nel gruppo di controllo, la blastocisti trasferita sarà invece scelta sulla base della miglior morfologia della blastocisti stessa. Lo studio sarà molto più articolato perché nei due gruppi sarà eseguita la determinazione delle 27 citochine nei fluidi follicolari e lo score morfologico. Questo permetterà inoltre di studiare sulla totalità delle pazienti le associazioni tra il tasso di fertilizzazione, la morfologia della blastocisti, e l'impianto con le citochine, per la stimolazione FSHr ed LHr, al fine di determinare se alcune citochine (in particolare G-CSF) possono essere dei fattori predittivi.

Lo studio descritto prevede diversi obiettivi:

1. Determinare se il G-CSF, in modo univoco o in combinazione con altre delle 26 citochine dosate nei FF degli ovociti prelevati da donne che si sottopongono a protocollo ICSI dopo stimolazione ovarica con FSHr ed LHr, correla con il tasso di fertilizzazione degli ovociti per ogni paziente e diventare un fattore predittivo della fertilizzazione.
2. Determinare se nei protocolli ICSI dopo stimolazione ovarica con FSHr ed LHr, il G-CSF in modo univoco o in combinazione con altre delle 26 citochine dosate nei FF correla con la morfologia dell'embrione e quindi se uno o più dei 27 analiti dosati con la nostra tecnologia di quantizzazione correlano con il miglior score della blastocisti e possa diventare un fattore predittivo della morfologia della blastocisti.
3. Confermare se il G-CSF in associazione o no con l'IL-15 nei cicli ICSI dopo stimolo ovarico FSHr e LHr che prevede il trasferimento della blastocisti a 5 giorni nella cavità uterina, mantiene le sue caratteristiche di biomarker di competenza ovocitaria e quindi predittivo dell'impianto (positività al dosaggio delle beta-hCG e presenza ecografica della camera gestazionale).

4. Mettere in evidenza se altre citochine oltre al G-CSF ed all'IL-15 nei cicli di ICSI stimolati con FSHr e LHr, possono essere predittive dell'impianto (con positività al dosaggio delle beta-hCG e presenza ecografica della camera gestazionale) in questo protocollo sperimentale e divenire dei biomarkers predittivi dell'ovociti con il miglior potenziale di impianto.
5. Determinare se la presenza di una o più delle 27 citochine dosate nei fluidi follicolari possa essere associata ad una tipologia d'infertilità. In particolare determinare se l'infertilità femminile non di origine tubarica, ma legata ad un insufficienza follicolare, possa essere messa in evidenza da un biomarcatore citochinico misurato nel fluido follicolare.

3. MATERIALI E METODI

3.1 Pazienti

In questo studio di tipo "prospettivo" sono state arruolate N=49 donne che si approcciavano alle tecniche di PMA al centro di procreazione assistita Demetra srl (Firenze) in convenzione con la Regione Toscana, per un protocollo ICSI. Le pazienti, dopo aver letto, compreso e firmato il consenso informato, sono state reclutate in base ad una serie di criteri.

I criteri di inclusione comprendono un body mass index (BMI) normale (18-26), parametri di riserva ovarica normali secondo dosaggio di FSH e ormone anti-mulleriano (AMH) al terzo giorno del ciclo mestruale (FSH<10 mU\ml ; E2<80 pg\ml; AMH>1,5ng\ml), e normale conta dei follicoli antrali (AFC>6).

Sono escluse dallo studio donne affette dalla sindrome dell'ovaio policistico, donne che abbiano subito chirurgia a livello ovarico, affette da endometriosi pelvica e donne affette da scarsa riserva ovarica (POR) secondo i criteri ESHRE 2010.

Lo studio prevedeva l'arruolamento di 100 donne, ma a causa della difficoltà di ottenere un consenso, purtroppo è stato possibile arruolarne solo 49.

Le donne sono state suddivise in modo randomizzato in 2 gruppi: nel "gruppo 1" (N=24 pazienti) la blastocisti che verrà trasferita nella cavità uterina sarà scelta in base ai criteri morfologici standard, nel "gruppo 2" (n=25 pazienti) la blastocisti che sarà trasferita sarà scelta in base alla valutazione dei livelli delle citochine G-CSF e IL-15, più alti e più bassi rispettivamente nel fluido follicolare derivante dallo stesso

follicolo da cui è stato aspirato l'ovocita che è stato fecondato con tecnica ICSI, paragonandoli a quelli della corte dei fluidi follicolari aspirati nella paziente.

Le cause dell'infertilità delle pazienti arruolate (Tab.1) sono: fattore femminile, fattore maschile , idiopatica, tubarica e fattore femminile e maschile presenti contemporaneamente.

Tipologia infertilità	**Num di pazienti**	**Età**
Fattore femminile	9	36±3
Fattore maschile	11	34±5
Idiopatica	10	35±3
tubarica	9	34±3
Fattore femminile e maschile	10	36±3

Tab.1 Caratteristiche delle coorti di pazienti del nostro studio in base alla tipologia di infertilità. Non ci sono differenze statisticamente significative per il parametro "età" i gruppi quindi sono statisticamente paragonabili .

3.2 Stimolazione ovarica con FSHr e LHr e raccolta dei fluidi follicolari

Le pazienti stimolate per il primo ciclo con protocollo corto standard (GnRHAnt) e solo FSHr, che avevano avuto una bassa risposta ovarica, Fort <50% (FORT Criteria, 2012; FORT = PFCX100 / AFC), sono state trattate per il secondo ciclo con protocollo corto standard (GnRH-Ant) e FSHr più LHr (Merck Darmstadt,

Germania), secondo lo standard stabilito dalla pratica clinica del centro di procreazione. Ovviamente le dosi dei due ormoni sono variate da paziente in paziente e dipendono dalla risposta individuale della crescita follicolare.

Dopo stimolazione ovarica le pazienti sono state monitorate per la risposta ovarica attraverso ecografia transvaginale e dosaggio di estradiolo nel plasma. Quando almeno tre follicoli raggiungevano il diametro di 18 mm, la paziente veniva stimolata con 5000 UI di gonadotropina corionica umana (hCG) per indurre la maturazione del follicolo.

Gli ovociti maturati sono stati aspirati dall'ovaio (pick up) dopo 36 ore dalla somministrazione di hCG.

L'aspirazione degli ovociti insieme al fluido follicolare avviene transvaginalmente in anestesia generale o locale mediante monitoraggio con ecografia intravaginale secondo le linee guida, 36 ore dopo la somministrazione di hCG.

Ogni follicolo viene aspirato mediante l'utilizzo di una siringa da 10 ml. La normale procedura clinica di aspirazione degli ovociti è stato modificata per permettere di aspirare ogni fluido follicolare singolarmente (Fig.3). Ad ogni fluido follicolare raccolto corrisponde un ovocita derivante dallo stesso follicolo.

I campioni di fluido follicolare aspirati dall'antro del follicolo antrale, sono stati raccolti in criotube sterili. I campioni di fluido follicolare aspirati senza la presenza dell'ovocita sono stati scartati. Ogni fluido follicolare è stato centrifugato per eliminare i detriti cellulari e aliquotato in criotube sterili correttamente siglati con l'identificativo della paziente (ID), il numero corrispondente all'ovocita a cui apparteneva e la data del prelievo. Appena prelevati sono stati raccolti dal nostro

laboratorio per effettuare subito a fresco l'analisi delle 27 citochine tramite multiplex-bead based assay.

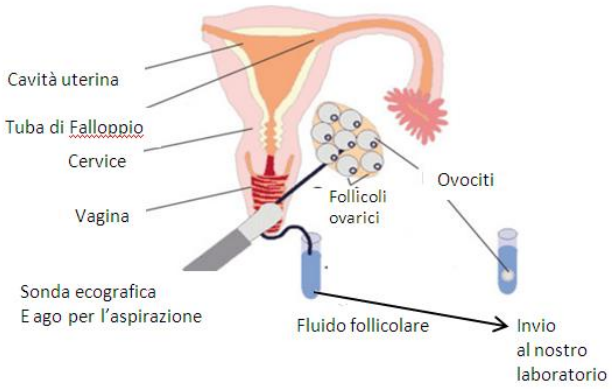


Fig.3 Aspirazione e raccolta degli ovociti e dei singoli fluidi follicolari

3.3 Fertilizzazione degli ovociti e trasferimento dell'embrione nella cavità uterina

A seguito del pick up ovocitario le cellule della corona radiata e del cumulo ooforo sono state rimosse come da pratica del centro Demetra. Le cellule della corona e del cumolo ooforo sono state sottoposte a trattamento con ialuronidasi 80 UI (Irvine Scientific, Santa Ana, CA, Stati Uniti d'America). Lo spermatozoo è stato micro-iniettato all'interno del citoplasma dell'ovocita sotto guida microscopica mediante un micromanipolatore. Lo spermatozoo è stato mantenuto in terreno con polivinilpirrolidone PVP al 7% (Irvine Scientific, Santa Ana, CA, Stati Uniti d'America) che opera un'azione di rallentamento della motilità spermatica. Sono stati ovviamente inoculati tutti gli ovociti prelevati. Gli ovociti inoculati sono stati mantenuti in coltura in 50 microlitri di terreno Continuous Single Culture™

Complete (CSCM-C) (Irvine Scientific, Santa Ana, CA, Stati Uniti d'America) in olio a 37°C in presenza di CO2 al 5%. In seguito è stato valutato dall'operatore al microscopio l'aspetto degli embrioni. A 46 ore sono stati valutati il grado di frammentazione (grado A :0-5%, grado B: >5 e <15%, grado B/C: >15 e <30%, grado C: >30% e <50%. grado D >50%; grado E=embrione non evolutivo), il numero di blastomeri (grado A :4, grado B: 2-4, grado B/C: 2-6, grado C:2-6. grado D:2-6; grado E=embrione non evolutivo) e il regolare aspetto di ogni blastomero (regolare per il grado A e B, irregolare per il grado C, D ed E). La qualità della blastocisti, stimata al giorno 5, viene valutata da tre parametri: la sua grandezza (numero che varia da 1 a 6), la qualità della massa interna (grado A: cellule molto compatte, grado B: diverse cellule raggruppate in modo libero, grado C: poche cellule) e la qualità del trofoectoderma (grado A: molte cellule appiattite in strato coeso, grado B: poche cellule epitelio lasso, grado C: pochissime cellule molto larghe). Gli embrioni sono stati trasferiti al giorno 5 in stadio di blastocisti. I criteri per la valutazione degli embrioni/blastocisti utilizzati dal centro di procreazione sono rappresentati dai criteri di Gardner (Gardner DH, 2000).

3.4 Dosaggio delle citochine e chemochine nei fluidi follicolari mediante tecnica multiplex bead-based assay

La tecnologia multiplex bead-based assay permette di misurare simultaneamente diverse tipologie di molecole in un unico pozzetto, in un volume ridotto di campione. Nel caso del nostro studio sono state dosate 27 citochine e chemochine nei fluidi follicolari comprese il G-CSF e l'IL-15. La tecnologia multiplex bead-based assay utilizza biglie magnetiche che vengono colorate internamente con una miscela

di due coloranti uno che emette nello spettro del rosso e uno che emette nello spettro dell'infrarosso. A biglie con spettro di emissione diverso sono legati degli anticorpi monoclonali anti-citochine fornite da ditte farmaceutiche. Il kit utilizzato in questo studio è il 27-plex human cytokine (Biorad Hercules, California CA Stati Uniti d'America) che analizza la presenza delle seguenti molecole: Interleuchina(IL)-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 IL-13, IL-15, IL-17, Eotaxin, Fibroblast Growth Factor (FGF), G-CSF, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), IFN-G, Interferon gamma-induced protein 10 (IP-10), Monocyte Chemoattractant Protein 1 (MCP-1), Macrophage Inflammatory Proteins-1α (MIP-1α), Macrophage Inflammatory Proteins-1β (MIP-1β), Platelet-derived growth factor (PDGF), Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), Tumor necrosis factor-α (TNF-A), Vascular Endothelial Growth Factor (VEGF). Ogni saggio è stato effettuato mediante le informazioni fornite dalla ditta.

La miscela delle biglie legate ad anticorpi anti-citochine (50µl) (Biorad Hercules, California CA, Stati Uniti d'America) viene incubata in un pozzetto di una piastra da 96 (Greiner bio-one, North Carolina NC, Stati Uniti d'America), con 50 µl di liquido follicolare e 50 µl della curva standard di riferimento con concentrazioni scalari (1:4) di tutti gli analiti analizzati (Fig.4A) . Dopo un'incubazione di 30 minuti su di un agitatore orbitale (800±50 rpm) a temperatura ambiente e 3 lavaggi per la rimozione delle proteine non legate, si aggiunge 25 µl pozzetto della miscela di anticorpi monoclonali anti-citochine biotilinati (Biorad Hercules, California CA, Stati Uniti d'America). Dopo un'incubazione di 30 minuti su di un agitatore orbitale (800±50 rpm) a temperatura ambiente e 3 lavaggi, si aggiunge 50 µl per pozzetto della streptavidina coniugata con la ficoeritrina che ha la funzione di reporter di

fluorescenza. Dopo un'incubazione di 10 minuti su di un agitatore orbitale (800±50 rpm) a temperatura ambiente e 3 lavaggi, vengono aggiunti 125µl di Assay buffer (Biorad Hercules, California CA, Stati Uniti d'America) e la piastra viene poi letta dallo strumento, Bioplex (Biorad Hercules California CA, Stati Uniti d'America) (Fig.4B), opportunamente settato. Il sistema prevede l'aspirazione del singolo pozzetto e il passaggio delle biglie in singola stringa attraverso due laser che eccitano ogni biglia. Un laser emette nel rosso (a 635 nm) e registra quale biglia lo strumento sta analizzando. L'altro laser emette nel verde (a 532 nm) ed eccitando la ficoeritrina genera il segnale reporter che verrà inviato ad un fotomoltiplicatore e restituirà la quantità relativa di quell'analita. Un software (Bioplex manager 4.0) restituirà i dati di analisi in termini di intensità di fluorescenza media (MFI), che saranno trasformati in pg\ml. La percentuale del coefficiente di variazione è, inferiore al 5% per la variazione intra-saggio e minore del 10% per quello inter-saggio.

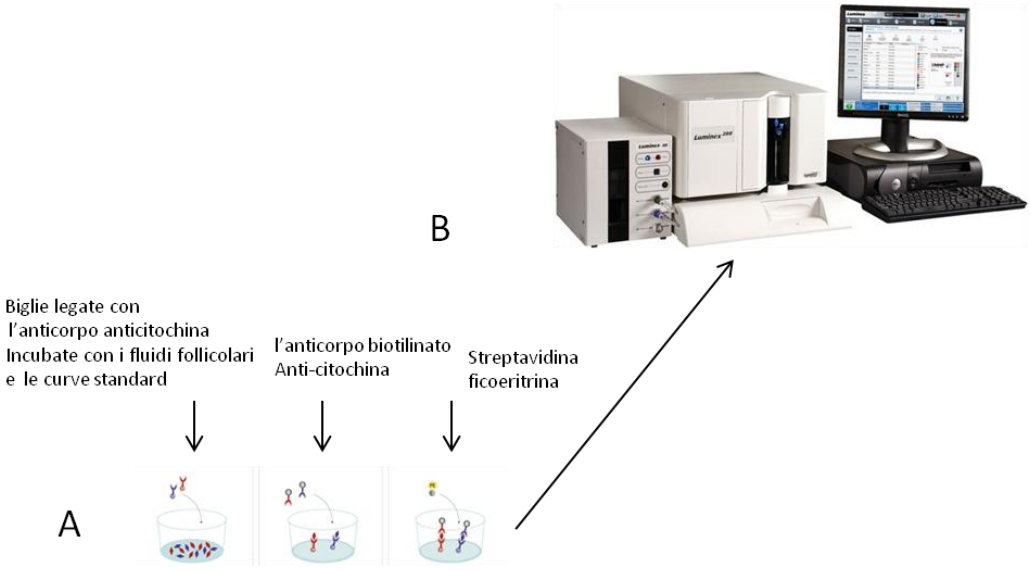


Fig.4 Principio del saggio multiplex bead based assay (A) e Bioplex 200 (B)

Durante lo studio la ditta Biorad che commercializza il kit 27-plex ha cambiato alcuni componenti nel kit. Quindi in circa la metà delle pazienti di ogni gruppo i dosaggi sono stati eseguiti con un lotto diverso, rispetto a quello utilizzato per dosare i fluidi follicolari delle pazienti di entrambi i gruppi arruolate all'inizio dello studio.

3.5 Database

E' stato costruito un database che raccoglie tutte le informazioni relative alle pazienti, contenente il loro ID (identificativo per la privacy), l'età, la data delle ultime mestruazioni, la data del pick up, la tipologia di infertilità, il numero di ovociti recuperati, il numero e l'identificativo degli ovociti che sono andati in metafase II, gli ovociti fertilizzati, il tasso di fertilizzazione (espresso come il rapporto tra numero di embrioni vitali ottenuti dopo fertilizzazione e il numero totale degli ovociti fertilizzati), il numero identificativo delle blastocisti ottenute e il numero della blastocisti trasferita, la positività o meno alle beta-hCG e la presenza o assenza di camera gestazionale identificata mediante controllo ecografico. A tutti questi dati anagrafici e clinici si aggiungono tutti i dati relativi ai dosaggi. Per ogni fluido follicolare sono presenti i valori quantitativi di 27 citochine e chemochine (IL-1β, Il-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 IL-13, IL-15, IL-17, Eotaxin, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α, VEGF).

3.6 Analisi statistica

I livelli di citochine nei fluidi follicolari individuali sono stati analizzati su molteplici piani statistici mediante il software SPSS. Innanzi tutto è stata fatta un analisi di distribuzione del dato, attraverso valutazione della normalità secondo il test di Kolmogrov-Smirnov e confermati mediante il test di Shapiro-Wilk. Successivamente è stata effettuata un'analisi con il test non parametrico di Mann-Whitney nelle varie categorie di variabili. E' stata valutata la correlazione mediante analisi multivariata delle varie citochine tra di loro e di ogni citochina con il tasso di fertilizzazione, con lo score morfologico e con l'avvenuta gravidanza. E' stata infine costruita una curva ROC per definire se una delle molecole da noi analizzate potesse essere un fattore predittivo di impianto. Per costruire la curva ROC abbiamo preso in considerazione due categorie "assenza di camera gestazionale" e "presenza di camera gestazionale". La discriminazione tra queste due categorie rispetto alla concentrazione delle 27 citochine misurate in ogni fluido follicolare individuale è stata analizzata mediante la costruzione di una curva ROC con il software di analisi statistica SPSS. Per disegnare la curva ROC è stata valutata la sensibilità, la specificità e la proporzione dei falsi positivi (1-specificità) relative ad una serie di valori soglia della molecola presa in analisi. La scelta dei valori soglia viene effettuata dal software mediante algoritmo specifico. Il calcolo dell'area sotto la curva misura l'accuratezza con cui una citochina può riuscire a discriminare tra un ovocita che darà origine ad una blastocisti che si impianterà nella cavità uterina e darà luogo ad una gravidanza, da un ovocita che non porterà a gravidanza una volta fertilizzato e coltivato per ottenere una blastocisti a 5 giorni. Il valore di una curva ROC varia da 0 a 1. Se si ottiene un valore di 0,9-1 la discriminazione è perfetta, nulla per 0,5-0,6 o inferiore. Un valore p inferiore a 0,05 risulta significativo.

4. RISULTATI

4.1 Pazienti

Nell'arco di questo studio sono state reclutate 49 donne infertili trattate mediante protocollo ICSI. Le pazienti avevano una normale riserva ovarica, ed un età nel range di 30-40 anni. Le donne sono state suddivise in modo randomizzato in 2 gruppi: 25 pazienti nelle quali è stata trasferita una singola blastocisti sulla base dei livelli di G-CSF e IL-15 nel fluido follicolare dell'ovocita corrispondente e 24 pazienti nelle quali la blastocisti trasferita è stata scelta in base allo score morfologico.

Non ci sono differenze significative nel parametro "età" tra i due gruppi di pazienti studiate (Tab.2) e quindi i due gruppi sono risultati statisticamente paragonabili. Durante lo svolgimento del progetto il kit di determinazione delle citochine (27 human cyrtokine, Biorad, Hercules California CA, Stati Uniti d'America) ha subito una variazione nei reagenti utilizzati (anticorpi monoclonali anti-citochine) e questo ha determinato un'inaspettata variazione nella quantificazione delle citochine nei campioni. Prima quindi di procedere all'analisi dei dati abbiamo dovuto valutare se i due lotti fossero equiparabili. Entrambi i gruppi 1 e 2 risultano comparabili a livello statistico per quanto riguarda: quantità di pazienti analizzati, età, numero di ovociti, percentuale di fertilizzazione e di formazione di blastocisti (Tab.2). I due lotti come numero di casi selezionati, utilizzati per l'analisi delle citochine, sono ugualmente distribuiti (Tab.2). Purtroppo però dall'analisi dei due lotti sono emerse delle criticità. I livelli del G-CSF e dell'IL-15 presentano grande variabilità tra i due lotti e sono risultati essere molto dispersi. Nonostante la diversità del campione siamo

comunque andati a valutare se ci fossero differenze tra le citochine dosate. Non si evidenziano differenze tra i livelli delle citochine nei fluidi follicolari dei due gruppi, e si è proceduto comunque ad analizzare il tasso di fertilizzazione e di impianto. La percentuale di positività alle beta-hCG è maggiore nel "gruppo 2" sia considerando solo le beta-hCG sia considerando anche l'avvenuto impianto, ma la differenza tra i due gruppi non raggiunge la significatività. Le criticità emerse dallo scarso numero di pazienti reclutate, unite alla variabile ulteriore del cambio del lotto del kit in utilizzo, hanno reso impossibile poter avere un dato confidente. Nell'ottica di ampliare la casistica dello studio prospettivo (proseguendo il reclutamento), siamo comunque andati ad analizzare se, considerando i due gruppi come un unico gruppo di pazienti, il G-CSF si riconfermasse come marcatore predittivo della competenza ovocitaria e se altre citochine emergessero come biomarker del tasso di impianto. E' stato possibile effettuare questo tipo di analisi dato che il nostro laboratorio ha dosato le citochine nel fluido follicolare di entrambi i gruppi delle pazienti.

		Gruppo 1		Gruppo 2		
N di pazienti		24		25		p
Età (media, ds)		35	4	34.5	4	0.932
Lotto del kit (n°,%)	1	16	66	16	64	0.845
	2	8	33	9	36	
N° OVO RECUPERATI (mediana, IRQ)		10.5	6-13	9	7.5-12.5	0.689
Fert% (mediana, IRQ)		77.5	66-92	77	63-93	0.984
Blastocisti (n, %)		56	23	62	24	0.759

Tab.2 Età (media±DS) dei due gruppi di pazienti, numero (n° e percentuale) di pazienti analizzate con i due diversi lotti del kit di dosaggio delle citochine (lotto 1 e lotto 2), n° ovociti recuperati (mediana, scarto interquartile), percentuale di fertilizzazione (mediana, scarto interquartile) e n° di blastociti ottenute nei due gruppi di pazienti oggetto di studio (numero, percentuale).

4.2 Fluidi follicolari individuali raccolti ed analizzati

Sono state arruolate 49 pazienti suddivise in modo randomizzato in: 24 pazienti del gruppo 1, e 25 pazienti del gruppo 2 come mostrato nella tabella 3. All'interno dei 2 gruppi di pazienti sono stati analizzati un totale di 496 fluidi follicolari (gruppo1=242 ; gruppo2=254), 132 blastocisti totali ottenute (gruppo1=56; gruppo 2=76), 18 blastociti con positività alle beta-hCG (gruppo 1=8 ; gruppo 2=10) e 14 blastocisti totali impiantate nella cavità uterina (gruppo 1=6; gruppo 2=8) (Tab.3).

	Gruppo 1	**Gruppo 2**	**Totale**
Pazienti arruolate	24	25	49
Fluidi Follicolari analizzati	242	254	496
Blastocisti analizzate	56	76	132
Blastocisti con Beta hCG+	8	10	18
Blastocisti impiantate nella cavità uterina	6	8	14

Tab.3 Numero di campioni analizzati nello studio, quantità dei campioni di fluidi follicolari analizzati, numero di blastocisti con caratteristiche di impianto dei due gruppi di pazienti.

4.3 Citochine nei fluidi follicolari e tasso di fertilizzazione

Essendo una distribuzione campionaria si rende necessario prima di ogni analisi determinare se il campione è distribuito normalmente oppure no. Per verificare la normalità della distribuzione è stato utilizzato un test parametrico di Kolmogorov-Smirnov, con un'ulteriore conferma utilizzando il test di Shapiro-Wilk anch'esso test di normalità per piccole distribuzioni campionarie. La netta e forte significatività ($p=0.0001$) di entrambi i test hanno chiaramente indicato come il dato non sia distribuito normalmente e quindi il test statistico da noi applicato per l'analisi dei dati è di tipo non parametrico (test di Mann-Whitney).

Abbiamo valutato se i livelli di citochine nei fluidi follicolari fossero correlate al tasso di fertilizzazione degli ovociti. Il tasso di fertilizzazione definisce quanti ovociti vengono effettivamente fertilizzati rispetto a tutti gli ovociti che vengono inoculati con lo spermatozoo durante protocollo ICSI. Il tasso di fertilizzazione varia dal 33% al 100% nel nostro studio.

Abbiamo analizzato le differenze nei livelli delle citochine nei fluidi follicolari nel caso in cui il tasso di fertilizzazione era maggiore dell'80% (tasso di fertilizzazione elevato) e nel caso in cui il tasso di fertilizzazione era minore dell'80%.

Con un "cut off" di fertilizzazione rappresentato dall'80% i livelli di IL-4, IL-8, IL-9, eotassina, IFN-γ, IP-10, RANTES, TNF-α e VEGF sono risultati significativamente più elevati nei FF di ovociti con tasso di fertilizzazione maggiore dell'80%, mentre

l'IL-7 e l'MCP-1 sono risultati statisticamente più bassi negli ovociti con tasso di fertilizzazione maggiore dell'80 % (Fig.5).

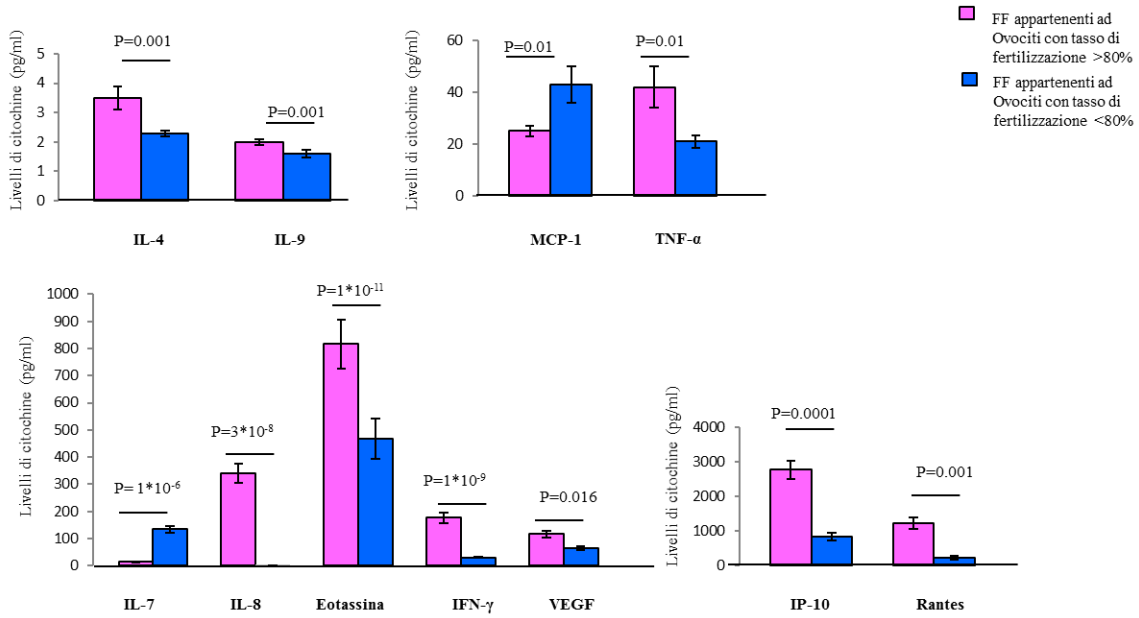
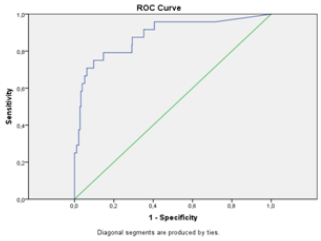


Fig.5 IL-4, IL-9, MCP-1, TNF-α, IL-7, IL-8, Eotassina, IFN-γ, VEGF, IP-10 e RANTES dosati nei FF derivati da follicoli con ovociti che mostrano un tasso di fertilizzazione elevato (>80%) rispetto a quelli che mostrano un tasso di fertilizzazione inferiore (<80%).

Infine un'analisi mediante curve ROC ha valutato se una o più delle 27 citochine misurate potesse essere un efficace parametro di identificazione dell' ovocita con tasso più alto di fertilizzazione dopo l'iniezione dello spermatozoo nel citoplasma della cellula. Tra le 27 citochine il VEGF ($p=2*10^{-10}$) è risultato efficace come marcatore predittivo di fertilizzazione come mostrato dalla figura 6. Il valore di cut off che ci indica il valore soglia al di sopra del quale il nostro test risulta attendibile, risultato attraverso il calcolo dell'indice di Youden, è di 69 pg/ml per il VEGF.

Test Result Variable(s)	Area
VEGF	,883



VEGF $p=2*10^{-10}$

Fig.6 IL VEGF rappresenta un marcatore predittivo di un alto tasso di fertilizzazione. Analisi mediante curva ROC del VEGF dosato nei FF in base al tasso di fertilizzazione. AUC_{ROC} è di è di 0.883 per VEGF ($p=2*10^{-10}$).

4.4 Rapporto tra le citochine nel fluido follicolare e morfologia della blastocisti

Per studiare la variazione del livello di citochine presenti nei FF in funzione della morfologia, abbiamo creato 2 gruppi di blastocisti il gruppo delle blastocisti con score morfologico "A/B" cioè quello delle blastocisti con morfologia migliore e il gruppo delle blastocisti con score morfologico "C", che presentano una morfologia peggiore.

Le uniche due citochine che se presenti ad alti livelli si associano ad una morfologia A\B (di livello superiore) sono IL-1Ra (p=0,014) e il già noto G-CSF (p=0,02) (Tab.4).

	Score morfologico della blastocisti A/B	Score morfologico della blastocisti C	P
IL-1β	6.29±5.67	5.98±5.64	
IL-1Ra	3174.91±2679.2	145.9±75.94	0.014
IL-2	8.24±4.16	0.35±0.19	
IL-4	2.96±0.97	0.96±0.21	
IL-5	5.94±5	20.68±19.55	
IL-6	16.27±10.31	24.73±21.98	
IL-7	6.46±1.94	27.24±19.15	
IL-8	660.54±419.4	323.49±159.82	
IL-9	7.07±5.31	1.7±1.14	
IL-10	140.93±131.71	26.51±25.32	
IL-12	8.4±5.3	29.67±26.71	
IL-13	5.7±3.95	6.69 ± 4.49	
IL-15	2.7±0.9	2.50±0.	
IL-17	4.68±3.37	0.32±0.24	
EOTAXIN	739.47±260.3	611.19±321.18	
FGF	8.45±2.81	5.05±3.32	
G-CSF	28±8,5	16±4	0.02
GM-CSF	0.19±0.19	0±0	
IFN-γ	84.83±38.67	134.13±91.42	
IP-10	635.43±191	770.22±373.12	
MCP-1	116.21±57.76	169.37±146.61	
MIP-1α	0.49±0.13	0.28±0.12	
MIP-1β	3.54±0.87	5.75±2.38	
PDGF	77.23±32.74	25.31±18.54	
RANTES	291.68±132.19	58.06±26.59	
TNF-α	23.84±6.09	15.21±5.62	
VEGF	191±68	170.7±144.08	

Tab.4 IL-1Ra e G-CSF nei fluidi follicolari si associano ad uno score morfologico della blastocisti con score morfologico migliore. Analisi mediante test non parametrico di Mann-Whitney, delle 27 citochine nei FF individuali in base alla morfologia migliore (A/B) o peggiore (C).

Per il G-CSF è stato possibile costruire una curva ROC discriminante tra morfologie di blastocisti A\B e C, ed il G-CSF è risultato un fattore predittivo della morfologia A\B (migliore). L'area sotto la curva è risultata pari a 0,7 con un $p<0.05$ (Fig.7). Il valore soglia al di sopra del quale il nostro test risulta attendibile, risultato attraverso il calcolo dell'indice di Youden, per il G-CSF è di 21 pg/ml.

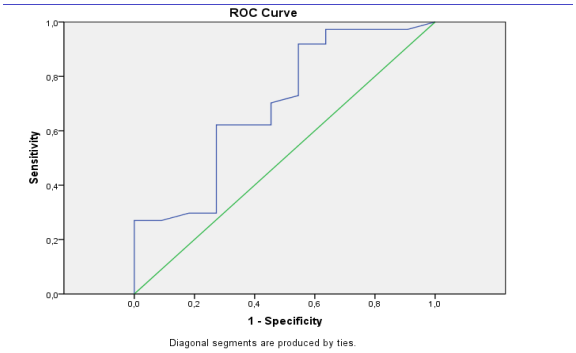


Fig.7 G-CSF, nel fluido follicolare è un fattore predittivo della morfologia A o B (score morfologico più elevato). AUC_{ROC} è di 0,7 ($p<0.05$) per il G-CSF.

4.5 Rapporto tra citochine nel fluido follicolare ed avvenuto impianto della blastocisti

Al fine di analizzare l'associazione tra le citochine dosate nei FF e l'impianto della singola blastocisti, abbiamo eseguito un'analisi statistica delle 27 citochine dosate nei FF in funzione della positività alle beta-hCG e alla presenza della camera gestazionale che indica l'avvenuto impianto della blastocisti. Solamente il G-CSF ($p=0,035$) e l'IL-15 ($p=0,003$) sono risultate significativamente più elevate nei fluidi follicolari appartenenti a follicoli da cui provengono gli ovociti che hanno generato le blastocisti trasferite nella cavità uterina che si sono impiantate (Fig.8).

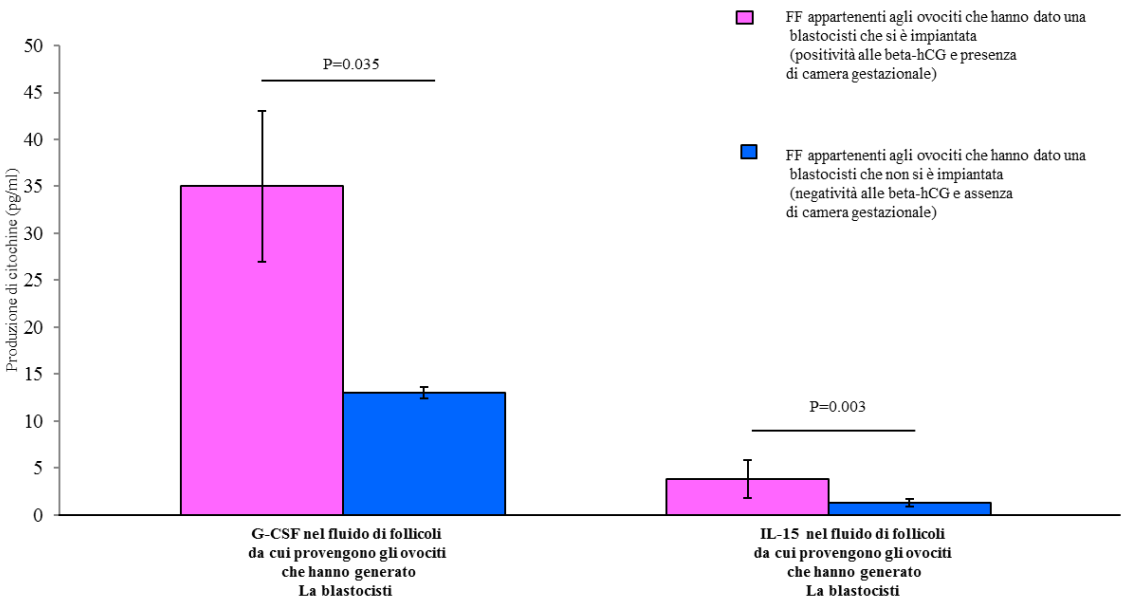


Fig.8 I livelli di G-CSF e IL-15 nei FF sono associati alle blastocisti (derivate dagli stessi follicoli) che si sono impiantate nella cavità uterina. Analisi mediante test di Mann-Whitney dei livelli quantitativi del G-CSF e dell'IL-15 nei FF associati alle blastocisti trasferite nella cavità uterina rispetto alla positività alle beta-hCG e alla presenza di camera gestazionale .

E' stata costruita una curva ROC per determinare se una delle 27 citochine del FF potesse essere un marcatore predittivo dell'impianto. L'unica citochina che si è rivelata essere marcatore predittivo dell'impianto della blastocisti è il G-CSF presente nel FF mostrato in figura 9. Il valore dell'area sotto la curva è di 0.701 con una significatività pari a p=0,035. Il valore soglia al di sopra del quale il nostro test risulta attendibile, risultato attraverso il calcolo dell'indice di Youden, per il G-CSF è di 24 pg/ml.

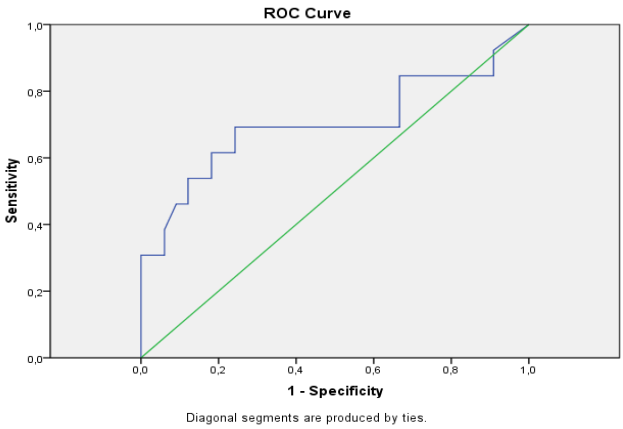


Fig.9 G-CSF nei FF marcatore predittivo dell'impianto della blastocisti. Curva ROC per il G-CSF nel FF rispetto alla positività alle beta-hCG e alla presenza della camera gestazionale. AUC_{ROC} è di 0,701 per il G-CSF (p=0,035)

4.6 Rapporto tra citochine nei FF e tipologia di infertilità della coppia sottoposta a ICSI

Infine siamo andati ad analizzare i dati ottenuti sulle 27 citochine dividendo le nostre pazienti in diversi gruppi in base alla tipologia di infertilità (fattore femminile, fattore maschile, idiopatica, tubarica e fattore femminile e maschile) (Tab.1). Non abbiamo evidenziato differenze statisticamente significative per il parametro "età" tra i diversi gruppi che risultano così statisticamente paragonabili.

L'analisi dei livelli di citochine nei FF tra i diversi tipi di infertilità ha rivelato una diminuzione di G-CSF nel fluido follicolare di donne con un fattore legato all'infertilità femminile (Fig.10), mentre nessuna differenza è stata riscontrata per le altre citochine nel FF e per gli altri tipi di infertilità. La ridotta presenza di G-CSF nel FF sembra quindi essere associata ad un'infertilità femminile non di origine tubarica.

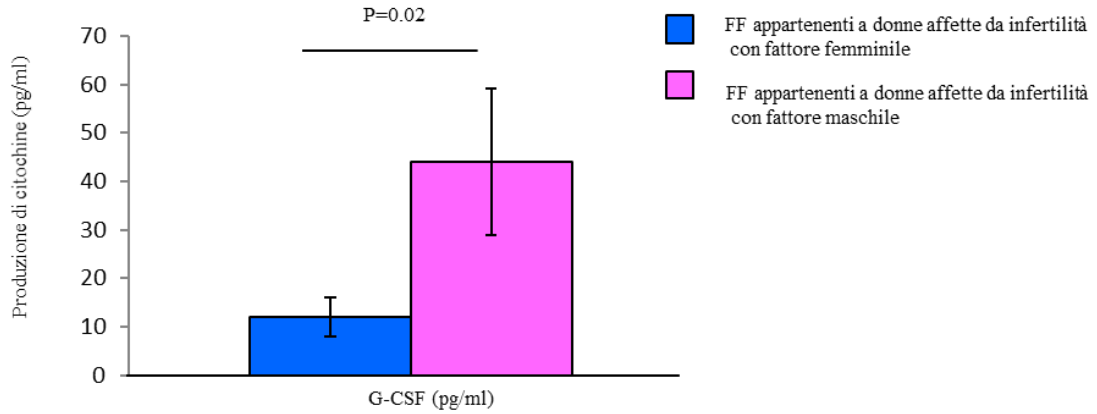


Fig.10 G-CSF misurato nei FF di donne con fattore femminile di infertilità e nei FF di donne con fattore maschile di infertilità.

5. DISCUSSIONE

L'attuale assenza di un criterio che definisca la competenza ovocitaria, rappresenta il limite delle tecniche di procreazione medicalmente assistita ed in particolare dell'applicazione della politica del trasferimento del singolo embrione. Tale politica è un obbiettivo che si pone la comunità medico-scientifica nell'ottica di annullare le complicanze legate alle gravidanze multiple dovute a cicli di procreazione medicalmente assistita, per i quali 2 o 3 embrioni sono trasferiti nella cavità uterina al fine di aumentare il tasso di impianto.

Il nostro studio prospettico randomizzato si è basato sulla valutazione del G-CSF e dell'IL-15 nel fluido follicolare individuale, come criterio per selezionare, prima della fertilizzazione, l'ovocita con il maggior potenziale di generare un embrione con elevato tasso di impianto nei cicli ICSI, dopo stimolazione ovarica con FSHr e LHr.

Questo stimolo è stato scelto perché abbiamo dimostrato essere adatto ad aumentare i livelli di G-CSF nei FF delle donne (dati non mostrati).

Lo studio prevedeva il transfer di una singola blastocisti nella cavità uterina (SET) scegliendo la blastocisti sulla base di 2 criteri diversi. Il primo criterio era la morfologia della blastocisti (A, B o C), quindi il criterio classico applicato dalla maggioranza dei centri di PMA ad oggi. Il secondo criterio era basato sui livelli di G-CSF nei FF, che dovrebbero essere più alti tra quelli ottenuti nella coorte di FF aspirati dalle pazienti e sui livelli di IL-15 nei FF che invece dovrebbero essere più bassi tra quelli ottenuti nella stessa coorte (Ledee N, 2008, 2011).

La selezione dell'ovocita basata sulla determinazione dei livelli di citochine nei FF è apparsa a molte pazienti come una metodologia che esce dagli schemi della tradizionale pratica clinica, e quindi non ancora affidabile per indurre la donna a sceglierla in sicurezza.

Quindi non è stato semplice ottenere il consenso da parte di molte pazienti. Durante l'arco dei 3 anni di tesi soltanto 49 pazienti (25 per il "gruppo delle citochine" nei FF e 24 per il "gruppo della morfologia della blastocisti") sono state arruolate, mentre lo studio prevedeva un arruolamento di 100 pazienti.

Abbiamo intenzione di continuare il reclutamento e ampliando la casistica, pensiamo di poter confrontare i due gruppi di pazienti, confidenti che il risultato abbia un valore prognostico più robusto data una maggiore taglia del campione.

In effetti, con questa casistica non abbiamo ottenuto alcun dato significativo. Tale analisi inoltre non è risultata conclusiva, dato che si è manifestata anche una criticità a livello tecnico. Il kit per il dosaggio delle citochine (Biorad, Hercules California CA, Stati Uniti d'America) ha subito una variazione di lotto, con modificazioni di alcuni reagenti, a metà dello studio. Questo ha provocato una modificazione dei livelli di citochine determinate nei FF, in special modo dell'IL-15 e del G-CSF. In effetti lo studio statistico ha rivelato una differenza nella determinazione delle citochine per i 2 diversi lotti di kit Biorad. Unito alla piccola taglia del campione questa criticità ha reso impossibile comparare i due gruppi per lo studio prospettico randomizzato, ma ha permesso di ottenere molteplici risultati che mettono in evidenza come la determinazione di citochine nei FF possa migliorare i risultati delle tecniche di PMA.

Abbiamo infatti analizzato i dati considerando che il nostro campione è rappresentato da un unico gruppo di donne che hanno approcciato la procreazione medicalmente assistita con protocollo ICSI, dopo stimolazione ovarica con FSHr in associazione a LHr. Abbiamo condotto l'analisi dei dati a più livelli. Abbiamo esaminato se una o più citochine fossero predittive del miglior tasso di fertilizzazione dell'ovocita, del migliore score morfologico della blastocisti e infine del successo dell'impianto dopo un solo protocollo di stimolazione con FSHr in associazione a LHr, mai ancora studiata prima. Ad oggi sono stati sempre studiati dei protocolli di stimolazione diversi, che sono stati combinati insieme nei vari studi.

5.1 Citochine nei FF come biomarker di fertilizzazione

Secondo le stime dei vari registri internazionali (Americano ed Europeo) il tasso di fertilizzazione nei centri di PMA si aggira intorno al 75%. Il tasso di fertilizzazione in un protocollo ICSI, definisce quanti ovociti vengono effettivamente fertilizzati rispetto a tutti gli ovociti che sono stati inoculati. Abbiamo scelto un tasso di fertilizzazione dell'80% come cut off tra i due gruppi, quello che comprendeva i fluidi che corrispondevano a ovociti con un tasso di fertilizzazione maggiore dell'80%, e il gruppo dei fluidi follicolari che corrispondevano a ovociti con un tasso di fertilizzazione minore dell'80%. E' stato scelto un cut off dell'80% poiché, essendo superiore al tasso medio registrato a livello internazionale, potessimo essere confidenti sulla robustezza del dato ottenuto. I livelli dell'IL-4, dell'IL-9, del TNF-α, dell'IL-8, dell' Eotassina, dell'IFN-γ, del VEGF, dell'IP-10 e del Rantes sono risultati più elevati nei fluidi follicolari associati ad ovociti con un tasso di fertilizzazione maggiore dell'80%. Mentre i livelli dell'MCP-1 e dell'IL-7 sono

risultati più bassi nei fluidi follicolari associati ad ovociti con un tasso di fertilizzazione superiore all'80%.

Il fluido follicolare aspirato contiene tutti i metaboliti che si sono accumulati durante lo sviluppo e la maturazione del follicolo. Il processo infiammatorio che avviene durante la crescita follicolare, l'ovulazione e la formazione del corpo luteo potrebbe dipendere dai leucociti presenti nel follicolo e nel cumulo ooforo (Piccinni MP 2002, 2007). Questi leucociti, in particolare i linfociti T ma anche i macrofagi, producono citochine. Le citochine sono regolatori della fisiologia che contribuiscono alla creazione di un microambiente che potrebbe supportare la crescita e la maturazione del follicolo (Piccinni MP, 2007; Guzeloglu-kayisli U , 2009; Field S, 2014; Sarapik A, 2012). La qualità dell'ovocita è molto legata alla composizione del microambiente follicolare (Field S, 2014). Il fluido follicolare, essendo ricco di fattori di crescita e di citochine nonchè data la sua prossimità all'ovocita, potrebbe influenzare il corretto processo di maturazione del follicolo (Dumesic D, 2015; Hashish N, 2015; Zamah A, 2015).

La figura 5 descrive l'aumento di livelli di IL-4 nei FF quando i tassi di fertilizzazione sono più elevati (>80%). L'IL-4, citochina prodotta dalla sottopopolazione T helper 2 (Romagnani S, 1991), è una citochina non solo essenziale al successo della gravidanza quando è prodotta dai linfociti T helper dell'endometrio perché assicura una tolleranza all'allotrapianto fetale (Piccinni MP, 2005, 2015; Lombardelli L, 2016), ma anche quando è presente nel fluido follicolare, dove sembra correlare con il successo della ICSI. In effetti è stato osservato che l'IL-4 aumenta con il tasso di fertilizzazione (Alhilali MJS, 2019). Questi dati confermano quelli del nostro gruppo. Questa citochina potrebbe essere stata prodotta dai linfociti T CD4+ presenti nel cumulo ooforo. Abbiamo in effetti riportato

precedentemente che i linfociti T helper presenti nel cumulo producono livelli alti di IL-4 e LIF in confronto ai linfociti T helper dell'ovaio o del sangue periferico di donne sottoposte a ICSI (Piccinni MP 2001, 2007).

I linfociti T CD4+ Th2 non producono solo IL-4, ma possono produrre anche IL-9, che abbiamo visto essere associata nei FF, come la IL-4, ad un tasso di fertilizzazione maggiore dell'80%. Inoltre il fattore chemiotattico per le cellule Th2, che quindi potrebbe attrarre queste cellule nel follicolo, l'Eotassina si associa a IL-4 e IL-9 nei FF derivanti da follicoli appartenenti ad ovociti aspirati che hanno mostrato un tasso di fertilizzazione più elevato (>80%). Questi dati suggeriscono che la presenza di citochine di tipo Th2 (IL-4, IL-9 e Eotassina) e quindi di cellule Th2 probabilmente presenti nel follicolo, forse a livello del cumulo ooforo, possa influenzare positivamente la fertilizzazione e quindi il successo della ICSI.

Altre citochine di tipo pro-infiammatorio si associano ad un tasso di fertilizzazione elevato (80%), come il TNF-α. (Fig.5). Inoltre delle chemochine come IL-8 e Rantes sono anch'esse associate ad un tasso di fertilizzazione elevato (>80%). Queste chemochine attraggono le cellule immunitarie (come i linfociti T attivati, macrofagi, polimorfonucleati e cellule dendritiche), nelle sedi di infiammazione o infezione.

Il TNF-α che è stato descritto nel fluido follicolare (Wang LJ, 1992; Bili H, 1998) ha un ruolo importante nell'ovaio. Risulta essere coinvolto in molti meccanismi necessari alla follicologenesi, e raggiunge un picco di produzione durante l'ovulazione in un normale ciclo naturale. Non sorprende la presenza di citochine pro-infiammatorie e chemochine che attraggono leucociti nel follicolo. Infatti il processo ovulatorio è stato descritto come un processo infiammatorio modulato da citochine pro-infiammatorie e chemochine come l'IL-6, l'IL-8, il TNF-α, e il sistema

dell'IL-1 (Gerard N, 2004). E' stato altresì dimostrato che i livelli di IL-8 associati all'IL-6 aumentano nei fluidi follicolari di donne che hanno sviluppato, in seguito al trattamento farmacologico per la stimolazione ovarica nella PMA, la sindrome dell'iperstimolazione ovarica (OHSS), che rappresenta la più severa complicanza di tale trattamento. L'IL-8 è stata proprio identificata sia a livello del fluido follicolare che a livello del siero di pazienti affetti da OHSS (Chen CD , 2000).

Il RANTES è stato descritto da alcuni gruppi come più elevato nei FF ottenuti da ovociti che hanno generato embrioni di alta qualità (Ledee N, 2008), mentre altri gruppi non hanno osservato un effetto positivo di questa chemochina, sulla qualità di embrioni o sul risultato del protocollo ICSI (Guerif F, 2007). Il suo ruolo quindi come discriminatore di ovociti con buon potenziale di impianto va maggiormente indagato, ma sembra invece essere associato ad un buon tasso di fertilizzazione.

Quindi le citochine pro-infiammatorie (TNF-α) e le chemochine associate (Rantes e IL-8), nel FF necessarie al processo infiammatorio dell'ovulazione, potrebbero anche influenzare l'esito di una fecondazione e quindi anche il tasso di fertilizzazione.

Potremmo ipotizzare che questi fattori prodotti dalle cellule immunitarie attratte in sede follicolare possano indurre l'ovocita secondario ad esprimere molecole in grado di favorire la sua fertilizzazione, come ad esempio il completamento della meiosi II raggiungendo lo stadio di ovulo maturo.

Nella fase periovultaoria si assiste ad un aumento della vascolarizzazione attraverso fattori angiogenetici tra cui il VEGF (Gutman G, 2008). Il VEGF sembra raggiungere il suo massimo livello quantitativo poco prima del picco dell'LH e potrebbe determinare la vascolarizzazione del follicolo periovulatorio. Questo fattore risulta predittivo della fertilizzazione dell'ovocita in cicli ICSI (Fig.6) nel

nostro studio. Infatti la presenza di VEGF sembra associarsi al successo della PMA. In effetti la presenza di polimorfismi nel gene *VEGF* aumenta il rischio di RIF (Recurrent implatation failure; quando l'impianto non avviene dopo 3 cicli di tecniche di PMA) (Turienzo A, 2018).

L'IFN-γ e l'IP-10, chemochina che attira i linfociti T CD4+ che producono proprio l'IFN-γ, chiamati Th1, sono risultati associati ad un tasso di fertilizzazione elevato (>80%).

L'IFN-γ principale citochina prodotta della sottopopolazione T helper 1, non è stato descritto nei FF (Gaafar TM, 2014; Boomsma CM, 2009). Un lavoro antecedente del nostro gruppo ha rilevato come livelli significativamente elevati di IFN-γ sono stati rilevati in FF di embrioni sottoposti a scissione precoce (Ledee N, 2008), ma in questo caso lo stimolo ovarico era diverso rispetto a questo studio e questo potrebbe aver giocato un ruolo sulla presenza delle citochine all'interno del FF. L'IFN-γ prodotto dalle cellule T di tipo Th1 è una citochina antagonista dei processi immunitari dell'IL-4, descritta precedentemente e prodotta dai linfociti Th2. L'IFN-γ potrebbe quindi essere presente nei FF per regolare le risposte mediate dai linfociti Th2 e quindi responsabile del controllo degli effetti dell'IL-4.

Nel nostro studio l'MCP-1 e l'IP-10 si muovono in senso opposto (l'una decrementa e l'altra aumenta rispettivamente) nei FF di ovociti che hanno un tasso di fertilizzazione maggiore dell'80%. Alcuni autori hanno riportato che una bassa concentrazione dell'MCP-1 e un'alta dell'IP-10 prodotte a livello delle secrezioni endometriali in donne sottoposte a tecniche di PMA, fossero legate all'impianto dell'embrione (Boomsma CM, 2009). Entrambe queste chemochine che giocano un ruolo nella migrazione delle cellule leucocitarie (Jones RL, 2004), sembrano essere

anche coinvolte nell'impianto, oltre che nella fertilizzazione (Drake PM, 2004). L'embrione ha in effetti i recettori per entrambe queste molecole (Dominguez F , 2003; Drake PM, 2004; Hanna J, 2006).

Oltre alla bassa concentrazione dell'MCP-1 nei FF associati ad un tasso di fertilizzazione alto (>80%), abbiamo osservato anche il decremento quantitativo dell'IL-7. L'IL-7 è una citochina che stimola la differenziazione delle cellule staminali ematopoietiche multipotenti in cellule progenitrici linfoidi e un fattore di crescita dei linfociti T. A livello del sistema riproduttivo è stata descritta come citochina coinvolta a livello della maturazione dell'ovocita. Il deficit dell'IL-7 è stato visto correlare con una scarsa conta ovocitaria e con ovociti di povera qualità (Ostanin AA, 2007). Quindi mentre sembra che la IL-7 debba essere diminuita per una migliore fertilizzazione, sembra che debba essere incrementata per la maturazione dell'ovocita.

5.2 Citochine nei FF e score morfologico della blastocisti derivata dalla fertilizzazione dell'ovocita associato allo stesso FF

In seconda analisi abbiamo valutato i livelli delle citochine presenti nei fluidi follicolari e lo score morfologico delle blastocisti che sono state poi trasferite nella cavità uterina. I gruppi che abbiamo paragonato mostravano una presenza di livelli significativamente più elevati dell'IL-1Ra e del G-CSF nelle blastocisti con morfologia migliore (score morfologico A\B) rispetto alle blastocisti con morfologia peggiore (score C) (Tab.4).

È ben noto come Il sistema della famiglia dell'IL-1 sia coinvolto nello sviluppo del follicolo. Questo sistema comprende anche l'IL-1Ra che rappresenta un fisiologico

recettore antagonista, che esplica la sua bioattività attraverso interazioni competitive del recettore per l'IL-1 (Arend WP, 1991).

L'IL-1Ra è stata già descritta come una citochina associata ai meccanismi che portano ad una gravidanza. Infatti è stata correlata ad un elevato tasso di successo dell'impianto (Spandorfer SD, 2000, 2003), e descritta nell'endometrio durante la fase secretoria del ciclo mestruale fisiologico (Simon C, 1993), e nell'embrione (Simon C, 1997).

Per quanto riguarda invece il G-CSF, questa citochina è stata ampiamente studiata nell'ambito della procreazione medicalmente assistita. E' stato dimostrato che i follicoli sono un importante sito di produzione di G-CSF. Il G-CSF qui prodotto raggiunge i suoi massimi livelli quantitativi prima dell'ovulazione (Salmassi A, 2004; Yanagi K , 2002). Quindi è possibile dedurre che la presenza di livelli elevati di G-CSF possa in ultima analisi essere specchio della corretta stimolazione ovarica che determina un'ampia crescita follicolare (Salmassi A, 2005). Abbiamo infatti dimostrato che lo stimolo ovarico influenza il profilo citochinico dei FF (lavoro in preparazione) in particolare FSHr insieme a LHr incrementa i livelli di G-CSF nei FF.

Come dimostrato dal nostro gruppo (Ledee N, 2008, 2011, 2011), il G-CSF dosato nei FF risulta essere un marcatore non invasivo predittivo del potenziale di impianto dell'embrione che deriva dall'ovocita corrispondente al follicolo da cui deriva il FF (Ledee N, 2010; 2008; 2011).

Nel nostro studio, precedentemente pubblicato (Ledee N, 2008), il G-CSF nei FF non sembrava essere predittivo del tasso di fertilizzazione, ma lo stimolo ovarico era differente e avendo dimostrato che questo influenzava il contenuto citochinico dei

FF, si potrebbe pensare che questa contraddizione possa essere legata alla tipologia di stimolo ovarico.

Il G-CSF è stato anche utilizzato in pazienti che si sottoponevano a fecondazione *in vitro* dopo ripetuti fallimenti di impianto, e il suo utilizzo si è rilevato essere un promettente metodo farmacologico per aumentare i tassi di impianto in donne con RIF (Wurfel W, 2010). Altri studi presenti in letteratura suggeriscono che il G-CSF possa riflettere la competenza ovocitaria e definire gli ovociti con potenziale di impianto elevato (Gaafar TM, 2014). Quindi si riconferma come il G-CSF sia una molecola presente nei fluidi follicolari e che sia predittiva del potenziale d'impianto dell'ovocita dopo fertilizzazione.

Abbiamo inoltre osservato che il G-CSF nel FF è un fattore predittivo della morfologia migliore della blastocisti (score morfologico A\B) (Fig.7), che suggerisce come un avvenuto impianto possa essere associato ad una adeguata morfologia della blastocisti.

5.3 Citochine del FF e impianto della blastocisti

Infine abbiamo considerato l'avvenuto o mancato impianto e correlato questi parametri ai livelli di citochine nei FF. L'analisi delle 27 citochine nei FF appartenenti ai follicoli con ovociti sviluppati in una blastocisti che si è impiantata nella cavità uterina, rispetto ai FF appartenenti ai follicoli con ovociti sviluppati in una blastocisti che non si è impiantata nella cavità uterina, ha messo in evidenza che, il G-CSF e l'IL-15 sono statisticamente più elevate nei FF che corrispondono agli ovociti che si sono sviluppati dopo fecondazione in una blastocisti che si è impiantata nella cavità uterina (Fig.8) . Quindi il G-CSF nei FF che si associa ad una migliore

morfologia della blastocisti, si associa ulteriormente ad una blastocisti che si impianta nella cavità uterina. Questi dati sarebbero una dimostrazione che lo score morfologico della blastocisti può essere un fattore predittivo ma non attendibile al 100% essendo dipendente dall'operatore, mentre la misurazione del G-CSF nel FF essendo un dato quantitativo oggettivo, risulterebbe maggiormente attendibile.

Mentre per il G-CSF questo dato è una marcata conferma del ruolo di questo mediatore in ambito di fecondazione assistita, il risultato sull'IL-15 è in contrasto con quello ottenuto precedentemente dal nostro gruppo (Ledee N, 2011), che indicava come alti livelli di G-CSF e bassi livelli di IL-15 fossero predittivi dell'impianto dell'embrione nei cicli naturali modificati con hCG. Questa discrepanza tra lo studio attuale e quello precedente potrebbe essere dovuta allo stimolo ovarico, che nel nostro studio attuale è rappresentato dall'FSHr in associazione all'LHr.

Inoltre grazie alla curva ROC abbiamo confermato che il G-CSF nel FF è un marcatore predittivo che permette di discriminare tra embrioni che si impiantano ed embrioni che non si impiantano (Fig.9).

5.4 Citochine nel FF e tipologia di infertilità

L'analisi condotta separando il nostro gruppo di pazienti dello studio in gruppi in base alla tipologia di infertilità, ha rilevato che Il G-CSF nei FF è risultato più elevato nelle donne reclutate per un protocollo ICSI quando l'infertilità di coppia era dovuta ad un fattore maschile piuttosto che a un fattore femminile (Fig.10).

Questo dimostra che quelle donne che hanno un infertilità non legata ad un fattore tubarico hanno un deficit follicolare caratterizzato da una ridotta produzione di G-CSF nel FF. Questi risultati suggeriscono che solo i livelli di G-CSF nel FF possono essere indicativi di una fertilità femminile alterata, mentre le altre 26 citochine misurate nei FF non hanno questa capacità. Il G-CSF nei FF risulta un buon marcatore di infertilità femminile non tubarica.

6. CONCLUSIONI

L'impianto dell'embrione nelle tecniche di procreazione medicalmente assistita resta la fase a più elevata criticità. Per ovviare a questa criticità nella normale pratica clinica vengono trasferiti più embrioni, andando incontro alla complicanza della gravidanza multipla e rendendo inattuabile la politica del singolo trasferimento di embrione obiettivo auspicabile da parte di tutta la comunità scientifica.

La politica della SET potrebbe essere raggiunta se si definisse una metodologia prognostica di competenza ovocitaria in modo tale da definire a monte l'ovocita da fertilizzare.

Il fluido follicolare rappresenta il microambiente dove l'ovocita matura e di conseguenza influenza la sua fertilizzazione e lo sviluppo precoce dell'embrione. Le citochine presenti al suo interno sembrano modulare i processi riproduttivi e possono essere determinanti nel definire la competenza ovocitaria.

In questo studio viene riconfermato come il G-CSF possa essere un marcatore prognostico di competenza ovocitaria anche per lo stimolo FSHr e LHr, mentre non si conferma il dato sull'IL-15 osservato precedentemente nei cicli naturali modificati con hCG.

Il nostro studio ha messo in evidenza che il G-CSF nei FF non solo si associa ad una morfologia della blastocisti migliore (score morfologico A\B) insieme all'IL-1Ra, ma risulta l'unico fattore predittivo della morfologia migliore (score A\B) delle blastocisti. Inoltre questa morfologia migliore della blastocisti, quando i livelli di G-CSF nei FF sono elevati, si associa ad un impianto di questa blastocisti e quindi il G-CSF risulta un fattore predittivo dell'impianto della blastocisti nella cavità uterina.

Mentre la fertilizzazione dell'ovocita non si associa a livelli di G-CSF nei FF, ma a molteplici citochine di tipo Th2, Th1, pro-infiammatorie e chemochine.

Solo il VEGF nei FF risulta essere predittivo della fertilizzazione.

Infine si dimostra per la prima volta che i livelli bassi di G-CSF nel FF risultano essere indicativi di una infertilità di coppia di origine femminile non tubarica. Quindi, il G-CSF sembra essere un marcatore di infertilità femminile di origine "follicolare". Questo aspetto dovrebbe essere approfondito.

Il G-CSF nel fluido follicolare potrebbe essere predittivo e quindi associato a patologie\deficit follicolari e sarebbe interessante valutare la sua quantizzazione come potenziale test diagnostico.

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8. PUBBLICAZIONI, PARTECIPAZIONI A CORSI E CONGRESSI NEL TRIENNIO DI DOTTORATO

Pubblicazioni

- Giudici F, Lombardelli L, Russo E, Cavalli T, Zambonin D, Logiodice F, Kullolli O, Giusti L, Bargellini T, Fazi M, Biancone L, Scaringi S, Clemente AM, Perissi E, Delfino G, Torcia MG, Ficari F, Tonelli F, Piccinni MP, Malentacchi C. Multiplex gene expression profile in inflamed mucosa of patients with Crohn's disease ileal localization: A pilot study. World J Clin Cases. 2019;7:2463-2476.
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Corsi

- Soft and Complementary Skills "Pillole di probabilità e statistica nell'era della scienza dei dati (Firenze 7-8\02\2019)
- Soft and Complementary Skills "Scrivere di scienza" (Firenze 6 e 13 09\2019)

Congressi

- Farmaci biologici nelle malattie immunomediate: una storia di 15 anni 10-11/03/2016; XXXII congresso SIAAIC Toscana 11-12/11/2016; XXX congresso nazionale SIAAIC 6-9/04/2017
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ORIGINAL ARTICLE

Observational Study

Multiplex gene expression profile in inflamed mucosa of patients with Crohn's disease ileal localization: A pilot study

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Abstract

BACKGROUND

Crohn's disease (CD) is a complex disorder resulting from the interaction of genetic, environmental, and microbial factors. The pathogenic process may potentially affect any segment of the gastrointestinal tract, but a selective location in the terminal ileum was reported in 50% of patients.

AIM

To characterize clinical sub-phenotypes (colonic and/or ileal) within the same disease, in order to identify new therapeutic targets.

METHODS

14 consecutive patients undergoing surgery for ileal CD were recruited for this

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study. Peripheral blood samples from each patient were collected and the main polymorphisms of the gene *Card15/Nod2* (R702W, G908R, and 1007fs) were analyzed in each sample. In addition, tissue samples were taken from both the tract affected by CD and from the apparently healthy and disease-free margins (internal controls). We used a multiplex gene assay in specimens obtained from patients with ileal localization of CD to evaluate the simultaneous expression of 24 genes involved in the pathogenesis of the disease. We also processed surgery gut samples with routine light microscopy (LM) and transmission electron microscopy (TEM) techniques to evaluate their structural and ultrastructural features.

RESULTS
We found a significant increase of Th17 (IL17A and IL17F, IL 23R and CCR6) and Th1 (IFN-γ) gene expression in inflamed mucosa compared to non-inflamed sites of 14 CD patients. *DEFB4* and *HAMP*, two genes coding for antimicrobial peptides, were also strongly activated in inflamed ileal mucosa, suggesting the overwhelming stimulation of epithelial cells by commensal microbiota. IFN-γ and CCR6 were more expressed in inflamed mucosa of CD patients with ileal localization compared with patients with colonic localization suggesting a more aggressive inflammation process in this site. Morphological analysis of the epithelial lining of Lieberkün crypts disclosed enhanced release activity from goblet mucocytes, whereas the *lamina* propria contained numerous cells pertaining to various lines.

CONCLUSION
We observed that the expression of ileal genes related to Th1 and Th17 activity is strongly activated as well as the expression of genes involved in microbiota regulation.

Key words: Crohn's disease; Ileum; Colon; Messenger ribonucleic acid; Th1/Th17; Microbiota; Inflammation

Core tip: Multiplex Gene Assay in specimens obtained from patients with ileal localization of Crohn's disease (CD) allowed the simultaneous analysis of messenger ribonucleic acid levels for 24 genes, known to be involved in the inflammation processes of CD pathogenesis. The result showed that the expression of genes related to Th1 and Th17 immune response is strongly activated as well as the expression of genes deputized to interact with the commensal microbiota, such as DEFB4 and HAMP, which code for antimicrobial peptides.

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INTRODUCTION

The pathogenesis of Crohn's disease (CD), one of the major inflammatory bowel diseases (IBD) together with ulcerative colitis (UC), has been extensively investigated. It is generally accepted that both genetic and environmental factors contribute to the etiology of the disease. In CD patients, strong associations between genes involved in maintaining intestinal barrier function, epithelial anti- microbial defence, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, and metabolic pathways have been identified[1,2].

Environmental risk factors involved in the progression of the disease include

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smoking, low-fiber and high-carbohydrate diet, gut microbiota (GM) alteration, and treatments with antibiotics or non-steroidal anti-inflammatory drugs[3].

CD is characterized by a transmural inflammation which can potentially affect any segment of the gastrointestinal tract[4]. However, recent studies reported a selective location in the terminal ileum in 50% of the patients and location in the colonic district in 20% of the patients. Ileum and colon district were involved in the remaining 30% of the patients. A different clinical course and surgical requirement was reported according to disease's localization but currently the reasons underlying the differences in the clinical course have not been defined. In addition, the immunological pathways involved in colonic inflammation are different from those involved in ileal inflammation[5].

The mutual interplay between GM and the immune system is involved in the pathogenesis and prognosis of intestinal diseases[6] as the GM is a key modulator of intestinal inflammation[7]. In CD patients, a reduced GM diversity and lower bacterial load in inflamed *vs* non-inflamed tissues was observed[8]. In addition, several evidences report that the small bowel is responsible for the systemic tolerance towards microbes. A recent study revealed that the ileum harbors a distinctive niche of the GM that differs more from the colonic[9]. This different GM composition could be attributed to the activation of distinctive immunological pathways.

In the present study, we used the multiplex gene assay[10,11] to analyze surgical specimens of CD patients with prevalent ileal localization.

MATERIALS AND METHODS

Patients

14 consecutive patients undergoing surgery for ileal CD aged 15 to 57 and hospitalized at the Surgery Unit of Azienda Ospedaliero-Universitaria Careggi, University of Florence, were recruited (Table 1). CD was diagnosed based on both histological and clinical/endoscopic criteria. Table 1 reports the clinical characteristics of the patients.

Peripheral blood samples from each patient were collected in EDTA tubes and genomic DNA was extracted using QIA-AMP DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany). The main polymorphisms of the gene *Card15/Nod2* (R702W, G908R, and 1007fs) were analysed in each sample[12].

mRNA extraction and multiplex Gene Assay

Tissue samples were taken both from the tract affected by CD and from the apparently healthy and disease-free margins (internal controls). The surgical specimens were opened longitudinally.

All samples were stored in RNA later (Qiagen, Germany) before homogenization. Then each sample was weighed and the appropriate lysis solution was added to a final volume of 150 μL containing 50% Lysis Mixture (Thermo-Fisher, MA, United States) and 1 g/L proteinase. The mixture was agitated for 30 min at 65 °C to lyse the cells. The lysate was stored at -80 °C for later use. We used a microarray panel of 24 genes implicated in CD etiopathogenesis[10]. We evaluated the expression of these genes in both non-inflamed and inflamed ileal biopsies. Table 2 indicates the panel of the examined genes, the number of Mendelian Inheritance in Man (MIM) (used as a reference), accession number and their corresponding encoded product and function. To improve the analysis of the results, the selected genes were divided into four groups according to their biological role: (1) Transport across epithelia: *ABCB1*, *SLC40A1*, *SLC22A4*, *SLC22A5*, *HAMP*; (2) Immune response: *CCR6*, *IL*-17F, IL-*17A*, *MICA*, *MYD88*, *STAT3*, *IL-23R*, *JAK2*, *IFNG*, *NOD2;* (3) Antimicrobial activity: *HAMP*, *CAMP*, *LRRK2*.*DEFB4*; and (4) Physiological activities: *STAT3*, *ESR1*, *LRRK2*, *TNFSF15*, *CARD14*, *DLG5 BMP2 ATG16L1*.

The messenger ribonucleic acid (mRNA) expression for *CCR6*, *IL-17A*, *IL-17F*, *BMP2*, *TNFSF15*, *ABCB1*, *IL-23R*, *DEFB4*, *CARD14*, *STAT3*, *SLC40A1*, *JAK2*, *SLC22A5*, *ACTB*, *ATG16L1*, *CAMP*, *DLG5*, *ESR1*,*CARD15*, *MICA*, *MYD88*, *SLC22A4*, *IFN-γ*, *LRRK2*, *HAMP*, *ACTB* (high expression housekeeping gene), *HPTR1* (low expression housekeeping gene) was measured using the QuantiGene® Plex assay (Thermo-Fisher, MA, United States).

A panel of oligonucleotide capture probes was covalently linked to carboxylated fluorescently encoded beads (Luminex, Bio-Rad, MA, United States). Each probe has a unique sequence of 15 bases. Each sample lysate diluted at 1:1 and 1:2 was mixed with the pooled capture beads in a round-bottom assay well and hybridized for 16 h at 54 °C (final volume in each well was 100 μL). The assay mixture was moved to a MultiScreen® Filter Plate (Millipore, Billerica, MA, United States) and unbound

Table 1 Clinical characteristics of patients with ileal Crohn's disease

Patient	Pt1	Pt2	Pt3	Pt4	Pt5	Pt6	Pt7	Pt8	Pt9	Pt10	Pt11	Pt12	Pt13	Pt14
Localization of CDa	L1	L1	L1	L1	L1	L1	L3	L1	L3	L1	L3-L4	L1	L3	L1
Age of CD onset (yr)	57	15	53	55	25	31	39	42	16	24	19	18	46	30
Surgery / relapse	1st surgery	1st surgery	Relapse	1st surgery	Relapse	Relapse	Relapse	1st surgery	Relapse	Relapse	Relapse	Relapse	Relapse	1st surgery
Disease behaviorb	B2	B2, B3	B2	B3	B2, B4	B2	B3	B2	B2	B2	B2	B2	B3	B2
Therapyc	C, F, I, B	F, C	C	No	F, C, I	C, F, I	I, B	F, C	C, I, F, B	C	F, C, B	F, C, I	C, F, B, I	F, C, I
Smoking statusd	No	Cur 20/15.5	No	No	1010	No	No	20/30	1010	Cur 10	1020	No	No	No
Genotype	wt	R702	wt	hzG881R	wt	wt	wt	wt	hzG881R	R702	No seq	No seq	wt	wt

Localization of Crohn Disease: L1: terminal ileum; L2: colon; L3: ileum colon; L4: upper G (gastrointestinal); Disease behavior: B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating; B4: perianal disease. Therapy: M: mesalazine; I: immunosuppressant; B: biologics; C: corticosteroids; Ab: antibiotics. Smoking status: No: non-smoker; Ex: ex-smoker; Cur: current smoker, no. cigs per day / no. yr. MIM: Mendelian inheritance in Man. 1: Transport across epithelia; 2: Immune response; 3: Antimicrobial activity; 4: Different physiological activities.

material was filter-washed from the wells by rinsing 3 times with wash buffer. The plate was hybridized with 100 μL/well of bDNA amplifier in Amplifier Diluent (Panomics, CA, United States) at 54 °C for 1 h. After the plate was filter- washed twice with wash buffer and incubated at 50 °C for 1 h with 100 μL/well of 5'-dT(Biotin)-conjugated label probe (Panomics, CA, United States) diluted in Label Probe Diluent (Panomics, CA, United States). After 2 washes, streptavidin-conjugated R-phycoerythrin diluted in SA-PE diluent (20 mmol/L Tris-HCl, 400 mmol/L lithium chloride, 1 mL/L Tween 20, 1 mL/L bovine serum albumin, and 5 mL/L Micr-O-protect) was added and the plate was shaken and incubated at room temperature for 30 min. We washed the beads to remove unbound SA-PE and then evaluated them with Bio-Plex® 200 system (Bio-Rad, MA, United States). The SA-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads. Expression of target-specific RNA molecules was calculated as the mean values from triplicate cultures and normalized against *Actin* gene (high expression housekeeping gene).

Polymorphism analysis

A standard non-enzymatic method, using the QIA-AMP® DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany) was used to extract Genomic DNA from peripheral blood leucocytes of all CD patients and healthy controls. In addition, DNA samples from 70 healthy Caucasian subjects (140 alleles) were analysed as controls. Three exon of the *CARD15/NOD2* gene (Exon 4, Exon 8, Exon 11), were amplified by PCR using pairs of primers derived from the published sequence of the gene (available upon request). Each exon is associated with the three main single-nucleotide polymorphisms (SNPs) (R702W-C2104T; G908R-G2722C; 1007fs-3020insC). These three main variants, associated with susceptibility to CD, represented 32%, 18%, and 31%, respectively, of the total CD mutations[13-15].

The BigDye® Terminator Cycle Sequencing kit (Applied Biosystems, CA, United States) was used to perform direct sequencing of PCR amplified products (SNPs rs87950, rs127951, and rs137955) of the *CARD15/NOD2* gene. The samples were analysed in an ABI Prism® 310 genetic analyzer (Applied Biosystems, CA, United States). The of the sequences were confirmed with the analysis of newly-amplified fragments and the sequencing of both DNA strands.

Statistical analysis

SSPS software vers. 10 (SPSS Inc., IL, United States) was used to perform the statistical analysis. All comparisons of genes mRNA expression in tissues (non-inflamed and inflamed areas) were performed by non-parametric assay (Mann-Whitney test, Wilcoxon test). Data are reported as mean and ranges unless otherwise stated. A P-value < 0.05 was accepted as statistically significant. Furthermore, to better characterize the different clinical CD phenotypes, we compared the results regarding the *CARD15*, *CCR6*, interferon gamma, and *IL-17A* genes to colonic CD patients previously examined for these same genes.

Table 2 Panel of the 24 genes investigated

Symbol	Complete name	Group	Accession number	mim	Gene product function (s)	Ref.
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Low expression housekeeping gene	M26434	308000	It plays a central role in the generation of purine nucleotides, chosen as a low expression housekeeping gene	[39]
ACTB	Actin betaprovided	High expression housekeeping gene	M28424	102630	Is involved in the cell motility, structure, and integrity	[40]
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member1	1	AF215636	604653	Exports iron from duodenal epithelial cells	[41]
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member1	1	M14758	171050	Transports various molecules across extra- and intra-cellular membranes. It belongs to a protein sub-family involved in multidrug resistance	[42]
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5	1	AF057164	603377	Transports several small organic cations in the liver, kidney, intestine. It is involved in elimination of drugs and environmental toxins	[43]
SLC22A4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	1	AB007448	604190	Polyspecific transporter of organic cations in the liver, kidney, intestine, and involved in the elimination of these molecules.	[44]
CCR6	Chemokine (C-C motif) receptor6	2	U68030	601835	Induces B-lineage maturation and antigen-driven B-cell differentiation	[45]
IL17A	Interleukin 17A	2	U32659	603149	Produced by Th17-type CD4+ cells. Regulates the activities of NF-kB and mitogen-activated protein kinases	[26]
IL17F	Interleukin 17F	2	AF384857	606496	Produced by Th17-type CD4+ cells. Stimulates the production of other cytokines, including IL6,IL8.It also inhibits angiogenesis by endothelial cells.	[46]
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	2-4	BC014482	102582	Activates transcription of cell growth and apoptosis' genes asresponses to inflammation	[22,47]

MICA	MHC class I polypeptide-related sequence A	2	L14848	600169	Acts as a stress-induced antigen broadly recognized by intestinal intra-epithelial gamma delta T cells.	[48,49]
MYD88	Myeloid differentiation primary response gene (88)	2	U84408	602170	Acts as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways	[50]
IL23R	Interleukin 23 receptor	2	AF461422	607562	Expressed on Th17 cells. Involvedin the IL23A signaling pathways with the receptor molecule IL12RB1/IL12Rbeta1.	[20]
JAK2	Janus kinase 2	2	3717	147796	Is involved in cytokine receptor signaling pathways and is required for responses to gamma interferon	[51]
IFNG	Interferon, gamma	2	3458	147570	It encodes a cytokine with antiviral,immunoregulatory and anti-tumor properties and activates macrophages	[52]
CAMP	Cathelicidin antimicrobial peptide	3	BC055089	600474	It is an antimicrobial protein (defensin)	[53]
CARD15	Nucleotide-binding oligomerization domain containing 2	2	AF178930	605956	Induces immune response to intracellular bacterial by recognizing the muramyl dipeptide (MDP)	[54]
DEFB4	Defensin, beta 4A	3	AJ314835	602215	Acts as an antibiotic peptide locally regulated by inflammation.	[55,56]
HAMP	Hepcidin antimicrobialpeptide	1/2/3	AF309489	606464	It is involved in iron transport, antimicrobial, defence and inflammatory responses	[57,58]
LRRK2	Leucine-rich repeat kinase 2	3/4	AK026776	609007	It is involved in autophagy and implicated in clearance of intracellular bacteria.	[59,60]
TNFSF15	Tumor necrosis facto (ligand) superfamily, member 15	4	AF039390	604052	Induces apoptosis in endothelial cells	[61]
CARD14	Caspase recruitment domain family, member 14	4	AF322642	607211	Regulates the molecular scaffolding process and activates NF-kappa B	[62,63]
ATG16L1	ATG16 autophagy related 16-like 1	4	AK000897	610767	Induces autophagy processes involved in degradation of cell organelles	[64]

ESR1	Estrogen receptor 1	4	X03635	133430	Involved in the metabolic pathway of the hormones and in several diseases including osteoporosis	[65]
BMP2	Bone morphogenetic protein 2	4	650	112261	Induces bone and cartilage formation	[66]
DLG5	Discs, large homolog 5	4	U61843	604090	It encodes for scaffolding molecules involved in cell-cell contact and in the maintenance of epithelial cell integrity. Its products are also involved in the transmission of extracellular signals	[67]

MIM: Mendelian Inheritance in Man. 1: Transport across epithelia; 2: Immune response; 3: Antimicrobial activity; 4: Different physiological activities.

Histological analysis

Once removed, tissue samples were rinsed in 0.1 M, pH 7.0 cacodylate buffer, the same used in prefixation and further steps of histological preparation. Samples were then placed in Karnovsky (1965)[16], aldehyde solution, and after 3 h prefixation (4°C), underwent prolonged washing in the buffer. Surgery specimens were reduced into approximately 20 mm^3 fragments that were post-fixed (1 h 30 min, 4°C) with 1% OsO4 in cacodylate. These specimens were washed in the buffer, dehydrated in graded ethanol series, soaked in propylene oxide, and embedded in Epon 812. Flat blocks were obtained after polymerization, which were reduced into semi-thin sections (1.5 μm thick), using an 8800 ULTROTOME III LKB equipped with glass knives. Semi-thin sections were stained with borax buffered 1% toluidine blue, and observed with a LEITZ DMRB, in order to collect LM digital images (JPG) for structural analysis. Subsequent ultrastructural observations were carried out on ultrathin sections, obtained with an ULTROTOME NOVA LKB, using a DIATOME diamond knife. Ultrathin sections with gold yellow to silver gray interference colour were selected and collected on uncoated 200-300 mesh copper grids to be electron-dense stained with a hydroalcoholic saturated solution (25 mg/mL) of uranyl acetate, followed by alkaline lead citrate (2 mg/mL). These sections where finally observed (80 KV) with a PHILIPS 201 TEM (BIO, UNIFI), and analogic images were collected, which were later acquired and stored as digital TIFF files using a DIMAGE SCAN DUAL (MINOLTA).

RESULTS

Expression of CD susceptibility genes in the inflamed ileum tissue

The simultaneous expression of 24 genes involved in the pathogenesis of CD was studied in surgical specimens from 14 CD patients with ileal localization of disease. The expression of genes in inflamed ileal mucosa was compared to that of non-inflamed ileal sites collected from the same patient. We observed a significant increase in mRNA levels of twelve genes compared to internal control (Figure 1).

Figure 1 shows that genes related to innate immune response (*NOD* 2, *ATG16L1*, *DEFB4*), and to adaptive immune response (*CCR6*, *IL17*A, *IL17F*, *IL23R*, *IFN-γ*) were significantly increased in inflamed mucosa of CD patients compared with non-inflamed sites. Moreover, the levels of mRNA for genes involved in physiological functions of epithelial cells, such as *JAK2*, *TNFSF15*, and *SLC22A4* were higher in inflamed mucosa compared to non-inflamed mucosa and the differences in expression reached statistical significance.

Detection of CARD15 polymorphism

DNA samples obtained from peripheral blood were sequenced to investigate the presence of polymorphisms of *CARD15/NOD2* gene. The results of this analysis showed that four patients (28.5%) included in this study are carriers of at least one of the polymorphisms investigated, suggesting that genetic factors might contribute to the dysregulated expression of *CARD15/NOD2* gene[17-19].

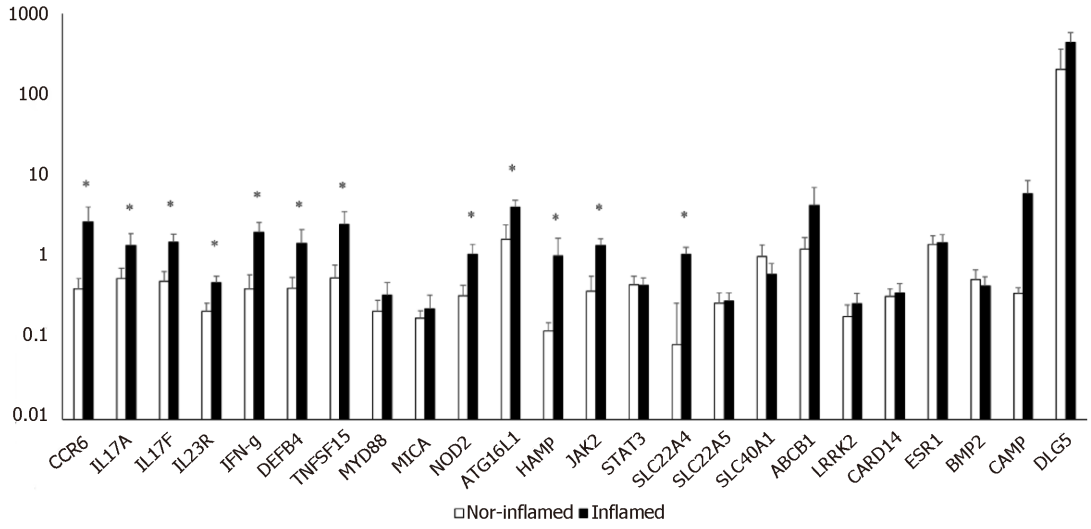

Figure 1 Quantitative evaluation of gene expression using multiplex gene assay in surgical ileum specimens of CD patients. The abscissa shows the genes evaluated in inflamed and not inflamed tissue; the axis of the ordinates shows the value of expression of the gene normalized to the housekeeping actin (gene/β actin ratio). The first seven genes are more closely related to immunity. *P* value is reported only when statistically significant ($P < 0.05$).

Morphological analysis

In order to observe the morphology of inflamed tissue samples, light microscopy (LM) and TEM micrographs were obtained. Both gut wall and Lieberkühn crypts retain usual features in both lining epithelium and *lamina propria*. Epithelial cells consist of constitutive enterocytes along with goblet mucocytes, whereas the underlying connective tissue contains large amounts of cells with wide morphological variety (Figure 2). Goblet cells are involved in impressive secretory processes, releasing a moderately opaque product into cryptal and gut lumen through gaps between enterocytes apices (Figure 3). As a consistent pattern, the lamina propria contains granulocytes and plasma cells.

DISCUSSION

Among the numerous genes that have been studied so far with respect to CD, strong and replicated associations have been identified with *NOD2*, *IL23R*, and *ATG16L1* genes[20]. Environmental factors like smoking, low-fiber and, high-carbohydrate diet, altered GM, and medications such as non-steroidal anti-inflammatory drugs interact with genetic background and induce abnormal inflammation and dysregulation of the immune response. Clinical symptomatology relates to such dysregulation.

The clinical course of CD is conditioned by several parameters such as disease location, extra-intestinal manifestation, and age at onset[21]. Strictures and fistulas are more frequent in patients with ileal disease, whereas Crohn's colitis remains uncomplicated for many years. On the whole, almost 80% of patients with CD require intestinal surgery, with a permanent stoma required by almost 10%. The presence of selected mutations in the *NOD2* gene (see, *e.g.*, 605956.0001-605956.0003) (IBD1; 266600) has been associated with susceptibility to ileum-localized CD[22]; patients homozygous for the 1007fs mutation had an early disease onset with long-segment ileal stenoses and entero-enteral fistulas; they frequently needed surgical intervention and had a high risk of recurrence[23,24]. Beside *NOD2* gene, huge genome-wide linkage-analyses and meta-analyses have described several CD susceptibility regions including IBD5 locus, DLG5, and autophagy- related 16-like 1 (ATG16L1) gene, JAK2, STAT3 interleukin-23 receptor (IL23R), SLC22A4 and SLC22A5 TNFSF15[14].

In this paper, we evaluated the expression of 24 genes that were associated to CD susceptibility[10]. mRNA was extracted from gut specimens obtained from patients with CD ileal localization of CD, undergoing surgery. We used a multiplex gene assay which directly quantifies the mRNA amounts without need of reverse transcription and gives a detailed picture of the inflammation process for each patient[11]. The same technique was used to quantify gene expression in colonic mucosa from surgical specimens or endoscopic bioptic fragments obtained by CD patients with predominant colonic (L2) location[10].

The analysis revealed a clear activation of immune-adaptive Th17 response in association with a Th1 response in inflamed mucosa of patients undergoing surgery

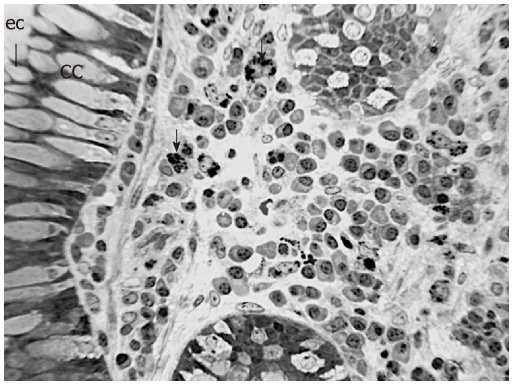

Figure 2 Representative Light microscope (LM) of the lamina propria between Lieberkühn crypts in inflamed ileal tissue of CD patient number 7b. Notice muciparous goblet cells in cryptal epithelium and large amount and variety of immune cells (arrows) in the connective tissue. Semithin (1 μm thick) section, toluidine blue staining; cc = goblet cell, ec = enterocyte. Scale bar (4 cm) = 70 μm.

suggesting a dysregulated and very aggressive immune-inflammatory response.

Here gene expression analysis of inflamed ileal mucosa revealed an increased expression of genes involved in adaptive immune response compared to non-inflamed tissue. In particular, we found a significant increase of *IL17A* and *IL17F*, *IL 23R* and *CCR6* gene expression suggesting an activation of a Th17 adaptive response[25,26] similar to that found in gut mucosa of patients with colonic localization. According with this hypothesis, three additional genes involved in Th17 differentiation as *JAK2*, *STAT3* and *TNFSF15*[27,28] were found to be overexpressed in inflamed ileal mucosa of CD patients compared to non- inflamed sites. Furthermore, as we expected, the expression of the antimicrobial peptides as defensin (DEFB4)[29] and Hepcidin 6 (HAMP)[30] were significantly increased in inflamed mucosa of CD patients compared with non-inflamed sites, suggesting the overwhelming stimulation of epithelial cells by commensal GM. Indeed, while the human β-defensin (HBD) 1 is constitutively expressed, other genes, like HBD2 (gene name DEFB4), show pathogen and/or inflammation dependent upregulation[31] while also being inducible by probiotic bacteria[32]. Conversely, HAMP transcription mediates the effects of host defence and inflammation. Shanmugam *et al*. provided persuasive evidence in support of an important role for the GM composition in influencing hepcidin expression during intestinal inflammation in mouse models of colitis[33].

As the position of the pathogenic tissue may condition not only the clinical course of the disease but also the probability to require surgery, we also compared with the same methodology (Quantigene 2.0) the expression of selected genes (IFN-γ, CCR6, IL17A, NOD2) involved in immune responses in inflamed mucosa with predominant ileal location with the one previously studied[10] in inflamed mucosa with colonic location. mRNA expression for IFN-γ γ and for the chemokine CCR6 appeared significantly higher in ileal site compared to colonic site (ileal CD = 2.7 ± 1.5; colonic CD = 0.2 ± 0.06; P = 0.01). The mRNA for IL-17 and NOD2 appeared to be expressed at higher levels in ileal site compared to colonic site, even if the difference is not statistically significant ($P \geq 0.05$). The significant differences in the expression levels of *IFN*-γ gene (higher expression in specimens from patients with ileal localization compared to patients with colonic localization) may suggest an increased damage of the ileal mucosa due to the simultaneous presence of Th1 and Th17 effector cells and/or the shift of Th17 cells to Th1 effectors functionally more aggressive than Th17 unshifted cells[34,35].

Furthermore, according to a worse clinical course of patient with ileal localization of CD compared with patient with colonic localization[36], the increased expression of IL17 and NOD2 in mucosal fragments from patients with ileal CD compared to patients with colonic CD is in agreement with the NOD2 –dependent regulation of immunity in mouse intestinal tract[37]. We suppose that the above differences between the two gut tracts (ileal and colonic) may be due to the Paneth cells at the bottom of the crypts of Lieberkühn in the small intestine, which produce antimicrobial peptides and hinder commensal GM and pathogenic bacteria to penetrate gut mucosa. Initially described as innate immune cells producing antimicrobial products, Paneth cells have recently been suggested to constitute a cardinal component of the intestinal stem cell niche. In fact, Paneth cells contribute to controlling the luminal flora as well as repairing the intestinal barrier following an insult. Genomic alterations that impede the Paneth cell compartment functionality can potentially increase the propensity to

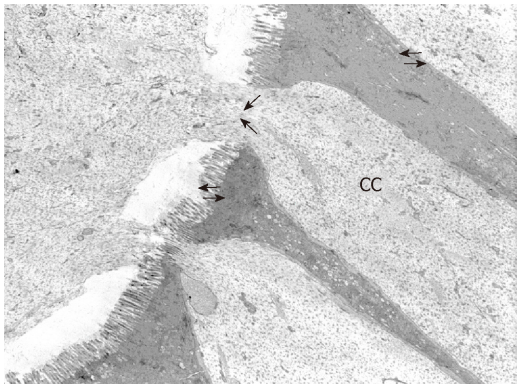

Figure 3 Representative TEM micrograph of Lieberkühn crypt wall and lumen that contains mucous product released (large arrows) by goblet cells (CC) in inflamed ileal tissue CD patient number 7b. Small arrows indicate transport processes involving apical and lateral surfaces of enterocytes. Scale bar (1 cm) = 1 μm.

develop CD[38].

As a consistent trait, cryptal globlet cells produce large amounts of mucus that performs the double role of barrier and holder of antimicrobial products. The microscopic anatomy analysis aims to provide some details that illustrate phenotypic features: the large cell variety in the *lamina propria* includes immune lines that represent a further defense tool. Although these morphological traits are not directly related to specific gene outputs, they illustrate the tissue responses to key gene deregulation.

As a pilot study, our study presents a low number of subjects investigated which may have influenced the statistical power of the results. To confirm these results, studies with a larger number of patients are needed. In addition, gene expression was evaluated with Multiplex Gene Assay only. This method directly quantifies the mRNA amounts without need of reverse transcription and gives a detailed picture of the metabolic processes for each patient but it should be validated by comparisons with additional techniques to evaluate gene expression.

One of the main purposes of our research is therefore to identify new molecules involved in metabolic pathways that could potentially represent new biological drugs to identify the appropriate therapy in relation to the clinical phenotype of the CD patient.

ARTICLE HIGHLIGHTS

Research background

The interplay of environmental, genetic and microbial elements influences the etiopathogenesis of Crohn's disease (CD). Differences in the clinical course of CD have recently been reported in patients with ileal or colonic localization of the inflammatory process.

Research motivation

Aim of this study was to define biochemical and histological differences in intestinal biopsies from patients with ileal or colonic localization of Crohn disease in order to identify new assays which can be useful for planning individual therapeutic strategies

Research objectives

Main objective of the current research was to investigate the expression of genes involved in immune-inflammatory pathways in gut mucosa from patients with ileal or colonic localization of CD and to correlate the results of gene expression with those obtained through a classical morphological analysis of surgical biopsies.

Research methods

A Multiplex Gene Assay was used to assess the simultaneous expression of 24 genes related to immune-inflammatory process and to CD pathogenesis. Structural and ultrastructural features of gut samples were also evaluated through Light microscopy (LM) and Transmission Electron Microscopy (TEM) techniques.

Research results

We observed a strong activation of genes involved in TH-1- and TH-17 immune response in patients with ileal localization of CD compared to patients with colonic localization. In addition, the expression of genes for antimicrobial peptides as DEFB4 and HAMP was found highly stimulated in ileal mucosa from CD patients suggesting a possible interference with microbial

commensals at this site.

Research conclusions

Our results indicate that patients with ileal localization of CD have a stronger activation of TH-1 and TH-17 immune-inflammatory responses compared with patients with colonic localization of the disease thus defining a clear subclinical phenotype of CD.

Research perspectives

These results may suggest that therapeutic strategies with biological drugs in CD patients can be differentiated depending on the location of the disease

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Medroxyprogesterone Acetate Decreases Th1, Th17, and Increases Th22 Responses via AHR Signaling Which Could Affect Susceptibility to Infections and Inflammatory Disease

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A synthetic progestin, medroxyprogesterone acetate (MPA), was used in a novel study to determine progestin effects on human purified macrophages and Th1, Th2, Th17, Th22 cells. MPA concentrations were equivalent to those in the serum of women after 6 and 9 months of progestin use. MPA has no effect on the proliferation of PBMCs and CD4+ T cell clones induced by immobilized anti-CD3 antibodies or by antigen (streptokinase). However, MPA decreases production and mRNA expression of IL-5, IL-13, IFN-γ, T-bet, RORC, and IL-17A but increases production and mRNA expression of IL-22 by CD4+ Th22 cell clones and decreases IL-22 production by Th17 cells. MPA inhibits RORC, but not T-bet and AHR, by Th17 cells but increases AHR mRNA and T-bet expression of established CD4+ Th22 cell clones. This suggests that MPA, at concentrations equivalent to those found in the serum of women after treatment for contraception and hormone replacement therapy, can directly inhibit Th1 responses (against intracellular bacteria and viruses), Th17 (against extracellular bacteria and fungi), Th2 (against parasites) but MPA therapy increases IL-22 produced by Th22 cells mediated by an increased expression of AHR and T-bet controlling inflammation. MPA could be responsible for the tissue damage limited by IL-22 in absence of IL-17A.

Keywords: hormone replacement therapy, contraception, medroxyprogesterone acetate, Th1, Th2, Th17, Th22, infection

INTRODUCTION

Different CD4+ T helper (Th) lymphocytes have been classified into different functional subsets based on their profile of cytokine production. Type 1 Th (Th1) cells produce interferon-gamma (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-β. They also promote the production of opsonizing and complement-fixing antibodies, macrophage activation, antibody-dependent cell cytotoxicity and delayed type hypersensitivity (1, 2). Type 2 Th (Th2) cells produce IL-4, IL-5, and IL-13 and provide optimal help for humoral immune responses, including IgE isotype switching and mucosal immunity, through mast cell and eosinophil differentiation and facilitation of IgA

synthesis. In addition, some Th2-derived cytokines, such as IL-4 and IL-10, inhibit several macrophage functions (1, 2). An additional subset of CD4+T helper cells beyond the traditional Th1 and Th2 cells has been identified more recently, i.e., the Th17 cell, which produces IL-17A, IL-17F, IL-21, IL-26, and IL-22 (3). The major role of Th17 is the protection against extracellular bacteria and fungi. These cells are also pathogenic in several murine models of chronic inflammatory disorders.

Th22 cells primarily secrete IL-22, IL-13, and TNF-alpha. Similar to Th17 cells, Th22 cells express CCR4, and CCR6, but they do not express IL-17, CCL20, IL-23R, CD161 (Th17 markers), IL-4 (Th2 marker), or IFN-gamma (Th1 marker). The expansion of IL-22-producing cells appears to be regulated by the aryl hydrocarbon receptor (AHR) transcription factor, although additional intracellular molecules involved in Th22 differentiation are still being investigated. Expression of the CCR4 and CCR10 skin-homing receptors on Th22 cells suggests these cells are likely recruited to the skin where they may contribute to host defense against microbial pathogens, and promote tissue repair or remodeling. Th22 cells may also be involved in the pathogenesis of inflammatory skin disorders such as psoriasis, atopic eczema, and allergic contact dermatitis (4, 5).

The development of Th1- or Th2-dominated responses depends on several factors, the most critical being cytokines produced in the microenvironment during antigen presentation. The differentiation of Th cells into polarized Th1 or Th2 cells can also be influenced by certain hormones. Some years ago, we showed that progesterone is a potent inducer of helper 2 (Th2) type cytokines (IL-4 and IL-5), leukemia inhibitory factor (LIF) and macrophage colony-stimulating factor (M-CSF) (6, 7). The ability of progesterone to suppress cell-mediated functions via the production of Th2-type cytokines has suggested that the high levels of progesterone in human placenta are of great relevance for the maintenance of pregnancy by promoting the tolerance of the fetus assimilated to an allograft (6–16). Recently, it has been shown that progesterone induces the expression of TSLP (an inducer of Th2-type responses) and inhibits the expression of Th17 related transcription factor RORγt, reducing the influx of neutrophils in murine vaginal gonococcal infection (17).

Synthetic progestins are selected for clinical use primarily to mimic the actions of endogenous progesterone, produced predominantly by the ovaries. The relatively short half-life of the reproductive steroids has, until recently, obviated the therapeutic goal of providing physiological hormone replacement unless parent compounds are pharmaceutically modified to prolong their action. MPA was developed to provide a safe, effective synthetic progestin to women requiring hormone therapy (HRT) and/or contraception. It has selective activity quite similar to progesterone itself (18). As expected, MPA has a more favorable bioavailability and a longer half-life than progesterone. Consequently, we were interested in the possibility that the synthetic progestin, MPA, which mimics progesterone effects could also influence T cell cytokine production.

Medroxyprogesterone acetate (MPA), a 17α-hydroxyprogesterone derivative, is a synthetic analog of the natural steroid progesterone. Since its development more three decades ago, MPA has been employed in the treatment of mammary and endometrial adenocarcinomas (19), as a supportive therapy in the anorexia/cachexia syndrome (20) and is also the most widely used injectable female contraceptive. Known as Depo-Provera, MPA is provided as a long-acting contraceptive (21), with at least 20 million current users worldwide (22). MPA is also the most commonly used progestin in the USA and Europe for hormone replacement therapy (23). The doses of MPA used in humans vary. A single 150 mg intramuscular injection every 3 months (24) creates circulating C_{max} MPA levels ranging from 0.32 to 3.7 ng/ml and C_{min} MPA levels of 0.04 to 1.31 ng/ml (25). After a single injection of 150 mg MPA, time to C_{max} is ∼9 days (Package insert, Depo-Subq Provera). Earlier estimates of MPA levels following a single sc injection of this MPA dose are consistent with current measurements: serum concentrations of MPA showed a brief initial elevation ranging from 1.5 to 3 ng/ml for a few days with a gradual decline to about 1 ng/ml for 2–3 months, decreasing gradually to 0.2 ng/ml during the sixth month to <0.02 ng/ml at about 7.5 to 9 months following administration (26). The blood level of MPA that suppresses ovulation is ∼0.1 ng/ml (26). Oral MPA has also been used in contraceptive preparations and most commonly for HRT (10 mg to 1.25 mg daily). The C_{max} for two PREMPRO tablets containing 1.5 mg MPA/tablet is 1.2 ng/ml which is achieved within 2.8 h (Package insert, PREMPRO). Additionally, very high doses of MPA (2,000 mg/day orally) have been used as endocrine therapy for hormone-related cancer (27). The plasma steady-state concentration of MPA with different regimens varied from 1 ng/ml in hormone therapy and contraception to more than 0.2 μg/ml in endocrine treatment of hormone-related cancer.

It is important to note that the scarce information regarding the effects of MPA on immune response has mostly been obtained from patients receiving high dose-schedules (28, 29). Studies carried out in patients receiving high doses of MPA showed that MPA either suppresses or has no effect on lymphocyte proliferation stimulated by mitogens (PHA, Con A) (28–30). High doses of MPA also reduce the production of IL-1β, IL-6, and TNF-α of PHA-stimulated peripheral blood mononuclear cells (PBMCs), providing further evidence that this progestin hinders the activity of cytokines that play a key role in the pathogenesis of the anorexia/cachexia syndrome, also explaining the clinical benefit of MPA treatment in cancer patients with this syndrome (28). It has been shown that blood mononuclear cells cultured in MPA at saturating ligand concentration, 10 μM (from 2×10^4 to 2×10^6 fold higher than in doses found in the serum of MPA users) produced significantly lower levels of IL-1α, IL-12p40, IL-10, IL-13, and G-CSF in response to BCG (31). The authors of this study (31) asserted without showing data that MPA only inhibited IFN-γ production at high but not at concentrations equivalent to serum levels in MPA users (31). Others reported that MPA 10^{-6} M (at doses 1,000 fold higher than those found in the serum of MPA users) inhibited the production of IFN-γ, IL-2, IL-4, IL-6, IL-12, TNF-α, macrophage inflammatory protein-1α (MIP-1α) by peripheral blood mononuclear cells and activated purified CD3+ T (32) MPA also reduced the production of IFN-α and TNF-α by plasmacytoid dendritic cells in response

to Toll-like receptor-7,−8, and−9 ligands (32). More recently, using two different murine Mycobacterium tubercolosis models, some authors studied the effect of MPA at doses found in serum of human users. They injected 1 mg/ml of Depot (D) MPA and found concentrations of MPA in the serum of mice from 1 ng/ml to 23 ± 6.90 ng/ml after 1 week and at 0.19 ng/ml after 16 weeks. They reported that DMPA concentrations altered both serum TNF-α, G-CSF and IL-10 in C57BL/6 mice and IFN-γ in BALB/c mice also altering the secretion of IFN-γ, IL-17, GM-CSF, IL-6, and MCP-1 by mononuclear cells from mediastinal lymph nodes stimulated by Mycobacterium tubercolosis antigens (PPD or ESAT6) (33). In mice low concentrations of MPA (10^{-9}M and 10^{-10}M), more similar to the ones found in the serum of MPA users were unable to induce the secretion of IL-4 and IL-2 by lymph nodes cells and did not exert a proliferating effect on lymph node cells of sheep red blood cells-immunized mice (34), but low dose MPA has the ability in mice to enhance *in vivo* and *in vitro* antibody production (IgM and IgG) (34).

AHR, is an orphan receptor which mediates the effects of a large number of synthetic and natural compounds including halogenated aromatic hydrocarbons like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (35). It regulates the expansion of IL-22-producing cells (Th22 and Th17 cells) and is involved in the regulation of a number of physiological processes in many organs, among them all organs of the female reproductive system (36).

Irregular cycles in AHR knockout mice and TCDD-treated rats are evidence for a regulatory function of AHR in the estrous and menstrual cycle (37). Considering that the development and function of the female reproductive system is mainly regulated by estrogens and progestins, a crosstalk between the AHR signaling pathway and sexual steroid hormones is likely. It has been shown that progesterone increases uterine AHR levels in rat endometrial epithelium (35), but apparently MPA does not induce significant changes in AHR transcript levels of endometrial stromal cells (38).

Interestingly, it was been shown that AHR ligands could have different effects on T cell-mediated responses. The AHR ligand TCDD exerts immunosuppressive mediated by AHR effects on the production of IL-2, IL-4, IL-5, and IL-6, whereas M50364, a synthetic compound with antiallergic effects increases IFN-γ but suppresses IL-4 and IL-5 production and the expression of GATA-3, a key transcription factor for Th2 cell differentiation (39). The fact that AHR can act on T helper responses suggested its effects in the development of inflammatory and autoimmune diseases. In fact TCDD administration confers protection from Experimental Autoimmune Encephalomyelitis (EAE), inhibiting Th17 cell differentiation (40). At the time of immunization systemic application of FICZ, another agonist of AHR, also reduced EAE pathology albeit to a lesser degree than TCDD. *In vitro* Th17 differentiation in the presence of AHR agonists, including TCDD, promoted IL-17 and IL-22 expression, by Th17 cells but did not induce Treg differentiation.

The role of MPA on human lymphocyte function has been investigated *1)* at higher concentrations of MPA than those found in the serum of MPA users (28) and, 2) on heterogeneous populations of peripheral blood and lymph node mononuclear cells (28, 31, 32, 34, 41). The observed effects of MPA on the supposed lymphocytes could be mediated by cytokines produced by a cell type present in the mononuclear cell fraction in response to MPA and not by the direct effect of MPA on T cells. We designed a study to examine the direct effect of MPA on human T CD4+ cells at concentrations equivalent to those found in serum of MPA users from 6 months to 9 months following administration [from 0.2 to 0.02 ng/ml (28)]. We determined the effect of MPA on the proliferation, production and mRNA expression of IFN-γ, IL-5, IL-10, IL-4, IL17, and IL-22 of human established CD4+ T cell clones, which cannot be contaminated by other cells present in the PBMC fractions and on Th2-, Th1-, Th22, and Th17-specific transcription factors (GATA 3, T-bet, AHR, ROR-C, respectively) mRNA expression. For the first time the effect of MPA on IL-22 and AHR expression by T helper cell subpopulations has been investigated.

MATERIALS AND METHODS

All the methods used for the study were performed in accordance with the relevant guidelines and regulations.

Donors

Twenty-seven healthy donors of peripheral blood agreed to participate to the study at AOU Careggi, Florence, Italy. They received verbal and written information about the aim and the design of the research, and all donors signed the informed consent and the study was approved by local ethic committee of AOU Careggi (n.115303). The 27 donors (age mean ± SD; 29.9 ± 0.9 years) were male (14) (mean age ± SD; 30.5 ± 4.2 years) and female (13) (mean age ± SD; 29.8 ± 4.1 years). There were no significant age differences between the groups of male and female donors. Donors who were enrolled had normal body BMI and had negative results for illnesses (infections, autoimmune and inflammatory diseases), exposure to communicable diseases, travel to disease endemic areas, pregnancy and lactation, medical, and surgical interventions, history of recent infections, currently under the influence of alcohol or drugs, or undergoing therapy with hormonal or anti-inflammatory therapy in particular.

REAGENTS

PHA was purchased from GIBCO Laboratories (Grand Island, N.Y.) and phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical Co. (St. Louis, MO). OKT3 (anti-CD3) mAb was purchased from Ortho Pharmaceuticals (Raritan, N.J.). Anti-CD4, anti-CD8 were obtained from Becton-Dickinson (Mountain View, Ca). Human recombinant IL-2 was a generous gift of Eurocetus (Milano, Italy). Human recombinant IL-12 was obtained from RD systems (Minneapolis, MN). FCS was from HyClone Lab Inc. (Logan, UT). 3H-thymidine was from Amersham (Buckinghamshire, UK). Highly purified MPA (6α-methyl-17α-hydroxyprogesterone acetate) and Mifepristone were purchased from Sigma Aldrich (St. Louis, MO). Streptokinase (SK) was purchased from Aventis Behring GmbH (Germany).

Generation of T Cell Clones

To generate T-cell clones, peripheral blood mononuclear cells (PBMCs) of normal subjects were seeded under limiting dilution conditions (0.3 cell/well) in 6 round-bottomed microwell plates containing 10^5 irradiated (9,000 rad) allogeneic PBMC (as feeder cells) and PHA (1% vol/vol) in a final vol of 0.2 ml complete medium supplemented with IL-2 (50 U/ml) and 10% FCS, as reported elsewhere (6). Growing microcultures were then supplemented, at weekly intervals, with IL-2 (50 U/ml) and 10^5 irradiated feeder cells. The phenotype distribution of T-cell clones was assessed by flow cytometer analysis.

Proliferation of T Cell Clones Stimulated by Immobilized Anti-CD3 Antibodies in the Absence and in the Presence of MPA

2×10^5 T cell blasts obtained from 7 CD4+ T cell clones, able to produce IL-4, IL-5, IL-13, IL-10, IFN-γ, IL-17A, IL-17F, IL-22, in 0.2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-mercaptoethanol and 10% FCS (Hyclone Laboratories Inc., Logan, UT) (complete medium) were stimulated in 96 U-bottomed plates with immobilized anti-CD3 antibodies in the absence or in the presence of MPA (0.02, 0.2 and 2 ng/ml) for 5 days. These concentrations were chosen on the basis of those found in the serum during contraception and HRT (26). After a 16-h pulse with 0.5 μCi 3H-TdR (Amersham International), cultures were harvested and radioactivity measured by liquid scintillation.

Proliferation of PBMCs Stimulated by SK in the Absence and in the Presence of MPA

2×10^5 PBMCs of 5 donors in 0.2 ml of complete medium in 96 U-bottomed plates were stimulated for 5 days with PHA and the antigen Streptokinase (SK) (500 U.I./ml) in the absence or in the presence of MPA (0.02, 0.2, and 2 ng/ml) for 5 days. After a 16-h pulse with 0.5 μCi 3H-TdR (Amersham International), cultures were harvested and radioactivity measured by liquid scintillation.

Induction of Cytokine Production by T Cell Clones in the Absence and in the Presence of MPA

To induce cytokine production, 2×10^5 T blasts from each 23 CD4+ T cell clone were cultured in the presence of immobilized anti-CD3 mAb (10 μg/ml) in the absence or the presence of MPA (0.02 and 0.2 ng/ml). After 36 h, culture supernatants were collected, filtered, and stored in aliquots at −70°C until used. For mRNA determination the cells were collected after 6 h.

Induction of the Cytokine Production of PBMCs Stimulated by SK in the Absence and in the Presence of MPA

2×10^5 PBMCs obtained from 15 donors in 0.2 ml of complete medium in 96 U-bottomed plates for 5 days were stimulated with the antigen (SK 500 U.I./ml) in the absence or in the presence of MPA (0.02 and 0.2 ng/ml). After 5 days supernatants were collected and stored in aliquots at −80°C until used.

Induction of Cytokine Production of Macrophages Stimulated by SK in the Absence and in the Presence of MPA

10^6 monocytes obtained from 7 donors were purified by adherence from PBMCs (mean ± SD; 92.59 ± 2.51%) in 1 ml of complete medium in 96 U-bottomed plates for 5 days with the antigen SK (10 μg/ml) in the absence or in the presence of MPA (0.02, 0.2, and 2 ng/ml). After 5 days supernatants were collected and stored in aliquots at −80°C until used.

Total RNA Extraction and Real Time Quantitative RT-PCR IL-4, IL-13, IL-5, IFN-γ, GATA-3, T-bet, IL-17, IL-22, ROR-C, AHR, and β-actin

Total RNA was extracted with RNAsy Kit and treated with DNase I (Qiagen, Hilden Germany) from 5 CD4+ T cell clones, and PBMC from 5 donors. cDNA was synthetized by using TaqMan Reverse Transcription Reagents (Applied Biosystem, Warrington, United Kingdom). Reverse Transcription–Polymerase Chain Reaction (RT-PCR) was then performed by using TaqMan methodology as described elsewhere (42). Quantitative analysis of IL-4, IFN-γ, IL-10, IL-13, IL-5, GATA-3, T-bet, IL-17, IL-22, ROR-C, AHR, and β-actin was performed by using Assay on Demand (Applied Biosystem, Warrington, United Kingdom). β-actin was used for normalization.

Quantitation of Cytokine Production by CD4+ T Cell Clones

The quantitative determination of IL-1beta, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IFN-alpha, TNF-alpha, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1-alpha, and MIP-1-beta was performed by a bead-based multiplex immunoassay (Biorad Laboratories, Hercules, CA). IL-17A, IL-17F, IL-21 and IL-22 were measured by a bead-based multiplex immunoassay (R&D, Minneapolis, MN) and a Bioplex 200 system (Biorad Laboratories, Hercules, CA) as we previously described (43, 44).

Statistical Analysis

Statistical analyses were performed using SPSS software (SPSS, Inc, Evanston, IL). Due to non-parametric distribution, all comparisons between cytokine concentrations in basal and stimulated conditions were performed by Wilcoxon test. Data are reported as median and ranges unless otherwise stated.

RESULTS

Effect of MPA on the Proliferation and on the Cytokine Profile of Peripheral Blood Mononuclear Cells (PBMCs)

We used PBMCs to mimic *in vitro* a T cell specific response to an antigen derived from a pathogen. We investigated the effect of MPA when the antigen presenting cells in the PBMC

fraction present an antigen to T cells and activate these T cells. Streptokinase (SK), a highly purified antigen extracted from a C-group beta-hemolytic streptococci culture and devoid of all other metabolic products of streptococci, was used as antigen.

Unstimulated PBMCs from 5 normal donors cultured in the presence of MPA at 0.02, 0.2, and 2 ng/ml showed no significant differences in their proliferative response compared PBMCs cultured in medium alone (data not shown).

Streptokinase (SK)- stimulated PBMCs from 5 donors in the presence of MPA at 0.02, 0.2, and 2 ng/ml showed no significant differences in their proliferative response compared to SK-stimulated PBMCs expanded in the absence of MPA (data not shown).

To provide evidence of the effect of MPA on PBMCs, we analyzed the ability of MPA to act on Th2-type cytokines (IL-4, IL-5, and IL-13), Th1-type cytokine (IFN-gamma) and Th17-type cytokines (IL-17A, IL-17F, and IL-22) production (**Figure 1**) by PBMCs from 10 different donors. There was no statistical difference in PBMCs cytokine production when cells were cultured without any stimulation in medium alone and in medium + MPA at 0.02, 0.2, and 2 ng/ml (data not shown).

The effects of SK stimulation alone over medium were not statistically significant for IL-4, IL-5, IL-13, IL-22, and IL-17F production in PBMCs, but significant for IFN-γ and IL-17A (**Figure 1**).

When PBMCs were stimulated with SK in the absence or presence of MPA, the levels of IFN-γ, IL-5, IL-13, IL-17A were significantly decreased whereas the levels of IL-22 were significantly increased in the presence of MPA compared to those found in the absence of MPA (**Figure 1**), indicating that MPA seems to modulate T cell cytokine production regardless the amplitude of the effect of SK alone. Thus, MPA seems to modulate the T cell cytokine production only after the stimulation of T cells by an antigen (here SK) presented by the antigen presenting cells in the PBMCs fraction.

We also attempted to confirm the previous results by examining with real time RT-PCR analysis of PBMCs of 5 additional donors stimulated with SK in the absence or in the presence of 0.02 and 0.2 ng/ml of MPA (**Figure 2**). As a control, the PBMCs were also stimulated with SK in the presence of IL-12, a potent inducer of Th1 differentiation (45). We found a significant increase of IFN-γ ($p = 0.043$) (**Figure 2**) in response to IL-12, suggesting that the culture conditions were satisfactory for the modulation of the PBMCs. PBMCs expressed lower levels of mRNA for IL-5, IL-13, IFN-γ, IL-17A and its transcription factor ROR-C (**Figure 2**). Higher levels of mRNA were found for IL-22 and the corresponding transcription factor AHR (**Figure 2**) when MPA was added to the culture medium. mRNA levels for IL-4 were not modified by MPA (**Figure 2**).

These results indicate that MPA can decrease some Th2-type cytokines (IL-5 and IL-13, but not IL-4), including a Th1 cytokine (IFN-γ) and a Th17-type cytokine (IL-17A). However, MPA increases IL-22, which can be produced by Th22 and Th17 cells.

Effect of MPA on the Cytokine Profile of Macrophages Derived From Peripheral Blood Monocytes

The negative effect of MPA on Th1, Th2, Th17-type cytokine production of PBMCs and its positive effect on Th22-type cytokine production could be due to the modulating effect of MPA on APCs present in the microenvironment of the T cells. However, the levels of these cytokines produced by SK-stimulated macrophages cultured in the presence of MPA were not significantly different than those of SK-stimulated macrophages cultured in the absence of MPA (**Figure 3**). Thus, in PBMC fractions the negative effect of MPA on Th1, Th2, Th17-type cytokine production of PBMCs and its positive effect on Th22-type cytokine production seem to be due to the action of MPA directly on T cells.

Moreover, cytokine production by macrophages in response to MPA could in turn modulate the T cell cytokine production. The negative effect of MPA on T cell-IFN-γ-production was not due to a decreased production of IL-12 and/or IFN-α and/or IL-18 by macrophages treated with MPA, i.e., the concentrations of IL-12, IFN-α, TNF-α, and IL-18 produced by purified macrophages from 7 different donors stimulated with SK showed no significant differences when cells were cultured either in the absence or in the presence of MPA (**Figure 5**). Thus, MPA acts directly on CD4+ T cells to decrease IFN-γ production and mRNA expression (**Figures 1**, **2**). However, the levels of IL-1α and IL-6 produced by SK-stimulated macrophages cultured in the presence of MPA (2 ng/ml) (data not shown) and the levels of MIP-3α (CCL20) in the presence of MPA at 0.02, 0.2, ng/ml (**Figure 3**) and 2 ng/ml (data not shown) were lower than those of SK-stimulated macrophages cultured in the absence of MPA. The decreased production of IL-1α and IL-6 by macrophages in response to MPA at 2 ng/ml, i.e., at a concentration higher than found in the serum of women treated with MPA after 6 and 9 months, was reported at 200 ng /ml (28). MIP-3α is the known ligand of CCR6, which is expressed on the cell membrane of Th17 cells. The interaction CCR6/CCL20 contribute to the trafficking of Th17 cells. Thus, the reduction of IL-17 production by T cells in the presence of MPA at doses found in the serum of human users could be associated with a reduction of the trafficking of Th17 cells. The levels of IL-23 essential for development of Th17, produced by SK-stimulated macrophages were not modified by the presence of MPA suggesting that MPA acts directly on CD4+ T cells by decreasing IL-17 production.

Direct Effects of MPA on the Proliferation and on the Cytokine Profile of Established CD4+ T-Cell Clones

To investigate the direct effect of MPA on the proliferative activity of unstimuled purified T cells, 7 T cell clones in medium alone and in medium plus MPA at 0.02, 0.2 and 2 ng /ml were analyzed for their ability to proliferate. No statistical difference of the T cell clones proliferative response was found when cells were cultured without any stimulation in medium alone and in medium plus MPA at 0.02, 0.2, and 2 ng/ml (data not shown).

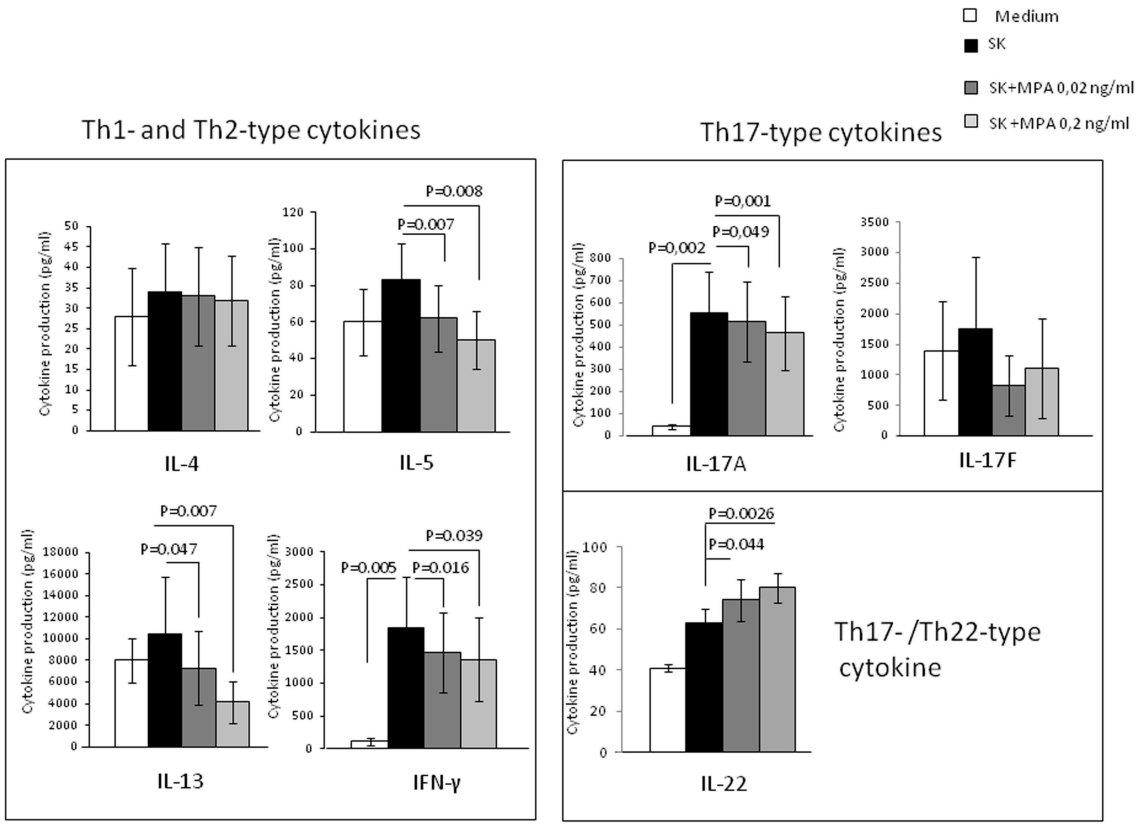

FIGURE 1 | Effect of MPA on the cytokine profile of peripheral blood mononuclear cells (PBMCs). PBMCs from 10 different donors were stimulated with SK in the absence or presence of MPA at 0.02 and 0.2 ng/ml to provide their ability to modulate Th2-type cytokine (IL-4, IL-5, IL-13), Th1-type cytokine (IFN-gamma) and Th17-type cytokines (IL-17A, IL-17F, and IL-22) production.

The direct effect of MPA on the proliferative activity of CD4+T cell clones stimulated with immobilized anti-CD3 antibody in the absence or presence of MPA at 0.02, 0.2, and 2 ng/ml was analyzed. According to the results obtained with PBMCs, no significant differences in the proliferative response were observed between stimulated T cell clones expanded in the presence or in the absence of MPA (data not shown).

To provide evidence of the direct effect of MPA on CD4+T helper cells, we examined the ability of MPA to act on a panel of cytokines (as listed in Methods). We also studied MPA effects on IFN-γ, IL-5, IL-4, IL-13, IL-17A, IL-22, and IL-10 mRNA expression by established CD4+ T-cell clones. Thirteen CD4+ T-cell clones were cultured in the presence of recombinant human IL-12, a Th1 inducer, which acts as a control of the functional activity of T cell clones. When the 13 CD4+ T-cell clones were cultured with IL-12, the levels of IFN-γ and mRNA for IFN-γ increased (**Figures 4A,B**) ($p = 0.02$ and $p = 0.005$, respectively) suggesting that the culture conditions were satisfactory for the modulation of CD4+ T cell clones.

There was no statistical difference between the T cell clones cytokine production when cells were cultured without any stimulation in medium alone or in medium plus MPA at 0.02, 0.2, and 2 ng/ml (data not shown).

When Thirteen CD4+ T-cell clones were cultured in the absence of any other cell type and stimulated with insolubilized anti-CD3 monoclonal antibody in the absence or presence of MPA the levels of IL-1beta, IL-1RA, IL-2, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17F, TNF-α, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1-α, MIP-1-β, IL-21 (data not shown), IL-22, and IL-4 (**Figure 4A**), were not significantly modified in the presence of MPA. In contrast, the levels of IFN-γ, IL-5, IL-13, IL-17A were significantly decreased in the presence of MPA compared to those found in the absence of MPA (**Figure 4A**). Thus, MPA seems to modulate the T cell cytokine production of T cells only when these cells are stimulated.

Real time RT-PCR analysis was used to determine MPA influence on IFN-γ, IL-5, IL-13, IL-17A, IL-4, IL-22, GATA-3 (a Th2-specific transcription factor) (46, 47) T-bet (a Th1-restricted transcription factor) (48), ROR-C (a Th17-specific transcription factor) and AHR (a transcription factor expressed by Th22 cells) mRNA expression of the CD4+T cell clones stimulated with immobilized anti-CD3 monoclonal antibody without or with MPA (**Figure 4B**). CD4+ T cell clones were also stimulated with immobilized anti-CD3 antibodies in the presence of IL-12. We confirmed an increase of IFN-γ in response to IL-12, suggesting that the culture conditions were satisfactory for the modulation

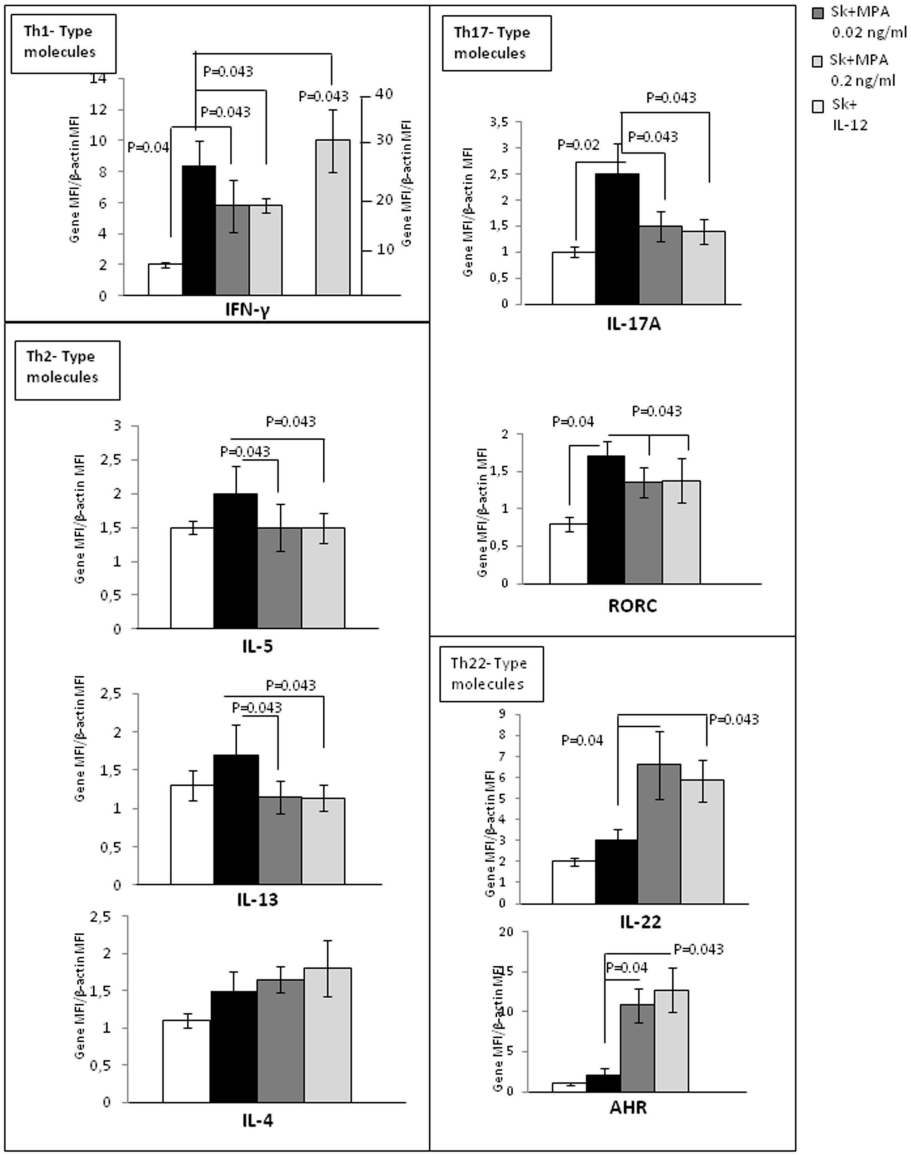

FIGURE 2 | Effect of MPA on the cytokine profile and transcription factor expression by peripheral blood mononuclear cells (PBMCs). mRNA expression of IL-4, IL-5, IL-13, IL-17A, IFN-γ, IL-22, AHR, and RORC performed by RT-PCR analysis of the PBMCs from 5 donors stimulated with SK in the absence or in the presence of 0.02 and 0.2 ng/ml of MPA was analyzed. PBMCs were also stimulated with SK in the presence of IL-12, which is a potent inducer of Th1 differentiation, to ensure that the culture conditions were satisfactory for modulation of the cytokine production of CD4+ T cell clones.

of CD4+ T cell clones (**Figure 4B**). The CD4+ T cell clones expressed lower levels of IFN-γ, IL-5, IL-13, IL-17A, ROR-C, and T-bet mRNA in the presence of MPA compared to the control, whereas the levels of transcripts for IL-4 and GATA-3 were not modified by the presence of MPA in the culture but the levels of AHR increased (**Figure 4B**).

Surprisingly, we found that the levels of IL-22 were increased when MPA is added to cultures of PBMCs, but not when it is added to the CD4+ T cell clones, although the levels of m RNA for AHR the transcription factor of Th22 cells producing IL-22 expressed by the same T cell clones were increased.

This apparent paradox could be explained by the fact that IL-22 is not only produced by Th22 cells, but also by Th17 cells, and there could be a differential production of IL-22 by Th17 and Th22 cells in response to MPA. AHR could be the key factor explaining the effect of MPA on the T cells. To investigate this possibility, we studied the effects of MPA on the cytokine profile of established CD4+ Th1- Th2-Th-17 and Th22-cell clones.

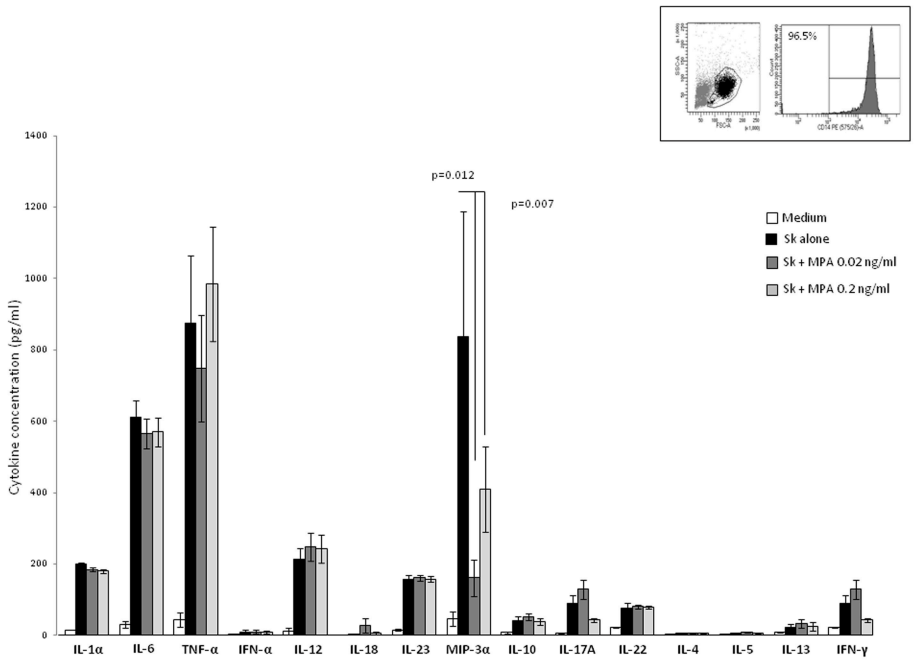

FIGURE 3 | Effect of MPA on the cytokine profile of macrophage. Macrophages from PBMC obtained from 7 donors purified by adherence stimulated for 5 days with the antigen SK in the absence or in the presence of MPA (0.02, 0.2, and 2 ng/ml). IL-1α, IL-6, TNF-α, IFN-α, IL-12, IL-18, IL-23, MIP3α, IL-10, IL-4, IL-5, IL-13 IL-17A IFN-γF, and IL-22 were measured in the supernatants.

Direct Effects of MPA on the Cytokine Profile of Established CD4+ Th1- Th2-Th17 and Th22-Cell Clones

To verify the direct effect of MPA on the different CD4+T cell subpopulations, 6 Th1 clones, 6 Th2 clones, 6 Th17/Th1 clones and 6 Th22 clones were stimulated with insolubilized anti-CD3 monoclonal antibody in the absence or presence of MPA (**Figures 5A,B**) and at protein level analyzed for IL-4, IL-5, IL-13, IL-22, IL-17A, IFN-γ. mRNA levels were determined for AHR, ROR-C, and T-bet.

In the presence of MPA levels of IL-4, IL-5, IL-13, IL-22, IL-17A produced by Th1 cells were not significantly modified. In contrast, the levels of IFN-γ produced by Th1 cells were significantly decreased in the presence of MPA compared to those found in the absence of MPA (**Figure 5A**).

The levels of IL-4, IFN-γ, IL-22, IL-17A produced by the Th2 cells were not significantly modified in the presence of MPA. However, the levels of IL-5 and IL-13 produced by Th2 cells were significantly decreased in the presence of MPA compared to those found in the absence of MPA (**Figure 5A**).

The production of IL-4 (data not shown), IL-5 (data not shown), IL-13, AHR and T-bet mRNA expression by Th17/Th1 cells were not significantly modified in the presence of MPA. Levels of IFN-γ, IL-22 and IL-17A at protein and mRNA levels (data not shown), and mRNA for ROR-C expressed by Th17/Th1 cells were significantly decreased in the presence of MPA compared to those found in the absence of MPA (**Figure 5B**).

The levels of IL-4, IL-5 (data not shown), IL-13 (data not shown), IFN-γ and IL-17A produced by the Th22 cells were not modified whereas the levels of mRNA for AHR, T-bet and IL-22, as well as levels of IL-22 protein ($p = 0.042$) were significantly increased in the presence of MPA compared to those found in the absence of MPA. Moreover, the levels of mRNA for ROR-C were not modified in Th22 cells in presence of MPA compared to those found in absence of MPA (**Figure 5B**).

These data confirmed the negative effects of MPA on the production of IFN-γ, IL-5, IL-13, and IL-17A, but supported the finding that Th17 and Th22 cells, which are able to produce IL-22, react differently to MPA; IL-22 production in response to MPA is increased in Th22 cells and decreased in Th17 cells. This could explain why the statistical analysis of IL-22 production by 13 different T- cell clones derived from different Th type subpopulations does not appear to be influenced by MPA (**Figure 4A**). The increased production of IL-22 by Th22 cells is probably compensated by the decreased production of IL-22 by the Th17 cells in response to MPA. Moreover, it seems that T-bet and AHR control the production of IL-22 by Th22.

DISCUSSION

We found that therapeutic concentrations of MPA, comparable to those present in the serum of women undergoing HRT or contraception, have no effect on the proliferation of CD4+ cells and several cytokines, (IL-1beta, IL-1Ra, IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17F). Neither do these doses of MPA

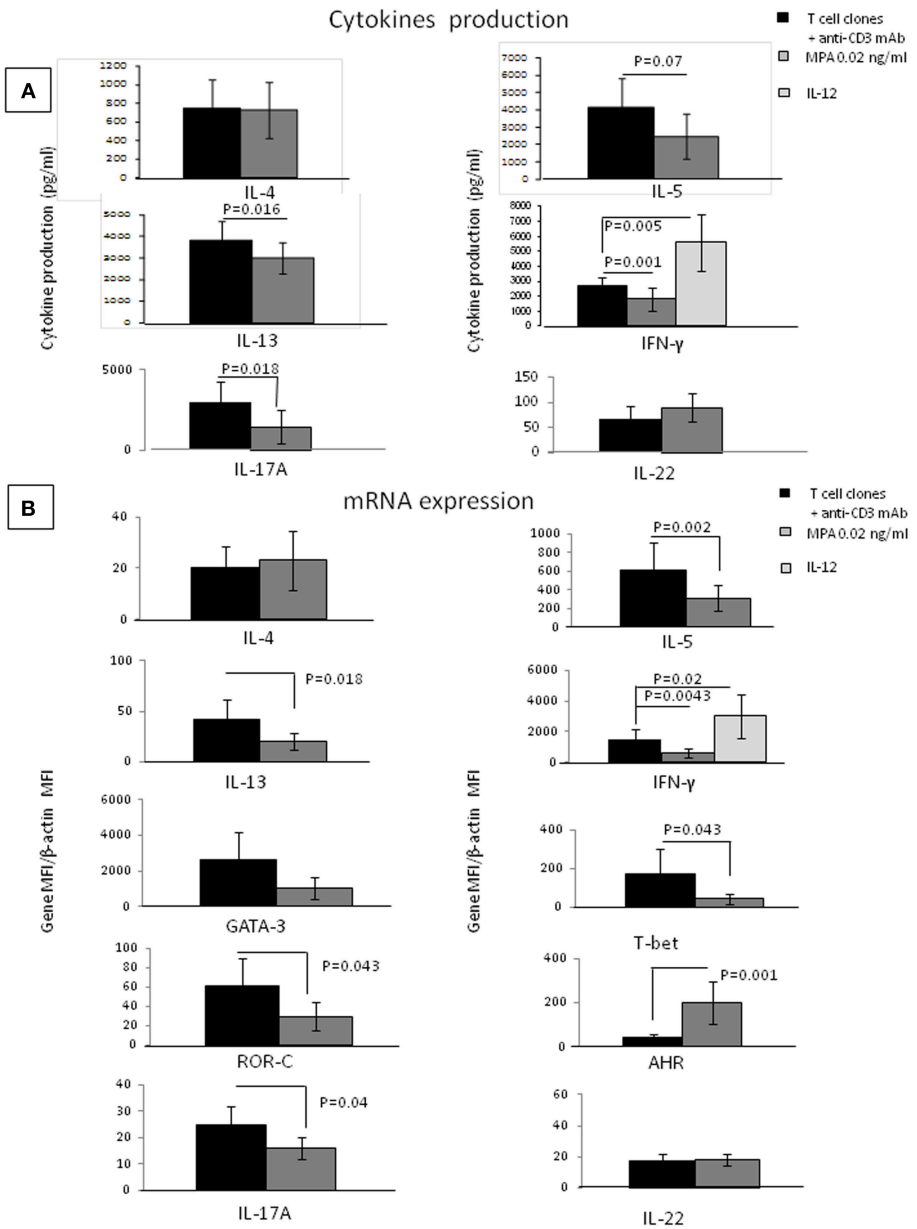

FIGURE 4 | Direct effects of MPA on the cytokine profile of established CD4+ T-cell clones. IL-4, IL-5, IL-13, IL-17A, IFN-γ, IL-17F, and IL-22 production were measured in the supernatant of 13 established T-cell clones (TCC) by multiplex assays **(A)**. mRNA expression for IFN-γ, IL-5, IL-4, IL-13, IL-17A, IL-22, GATA3, T-bet, and AHR was examined **(B)**. As a control, the 13 CD4+ T cell clones were also stimulated with immobilized anti-CD3 antibodies in the presence of IL-12, a potent inducer of Th1.

affect TNF-α, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1α, MIP-1β or IL-21 production by CD4+T cells. In contrast, MPA specifically decreases IFN-γ, IL-5, IL-13 and IL-17A production but increases IL-22 production by CD4+ T cells. These results were confirmed by examining MPA effects on mRNA levels. Furthermore, MPA decreased not only IL-17A production by Th17 cells but also MIP-3α (CCL20) by macrophages, which contributes to decreased trafficking of Th17 cells.

Interestingly, IL-22 production is decreased in Th17 cells treated with MPA but increased in Th22 cells. The decreased production of IL-17A and IFN-γ in the presence of MPA is associated with the decreased expression of ROR-C and T-bet, respectively, whereas the increased production of IL-22 by Th22 cells is associated with the increased expression of T-bet and AHR in response to MPA.

Thus, MPA, at doses equivalent to those found in the serum of women using MPA for contraception or HRT, could influence T

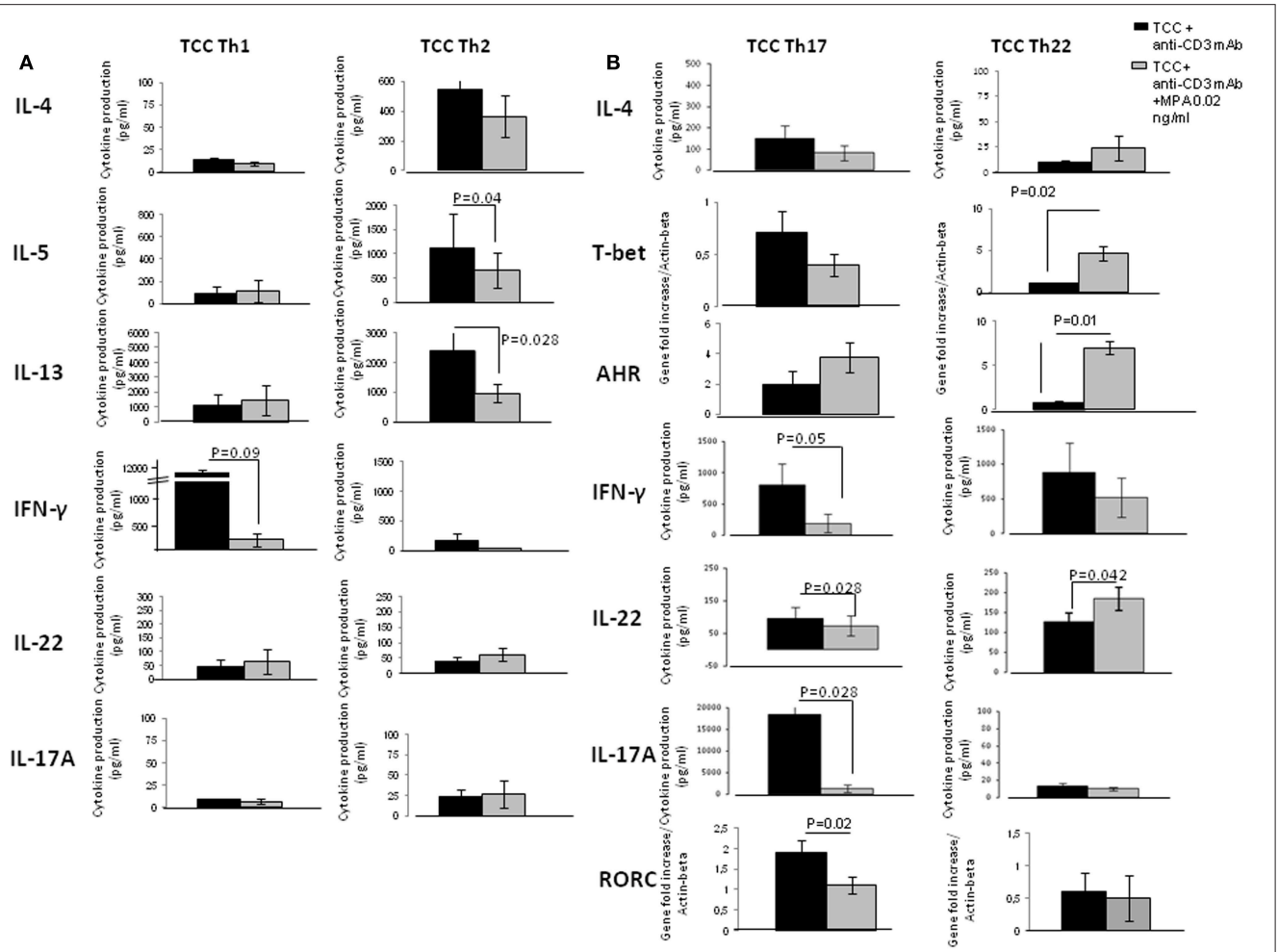

FIGURE 5 | Direct effects of MPA on the cytokine profile of established CD4+ Th1- Th2-Th-17 and Th22-type T cell clones (TCC). To provide evidence of the direct effect of MPA on different CD4+T cell subpopulations, 6 CD4+ Th1 T-cell clones, 6 CD4+ Th2 T-cell clones, 6 CD4+ Th17/Th1 T-cell clones and 6 CD4+ Th22 T-cell clones were stimulated with insolubilized anti-CD3 monoclonal antibody in the absence or presence of MPA at 0.2 ng/ml. The levels of IL-4, IL-5, IL-13, IL-17A, IFN-γ, and IL-22 were measured by multiplex assays in the supernatants **(A)** and of mRNA for AHR, RORC, and Tbet by RT-PCR **(B)**.

cell immune responses toward allograft rejection, infections and asthma as demonstrated *in vivo* in humans (49–56).

Indeed, an allograft rejection is characterized by a predominant production of IFN-γ (57, 58). Our results showing the depressed production of IFN-γ in response to MPA are consistent with data from animal model studies indicating that MPA prolongs survival of skin and renal allografts (49, 59, 60). More interestingly, in a trial of humans with intrafamilial renal transplants treated by addition of MPA to the immunosuppressive regimen, when renal function was stable at the time of initiation of MPA therapy, prednisone dosage could be lowered to an average of 37% of the previous dose (49). Thus, MPA has been shown to have immunosuppressive functions in human.

IFN-γ also plays an important role in adaptive and innate immune responses to viral and intracellular bacterial infection (61). The decreased IFN-γ production by T cells in response to therapeutic concentrations of MPA suggests this synthetic progestin could influence antiviral immunity. In fact, MPA is used to increase infectibility in mouse models of sexually transmitted diseases (62). Relative to progesterone, MPA was shown in these models to increase by ten-fold susceptibility to infection by Herpes simplex virus type 2 (HSV-2) (62). Unlike progesterone, MPA significantly decreases the immune response to intracellular pathogens (63). A Th1 response seems to be protective against HSV viral spread and tissue damage, but shifting to a Th2 response is associated with resolution of immunopathology (64, 65). MPA, by decreasing IFN-γ production by T cells, could be, at least in part, responsible for an increased susceptibility to infection by Herpes simplex virus. More importantly, it was reported that consistent MPA contraceptive use in 682 HSV2-negative women induced increased risk of HSV-2 seroconversion. In fact, incidence rate was 13.5 per 100 person-years in women consistently using

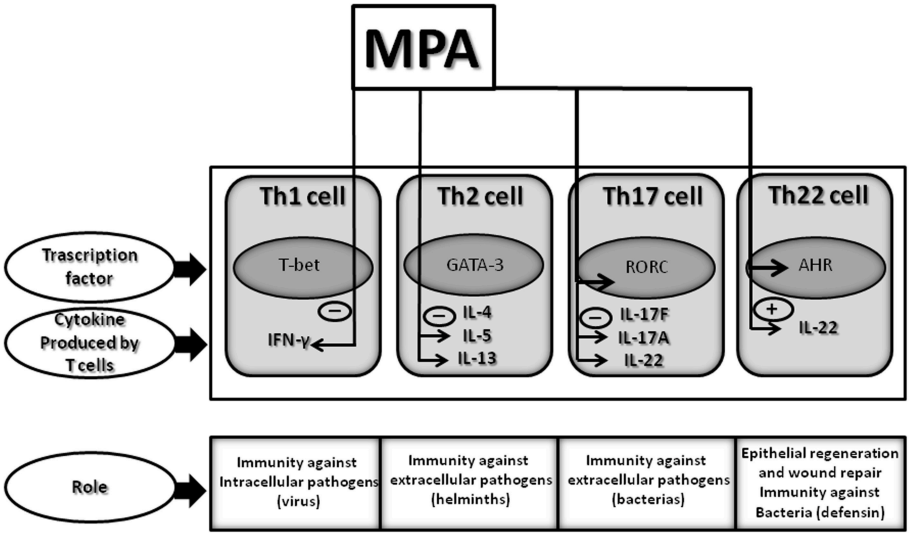

FIGURE 6 | Effect of MPA on the cytokine profile of established CD4+ Th1, Th2, Th17, and Th22 cells: summary of identified changes and potential outcomes. Therapeutic concentrations of MPA, comparable to those present in the serum of women undergoing HRT or contraception, decreases IFN-γ, IL-5, IL-13, and IL-17A production but increases IL-22 production by CD4+ T cells. Thus, MPA by decreasing IFN-γ by Th1 cells could diminish the T cell immune responses to intracellular pathogens (viruses); by decreasing IL-5, MPA could diminish the T cell immune responses to helminths; by decreasing IL-13 could affect allergic disorders; by decreasing IL-17A, MPA could diminish the T cell immune responses to extracellular pathogens; and by increasing IL-22 by Th22 cells MPA may contribute to the host defense against microbial pathogens, in addition to promoting tissue repair or remodeling.

DMPA (nine incident infections per 66.5 person-years) and 6.6 per 100 person-years in women who were neither pregnant nor using hormonal contraception (35 incident infections per 529.5 person-years) (52). Very recently, an updated systematic review incorporate studies published between January 2009 and June 2017. Thirty articles met the inclusion criteria and showed that Depo-medroxyprogesterone acetate (DMPA) increased the risk of HSV-2 (strong effect, few studies), whereas data on oral contraceptive use suggested it was associated with inconclusive findings for HSV-2 (53).

A Th1/Th2 cytokine imbalance is also critical to HIV-1 progression and pathogenesis. It was shown that addition of exogenous IFN-γ mediates antiviral activity against R5 HIV-1 thymocytes, which decreases viral replication in infected thymocytes (66). It was suggested that MPA suppresses both innate and adaptive arms of the immune system resulting in a reduction of host resistance to invading pathogens as HIV-1 (32). However, MPA was used in culture at doses 1,000 fold higher than the those found in the serum of MPA users. In the present study we show for the first time that doses of MPA equivalent to those found in women actually using MPA decrease IFN-γ production by T cells, which could influence HIV progression. Thus, MPA, by increasing shedding of HIV-1 DNA (67), increases the HIV transmission risk, and, by decreasing IFN-γ production by T cells, may also influence HIV viral replication. There is now a major concern in women who use long acting injectable hormonal contraceptives, particularly Depo-MPA with an increase of HIV-1 risk acquisition. In fact, different recent meta-analyses showed a significant association between use of Depo-MPA and the presence or acquisition of HIV+ in women (54–56). For this reason, the World Health Organization (WHO) published guidelines for hormonal contraceptive eligibility for women at high risk of HIV in March 2017. The guideline development group, through a consensus, made recommendations to change the medical eligibility criteria for contraceptive use from category 1 (condition for which there is no restriction for the use of the contraceptive method) to category 2 (condition where the advantages of using the method generally outweigh the theoretical or proven risks) for Depo-MPA among women at high risk of HIV acquisition (68).

It is important to recognize that MPA is the most commonly used progestin in the USA and Europe for hormone replacement therapy (23). In addition, MPA is the most widely used injectable female contraceptive (21), with at least 20 million current users worldwide (22). Of special interest in the developing world, with its high incidence of viral diseases and endemic malnutrition, we suggest that the choice of synthetic progestins used in contraception could have important implications for viral disease development. It is important to note, however, that the use of hormonal contraceptives, and in particular of MPA, does not significantly impact the effectiveness of antiretroviral therapy (25, 69).

As progesterone can induce an increased production of IL-5 and IL-4 by T cells, yet have no effect on T cell IFN-γ production (6), MPA is the only progestin yet analyzed that influences IFN-γ production by Th1 cells when used as a contraceptive or for HRT and also decreases IL-5 and IL-13 production without affecting IL-4 production. As a result of this activity MPA may also have an influence on allergic responses. Allergy is a disorder characterized by an increased ability of B cells to

produce IgE in response to certain groups of ubiquitous antigens (allergens) that can activate the immune system after inhalation, ingestion or penetration through the skin. IgE antibody synthesis results from the collaboration between Th2 cells and B cells, in which CD40/CD40L interaction is required (70). IL-4 and IL-13 produced by Th2 cells induce the production of IgE by B cells whereas Th1 cells produce IFN-γ that suppresses IgE synthesis (71). Th2 cells also produce IL-5 that favors the differentiation, activation and survival *in situ* of eosinophils. MPA by acting to decrease IL-5, IL-13 and IFN-γ production by T cells could have a negative effect on the differentiation of eosinophils, their activation and survival while having a positive effect on IgE production induced by IL-4. The latter activity would decrease the negative effect of IFN-γ on the IgE production. Asthma is a complex disorder characterized by intermittent, reversible airway obstruction, and by airway hyperresponsiveness and inflammation. Asthma may be divided into allergic (extrinsic) and non-allergic (intrinsic) asthma. Both allergic and non-allergic asthma are characterized by the presence in the bronchial mucosa of large numbers of activated eosinophils and of elevated concentrations of eosinophil-derived proteins, such as major basic protein and eosinophil cationic protein (72). In allergic asthma, the importance of Th2 cytokines, especially IL-5, in the induction of allergic pulmonary inflammation and airway hyperreactivity has been reported (73). Corticosteroid treatment in asthma, is associated in the downregulation of BAL cells expressing mRNA for IL-4 and IL-5 and in the upregulation of cells expressing mRNA for IFN-γ (74). MPA decreases IL-5 and IL-13 production and mRNA expression by T cells. This suggests that widely used concentrations of MPA can have a positive influence on the health status of patients with allergic and non-allergic asthma by decreasing IL-5 production by T cells, thus reducing eosinophilic infiltration of the lungs. Interestingly, after 6 months of HRT with transdermal 17-beta-estradiol and MPA, diminishing symptoms of asthma were observed and there was a reduction in the number of patients in whom it was necessary to use oral glucocorticoid therapy during exacerbation of asthma (50, 51). These patients were treated with 17-beta-estradiol associated with MPA. However, the diminishing symptoms of asthma cannot be due to the estradiol, which potentiates the development of asthma. In fact, in male mice treated with estradiol, eosinophil numbers increase in both blood and airways and the production of IL-5 and IL-13 by T cells is promoted (75). Thus, estradiol increases IL-5 produced by T cells, whereas MPA decreases IL-5. Therefore, the reduced asthmatic symptoms in patients treated with 17-beta-estradiol and MPA could be due to the reduction of IL-5 production mediated by MPA.

We found that MPA at therapeutic concentrations found in the serum of women upregulates AHR. AHR plays important physiological roles in many cells of the immune system, notably the Th17 and Th22 cells (76–78). Most of the current literature on AHR effects on immune system function is focused on the consequences of exposure to the high affinity ligand 2, 3, 7, 8 tetrachlorodibenzo-p-dioxin (TCDD). However, many recent studies in mice lacking AHR expression indicate that AHR activation affects important physiological functions in the absence of xenobiotic ligands (79). There is a range of potential physiological ligands for AHR including diet-derived AHR ligands (Quercetin present in apples and onions, Indol-3-carbinol present in many Brassicaceae, Resveratrol present in red wine, Curcumin), which strongly influence intestinal immune parameters (79). Our data seem to suggest that MPA could be another ligand for AHR. In agreement with the hypothesis that steroid hormones could affect AHR expression and could be a ligand of AHR, it was demonstrated that progesterone, as well as 17-beta-estradiol, regulate the AHR battery homeostasis in the rat uterus (37). Progesterone leads to an increase in uterine AHR levels, especially in endometrial epithelium. Only one demonstration of MPA influence on endometrial but not T cells showed that no significant changes were observed in AHR transcript levels in endometrial cells (35). While these results indicate that female sexual steroid hormones regulate the expression of the AHR battery in organs of the female reproductive system, no effectiveness of female hormones, in particular MPA, on the expression of the AHR battery in T cells has been previously reported.

The present study show that MPA, at concentrations found in the serum of women undergoing contraception or HRT, up-regulates IL-22 production by CD4+ Th22 cells through AHR and T-bet -induced signals, whereas IL-22 production by Th17 cells is down-regulated and Tbet and AHR are not modified by MPA. These data suggest that the differential production of IL-22 by Th22 cells and Th17 cells by MPA could be carried out through AHR-induced signals and T-bet-induced signals. In fact, it appears that IL-22 expression is due to the cooperation of AHR and T-bet-induced signals (78).

The ligand dependant-AHR activity is involved in the regulation of T cell-mediated immune responses (76, 77) and, as such, could be involved in shaping the course of autoimmune pathology. This suggest a link to environmental factors containing ligands of AHR that influence autoimmune disease. AHR-deficient mice developed a much milder form of EAE with many of these mice altogether protected from the onset of disease (76). However, the application of AHR agonists caused differential effects. The administration of the tryptophan metabolite 6-formylindolo(3,2-b) carbazole (FICZ), an endogenous AHR agonist, exacerbated disease (76), while systemic administration of TCDD had the same ameliorating effect on disease progression as AHR-deficiency (77). This led to the suggestion that AHR exerts its effects on immune responses in a ligand-dependent manner. The differential effects of different AHR agonists on autoimmune disease progression is probably due to their influence on the T helper responses responsible for the disease progression (39). Indeed, it has been demonstrated that the oral administration of a synthetic compound M50354, a AHR agonist, reduces the production of IL-4 and IL-5 by Ag-stimulated splenocytes and enhances the production of IFN-γ. TCDD, another AHR ligand, exerts suppressive effects on the production of IL-2, IL-4, IL-5, and IL-6. In contrast, M50367 did not affect the production of IL-2 and IL-6, but appeared to reduce Th2-mediated immune responses. M5037 suppressed the expression of a key transcription factor for Th2 cell differentiation, GATA-3, and the production of IL-4,

although it is not known whether activated AHR is directly involved in GATA-3 expression (39).

These data suggest that AHR may play an important role in the normal development and function of the immune system by down-regulating IFN-γ. In agreement, we showed that MPA has no effect on IL-4, upregulates AHR, downregulates IFN-γ production by T cells. Moreover, it was shown that in response to OVA immunization, high levels of IFN-γ mRNA were detected in lymphocytes from AHR Knock-out (AHR-/-) mice, but IL-4 mRNA levels in AHR-/- cells were similar to those in AHR+/+ mice (80).

The effects of AHR agonists on IL-22 production have been reported (81). AHR is down-regulated in intestinal tissue of patients with IBD; and AHR signaling via IL-22 inhibits inflammation and colitis in the gastrointestinal tract of mice (81). Intestinal lamina propria mononuclear cells in the presence of FICZ showed reduced levels of IFN-γ and up-regulated levels of IL-22 (81). We showed that MPA downregulates IFN-γ and upregulates AHR and IL-22 expression and production by T cells as does FICZ, the agonist of AHR.

Our results allow us to speculate that MPA could be an agonist of AHR in T cells, acting to decrease IFN-γ and, at the same time, IL-17A, upregulating IL-22 without any effect on IL-4 production.

It is important to note that AHR acts as an important co-factor in infections. For instance, AHR-deficient mice infected with Listeria monocytogenes, an intracellular bacterium, were more susceptible to infection but developed enhanced resistance to re-infection (82). Depending on the cell context analyzed and type of agonist used, AHR-driven signals could exert differential modulation of Th responses and act as initiators or attenuators of tissue- damage T cell-dependent inflammatory processes.

Through the AHR expression MPA, may potentially decrease immune function by decreasing T cell IFN-γ and IL-17A production, thereby influencing certain aspects of infection progression. By increasing IL-22 and decreasing IL-17A, MPA may be tissue protective (83). IL-22 induced by MPA could act as a protective hormone thereby counteracting the destructive effects of the immunoresponse. MPA may also limit the tissue damage observed in some autoimmune disorders, and may attenuate the inflammatory processes (81) in some autoimmune disorders.

The biology and pharmacology of progestins and their receptors are complex. Our understanding of their action in certain physiologic targets including the immune system continues to grow. The specific effects of hormones, including synthetic progestins, depend upon their preparation, dose, sequence of administration and context of treatment. The observation that ubquitously used doses of MPA influence the immune response of women adds yet another level of complexity to the design and prescription of hormone therapy, whether it be contraception or HRT, particularly in women with coexisting immune disorders and infections.

AUTHOR CONTRIBUTIONS

M-PP conceived the study and designed the experiments, analyzed all the data, supervised, and wrote the manuscript. MB participated in discussion and revision of the manuscript. LL, FL, and OK performed the experiments using PBMNCs and T cell clones cultures, performed the multiplex bead-based assays and RT-PCR. EM approved and authorized all the process.

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Transcriptomics and Immunological Analyses Reveal a Pro-Angiogenic and Anti-Inflammatory Phenotype for Decidual Endothelial Cells

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Abstract: Background: In pregnancy, excessive inflammation and break down of immunologic tolerance can contribute to miscarriage. Endothelial cells (ECs) are able to orchestrate the inflammatory processes by secreting pro-inflammatory mediators and bactericidal factors by modulating leakiness and leukocyte trafficking, via the expression of adhesion molecules and chemokines. The aim of this study was to analyse the differences in the phenotype between microvascular ECs isolated from decidua (DECs) and ECs isolated from human skin (ADMECs). Methods: DECs and ADMECs were characterized for their basal expression of angiogenic factors and adhesion molecules. A range of immunological responses was evaluated, such as vessel leakage, reactive oxygen species (ROS) production in response to TNF-α stimulation, adhesion molecules expression and leukocyte migration in response to TNF-α and IFN-γ stimulation. Results: DECs produced higher levels of HGF, VEGF-A and IGFBP3 compared to ADMECs. DECs expressed adhesion molecules, ICAM-2 and ICAM-3, and a mild response to TNF-α was observed. Finally, DECs produced high levels of CXCL9/MIG and CXCL10/IP-10 in response to IFN-γ and selectively recruited Treg lymphocytes. Conclusion: DEC phenotype differs considerably from that of ADMECs, suggesting that DECs may play an active role in the control of immune response and angiogenesis at the foetal-maternal interface.

Keywords: endothelium; decidua; skin; angiogenesis; inflammation

1. Introduction

Endothelial cells (ECs) form a continuous barrier between blood and tissues and act as a gateway to the traffic of molecules and cells across the vessel wall, playing an active role in homeostasis,

inflammation and immunity [1]. They also play an important role in the onset and maintenance of inflammation, being both target as well as source of cytokines, chemokines and growth factors [1]. ECs, although apparently similar to each other in function and morphology, represent a heterogeneous population of cells in terms of inflammatory mediators secretion, adhesion molecules modulation, leakiness and pro-coagulant activity [2]. The pathophysiological functions of ECs, which mainly relates to angiogenesis and inflammation, take place at the level of the microvascular bed, which constitutes the bulk of the overall endothelial surface [3]. The microenvironment as well as the epigenetics are involved in determining ECs heterogeneity, resulting in tissue-specific properties [4]. Skin is the primary interface between the body and the environment; skin injury or infections promptly result in the activation of an efficient inflammatory response. Dermal microvascular ECs are active regulators of the inflammatory process [5]. They contribute to the secretion of inflammatory mediators, bactericidal molecules, increasing leakiness and pro-coagulant activity, modulation of adhesion and transmigration of leukocytes through the expression of adhesion molecules and chemokines [6].

Decidua, the maternal part of human placenta, is derived from uterine endometrium through an inflammatory process that leads to the transformation of endometrial stromal cells [7]. Consequently, decidualization contributes to the activation of the local immune cells, such as Natural Killers (NK), macrophages and T lymphocytes. These immune cells play a dual role in the decidual environment, establishing immune tolerance and a mild inflammatory milieu, both being important for implantation and pregnancy outcome [8]. During the early stages of pregnancy, a state of mild systemic inflammation at the foetal-maternal interface is revealed by the presence of an activated vascular endothelium, leukocytosis and increased activation of immune cells such as monocytes. Increased plasma levels of inflammatory cytokines and chemokines, such as IL-8, IL-18, IL-12 and TNF-α are also observed [9]. Moreover, several immune cells, mainly uterine NK cells and macrophages, and to a lesser extent, dendritic cells and T cells, are diffusely embedded in the decidual microenvironment and contribute to the control of the immune response [10].

Decidual endothelial cells (DECs) are influenced by this special immunological and inflammatory environment established in the decidua and play a key role in controlling the traffic of leukocytes across the vessel wall, which needs to be tightly regulated in order to guarantee the success of the pregnancy. In addition, DECs are involved in angiogenesis, a necessary process for decidualization, which in turn, is an essential step in the maturation of new blood vessels in mammalian pregnancy.

The aim of this study was to characterize the phenotype of DECs in terms of expression of molecules contributing to angiogenesis and leukocyte recruitment, and to compare it with the microvascular ECs phenotype from human skin, which is normally geared to mount aninflammatory response. Adult Dermal Microvascular ECs (ADMECs) were chosen as a cellular model representingmicrovascular ECs that are physiologically involved in inflammatory responses. Although similar to the commonly used HUVEC, ADMECs are more responsive to inflammatory stimuli [11] and express different levels of endothelial markers [12].

2. Results

2.1. Decidual Endothelial Cells Express a Different Profile of Angiogenic Factors and Adhesion Molecules Compared to Endothelial Cell Isolated from Dermal Skin

Since DECs are the only ECs, which under physiological conditions, are able to synthesize the complement component C1q [13], an extensive characterization of DEC phenotype was carried out. Initially, a gene expression profiling using isolated primary human ADMECs and DECs was performed. The isolated cells were found to be 100% positive for the pan-endothelial cell markers VE-cadherin (CD144), von Willebrand Factor (vWF), and mesenchymal marker, vimentin, as assessed by immunostaining (Figure 1). A comparison of the gene expression profiles between DECs and ADMECs revealed 1909 transcripts that were differentially expressed (*t*-test *p*-value <0.01 and fold change $\geq$1), 1652 of which were up-regulated and 255 down-regulated in the DEC population (Supplementary Figure S1).

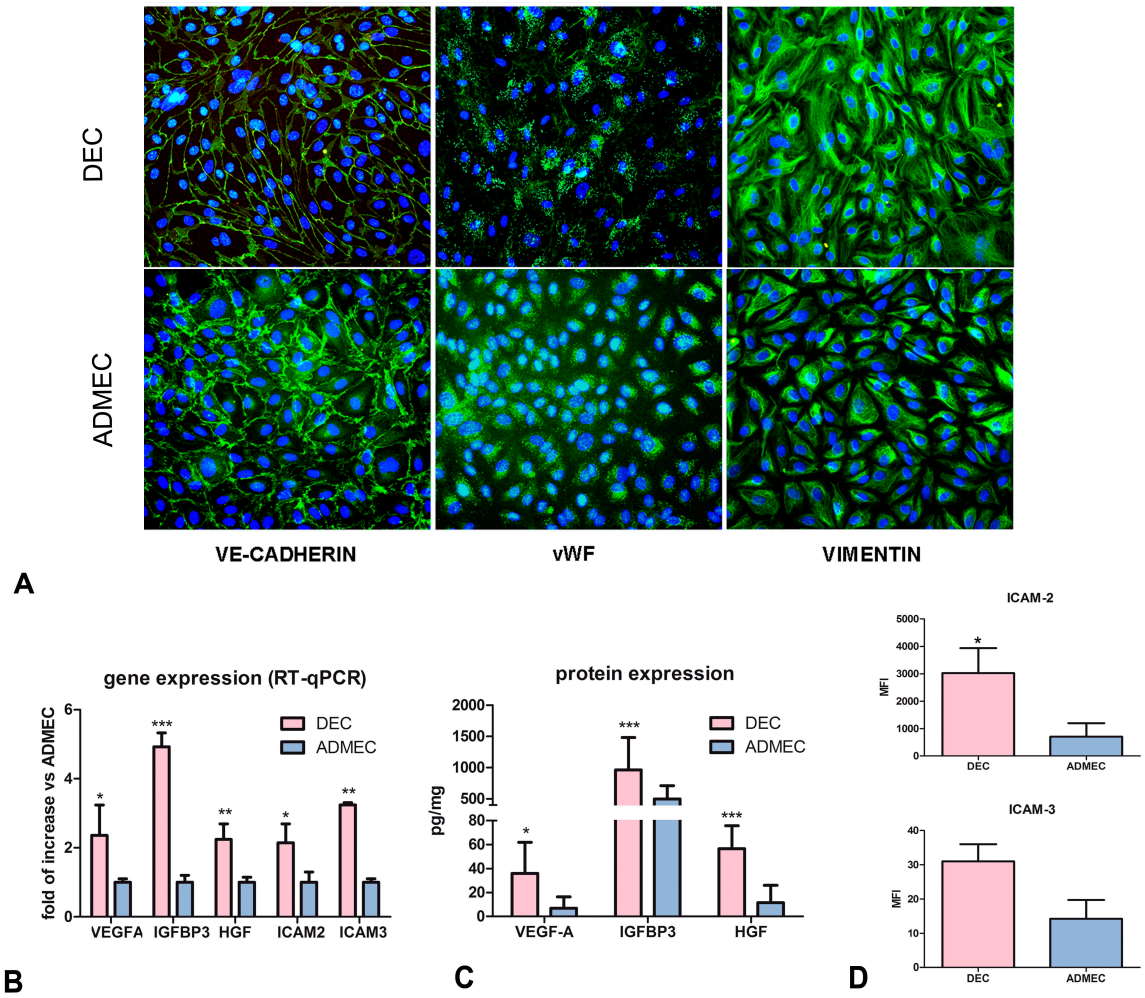

Figure 1. Phenotypic characterization of DECs and ADMECs. (**A**) Immunofluorescence analysis of vWF, VE-cadherin and vimentin on isolated and cultured DECs and ADMECs. Original magnification: 200×. (**B**) RT-qPCR of VEGF-A, HGF, IGFBP3, ICAM-2 and ICAM-3 genes differentially expressed by DECs and ADMECs. The data represent the mean ± SD of triplicate samples from five separate experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. (**C**) Evaluation of the production of VEGF-A, HGF and IGFBP3 proteins in the supernatants of a confluent monolayer of DECs and ADMECs after 4 h of culture using a commercial ELISA kit. The data represent the mean ± SD of triplicate samples from five separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. (**D**) Cytofluorimetric analysis for the expression of ICAM-2 and ICAM-3 in basal condition of freshly isolated DECs and ADMECs. The ECs were incubated with PE-conjugated mouse anti-human ICAM-2 and ICAM-3 mAb. PE-conjugated isotype-matched IgG2a or IgG1 were used as negative control, respectively. Data are represented as mean ± SD of the Mean Fluorescence Intensity (MFI) of five separate experiments.

Analysis of the gene expression profile (GEP) data revealed, besides other genes, interesting differences in the expression of genes involved in angiogenesis and leukocyte recruitment. In particular, we observed a 2.7 fold up-regulation of VEGF-A; a 2.1 fold increase in the expression of HGF, and even more pronounced up-regulation (11.6 fold) of IGFBP3 (Supplementary Figure S1). The expressions of VEGF-A, HGF and IGFBP3 transcripts were validated by RT-qPCR using microarray samples as well as five additional EC populations each obtained from other subjects (Figure 1B). The results presented in Figure 1B confirmed the difference in gene expression levels of these growth factors. The production of VEGF-A, HGF and IGFBP3 was also analysed at protein level by ELISA in the supernatants of five different populations of DECs and ADMECs (Figure 1C). The results shown in Figure 1C indicated that DECs produced higher amount of VEGF-A, IGFBP3 and HGF as compared to

ADMECs. The microarray and RT-qPCR analysis also revealed a higher expression of the constitutive adhesion molecules, ICAM-2 and ICAM-3, by DECs. These data were confirmed at the protein level by cytofluorimetric analyses (Figure 1D).

2.2. DECs Respond Feebly to Vasoactive Stimuli Leading to Vascular Leakage Compared to ADMECs

To investigate the unique characteristics of DECs in terms of inflammatory response, an endothelial permeability assay was carried out. ECs were grown to confluence onto a transwell (TW) insert. To measure the vascular permeability, ECs were treated with Platelet-Activating Factor (PAF), Histamine (HIS) or Bradykinin (BK). To determine the increase in ECs permeability, FITC-conjugated BSA was added to the upper chamber. The passage of FITC-BSA to the lower chamber was evaluated after 5, 15 and 30 min and the results were expressed as % of BSA leakage. As shown in Figure 2, ADMECs promptly responded to all three vasoactive stimuli. On the contrary, DECs were not responsive to vasoactive stimuli even after 30 min of incubation.

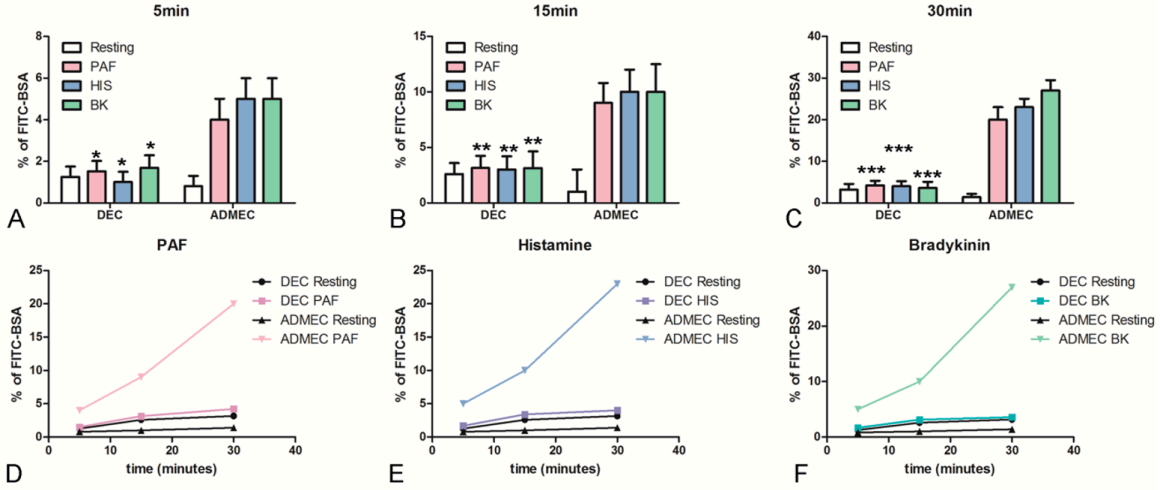

Figure 2. Permeabilizing activity of endothelial cells to classical vasoactive stimuli. The permeabilizing activity was evaluated kinetically, after 5 (**A**), 15 (**B**) and 30 min (**C**) adding PAF (**D**), HIS (**E**) or BK (**F**), to the upper chamber of the TW, measuring the amount of FITC-labeled BSA that leaked through a monolayer of endothelial cells into the lower chamber. The data represent the mean ± SD of duplicate samples from four separate experiments * $p < 0.05$; ** $p<0.01$; *** $p < 0.005$.

2.3. DECs Produce Lower Levels of Oxygen-Derived Reactive Molecules than ADMECs in Response to TNF-α and Histamine Challenge

We evaluated the capability of DECs and ADMECs to produce H_2O_2, an oxygen-derived reactive molecule endowed with bactericidal properties. Both ECs, cultured in 96 well plates, were incubated with Ampliflu Red reagent, a molecule that produces a fluorescent signal following its reaction with H_2O_2. As expected, ADMECs were able to produce high levels of H_2O_2 in response to both TNF-α and histamine stimulation. On the contrary, DECs maintained a lower level of H_2O_2 production following stimulation with TNF-α and histamine (Figure 3).

2.4. DECs, Compared to ADMECs, Are Weak Responders to TNF-α Stimulation with Respect to Chemokine Secretion, Adhesion Molecule Expression and Leukocyte Recruitment

Another important aspect of ECs function is their key role in controlling leukocyte trafficking across the vessel wall. It is well known that ECs, including ADMECs, promptly respond to TNF-α by enhancing adhesion molecule expression and cytokine secretion [14,15]. This results in leukocyte adhesion and transmigration into tissue spaces. We, therefore, analysed the expression of selected chemokines (IL-8, MCP-1, MIP-1α and RANTES) in resting as well as TNF-α stimulated ECs. ECs were incubated overnight with TNF-α and supernatants were collected. ECs were then lysed and

the total mRNA was extracted. The gene expression analysis (data not shown) and the chemokine quantification in the culture supernatant revealed a statistically significant weaker responsiveness of DECs to TNF-α stimulation (Figure 4A–D); only IL-8 production was comparable between DECs and ADMECs.

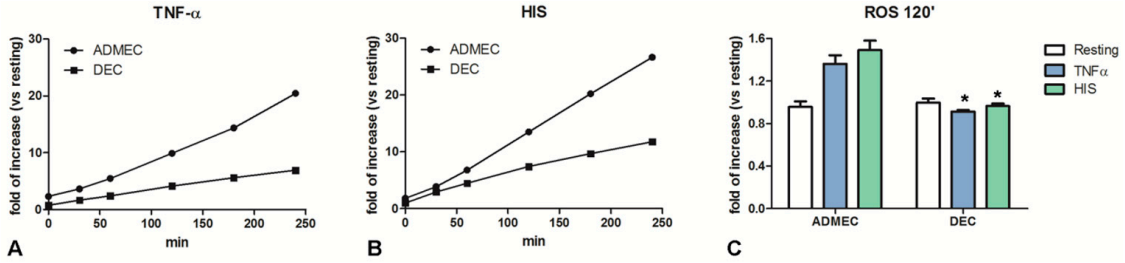

Figure 3. Production of intracellular ROS by endothelial cells. ROS production by endothelial cells exposed to TNF-α (**A**) or HIS (**B**). Following pre-treatment with TNF-α (100 ng/mL) or histamine (HIS; 0.1 μM), ECs were stained with Ampliflu Red for the evaluation of H_2O_2 production after 30, 60 120, 180 and 240 min. (**C**) Histograms represent the production of intracellular ROS by endothelial cells stimulated with TNF-α or HIS after 120 min. The data represent the mean ± SD of triplicate samples from five separate experiments; * $p < 0.05$.

The expression of ICAM-1, VCAM-1 and E-Selectin, which are inducible adhesion molecules involved in leukocyte recruitment, can be modulated by TNF-α. Monolayers of DECs and ADMECs were stimulated with TNF-α for 4 h (or 18 h for VCAM-1) and the expression of these adhesion molecules was evaluated by ELISA on the whole cells. The results are shown in Figure 4E and are expressed as fold of increase compared to resting condition. ADMECs, as expected, showed a significant up-regulation of ICAM-1, VCAM-1 and E-Selectin expression on their cell surface after stimulation with TNF-α, whereas DECs showed weaker surface expression of adhesion molecules.

In order to ascertain if feeble surface expression of adhesion molecules by DECs, following TNF-α challenge, coincided with a functional deficit in the leucocyte recruitment, we performed a trans-endothelial migration assay using Lympho-Monocytes (LM) isolated from peripheral blood. ECs were grown to confluence in the TW system and stimulated with TNF-α. Subsequently, peripheral blood LM were added to the upper chamber of the TW, allowed to migrate for 30 min, and then counted. As showed in Figure 4F, TNF-α stimulated DECs brought about a considerably reduced LM recruitment compared to ADMECs.

2.5. IFN-γ-stimulated DECs Are Potent Recruiters of Natural Killer and Regulatory T Cells

Chemokines such as CXCL9/MIG and CXCL10/IP-10 are important in controlling the recruitment and/or retention of NK cells in human decidua [16]. Since CXCL9/MIG and CXCL10/IP-10 are inducible by IFN-γ, we asked whether the production of these two chemokines by DECs could be modulated in response to IFN-γ. As shown in Figure 5, IFN-γ-stimulated DECs, but not ADMECs, secreted high amounts of both chemokines.

We performed a migration assay with lympho-monocytes (LM) using the supernatant of cells stimulated with IFN-γ (as chemoattractant) in order to establish the functional consequences of these chemokines produced by DECs (Supplementary Figure S2). In addition, we characterized the DEC-recruited cells that migrated to the lower compartment of the Boyden Chamber during the trans-endothelial migration assay. ECs were grown to confluence in the TW system and stimulated overnight with IFN-γ. Peripheral blood LM were then added in the upper chamber and allowed to migrate for 30 min. Migrated cells were stained for $CD45^+CD56^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^+FoxP3^+$ using appropriate monoclonal Abs. Interestingly, besides $CD45^+CD56^+$ NK cells (Supplementary Figure S3), DECs also exhibited a considerably enhanced ability to recruit Treg cells ($CD3^+FoxP3^+$), as compared to ADMEC (Figure 5C,D).

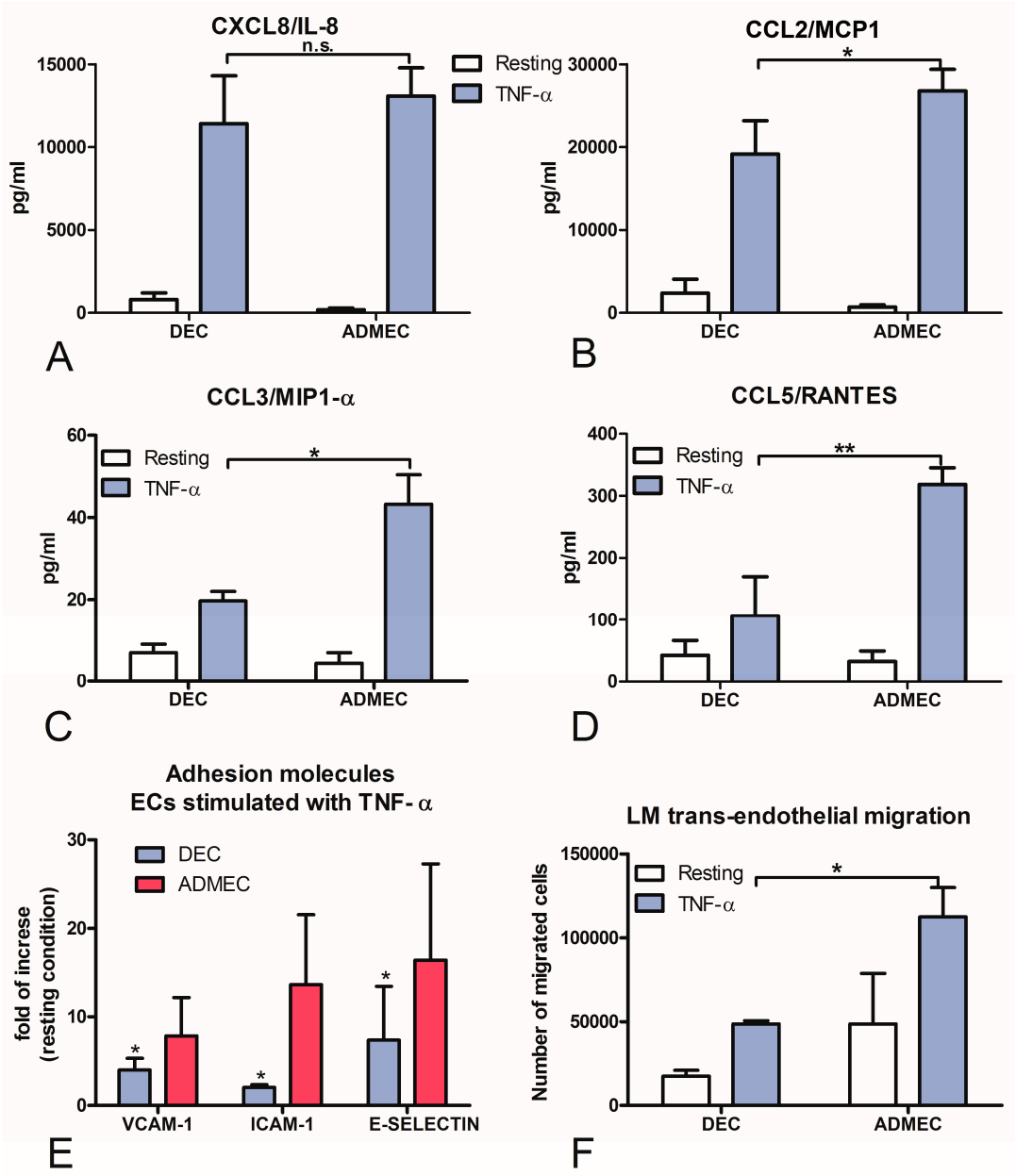

Figure 4. Secretion of chemokines and surface-expression of adhesion molecules by ECs stimulated with TNF-α. CXCL8/IL-8 (**A**), CCL2/MCP-1 (**B**), CCL3/MIP-1α (**C**) and CCL5/RANTES (**D**) production in DEC and ADMEC supernatants after 4 h incubation with TNF-α was measured using a beads-based multiplex immunoassay (Luminex®). (**E**) ELISA on the whole cells for the expression of ICAM-1, VCAM-1 or E-Selectin on DEC and ADMEC plasma membrane after 4 h or 18 h incubation with TNF-α. The data represent the mean ± SD of triplicate samples from five separate experiments * $p < 0.01$; ** $p < 0.005$. (**F**) Trans-endothelial migration of Lympho-Monocytes (LM) across untreated and TNF-α-treated DEC and ADMEC: migration is shown as the number of migrated cells. The data represent the mean ± SD of triplicate samples from three separate experiments * $p < 0.05$; n.s. not significant.

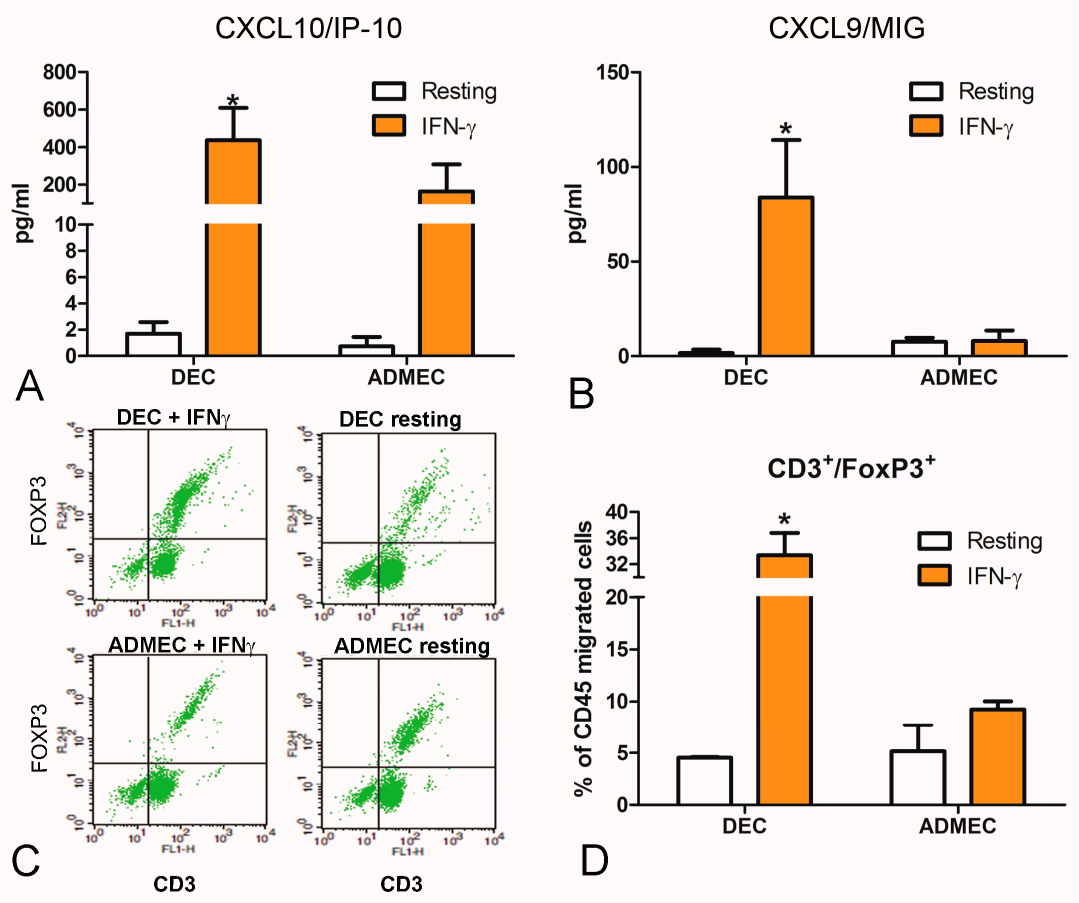

Figure 5. Secretion of chemokines and trans-endothelial migration of LM through EC stimulated with IFN-γ. The production of CXCL10/IP-10 (**A**) and CXCL9/MIG (**B**) in DEC and ADMEC supernatants after 4 h incubation with IFN-γ was measured using a beads-based multiplex immunoassay (Luminex®). The data represent the mean ± SD of triplicate samples from five separate experiments * $p < 0.01$. (**C**,**D**) Trans-endothelial migration of LM across untreated and IFN-γ-treated DEC and ADMEC. (**C**) Representative dot plots for flow cytometry of trans-endothelial migrated LM, stained for CD3 and FoxP3. (**D**) Quantitation of the percent of total migrated LM cells, positive for both CD3 and FoxP3 ($CD3^+FoxP3^+$) by flow cytometry. Migration was presented as the percentage of CD45 migrated cells. The data represent the mean ± SD of duplicate samples from three separate experiments * $p < 0.05$.

3. Discussion

In this paper, we report, for the first time, a detailed characterization of the phenotype of DECs that can differentially impact upon angiogenesis and leukocyte recruitment. As a point of reference, we used microvascular ECs derived from human skin which are normally robust participants during pro-inflammatory response.

Microvascular endothelial cells are active participants in and regulators of the inflammatory processes. They contribute to the process by (i) secreting inflammatory mediators and bactericidal molecules; (ii) increasing leakiness and pro-coagulant activity; (iii) modulating adhesion and migration of leukocytes through the expression of adhesion molecules and chemokines; and (iv) promoting pro-inflammatory cytokine expression [6].

In this study, we compared a range of immunological features of DECs and ADMECs, two types of microvascular ECs derived from very distinct anatomical sites. ADMECs are derived from a vascular location actively involved in the inflammatory process [5], whereas DECs are derived from decidua, where uncontrolled or heightened inflammatory milieu is unwarranted and can potentially lead to miscarriage or pregnancy related complications [17].

There are few studies concerning the characterization of DECs. Burrows and colleagues in 1994 described the basal level expression of adhesion molecules by immunohistochemistry, demonstrating that ICAM-1 is expressed by all vascular endothelium throughout the decidua [18]. However, it principally focused on differential marker expression ex vivo [18,19]. Alternatively, cells isolated from non-pregnant uterus were also employed to describe DEC properties [20–22].

We have previously shown that DECs are the only ECs able to express the complement protein C1q under basal physiologic conditions [13]. C1q, besides its well-known function as the recognition subcomponent of the complement classical pathway, is involved in trophoblast endovascular invasion [23]. The current study highlights a range of additional features of DECs. Most crucially, DECs produce a high amount of several important regulators of angiogenesis such as VEGF-A, HGF and IGFBP3. DECs also express high levels of the adhesion molecule ICAM-2 on their cell surface under basal conditions, and are the exclusive endothelial cell population that express ICAM-3, characteristics that have only been associated with the High Endothelial Venules (HEVs) so far [24].

DECs are unable to enhance inflammasome components in response to LPS stimulation [25]. More recently, comparing their immunological phenotype to that of other ECs such as HUVEC and ADMEC, DECs were found to be hypo-responsive to LPS stimulation in terms of IL-6, CXCL8/IL-8 and CCL2/MCP-1 production since they expressed low levels of TLR4 and manifested strong constitutive activation of the non-canonical NF-κB pathway and low responsiveness of the canonical pathway to LPS [26].

ECs play a pivotal role in regulating the selective passage of molecules that need to be recruited at the extravascular sites and participate in the control of fluid movement between luminal and ab-luminal sides of the vessels. Vascular permeability increase takes place mainly in post-capillary venules where the ECs are able to respond to different stimuli such as histamine, thrombin and bradykinin [27,28]. Indeed, ADMEC showed a prompt permeabilizing response to all the stimuli used (PAF, HIS, BK). DECs, however, were unable to show an increased vascular permeability. This unusual behaviour probably reflects some differences in receptor expression or intracellular signals triggered by receptor-agonist interaction. For BK, there are different types of receptors expressed on the cell surface and it has been demonstrated that only B2, and not B1, receptors are expressed at decidual level [29]. Moreover, as compared to other situations that involve specific modulation of vascular leakage, it has been shown that B1 receptor could also be important in the dermal vascular permeability [30], indicating a differential functional response between DEC and ADMEC. Besides vascular permeability, DECs are less efficient in the production of bactericidal molecules both in basal condition and in response to TNF-α and histamine stimulation.

Interestingly, while analysing DECs responses, an absent or mild modulation of the adhesion molecules, ICAM-1, VCAM-1 and E-selectin respectively, was observed when compared to ADMEC. Low or undetectable amounts of the chemokines, CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES, after stimulation with the pro-inflammatory cytokine TNF-α, were found in DECs. This is in contrast to the typical effect of TNF-α that normally induces endothelial activation, which is accompanied by an increase in adhesion molecule expression and cytokine production [31]. These data indicate that DECs are hyporesponsive to pro-inflammatory stimuli in comparison with other endothelial cells and could actively participate in the control of leukocyte infiltration/trafficking in decidua.

DECs appear to be unique in their ability to respond to IFN-γ, another pro-inflammatory cytokine, mainly produced in decidua by decidual NK cells [32]. DECs, but not ADMECs, produced a large amount of CXCL9/MIG and CXCL10/IP-10. DECs were shown to be able to express the mRNA for CXCL10/IP-10 also after progesterone treatment [33] and in response to trophoblast conditioned medium exposure [34]. The production of CXCL10/IP-10 was shown to be important for the recruitment of uterine NK cells in maternal decidua inducing the migration of peripheral blood NK cells derived from first trimester pregnant women, across DECs and decidual stromal cells [33]. In this paper, we also demonstrated that DECs stimulated with IFN-γ recruited a higher percentage of

$CD3^+FoxP3^+$ cells in comparison to ADMECs, although other markers (CD25, CD127) will be useful for a further characterization of Treg. Whether dysfunctional decidual endothelium can tip the balance away from the preponderance of Treg needs to be ascertained since maternal Tregs have been linked with a number of pregnancy-related complications. It is clear from this study that DECs are likely to be one of the lynchpins for proper recruitment and functioning of maternal NK cells, decidual macrophages and maternal Treg, each cell type being known to play a hierarchical role within the three trimesters of pregnancy.

4. Materials and Methods

4.1. Cell Isolation and Culture

Decidual first trimester biopsy specimens were obtained from healthy women (n = 8) undergoing voluntary termination of pregnancy at 8–12 weeks of gestation. Skin samples were obtained from women (n = 6) of fertile age undergoing reductive plastic surgery. The study was approved by the institutional review board of the Institute for Maternal and Child Health (IRCCS "Burlo Garofolo", Trieste, Italy. Approval 13 December 2009). I Informed consent was obtained from all women providing the tissue specimens. DECs and ADMECs were isolated and characterized as previously described [13]. Each population corresponded to a single woman. Both ECs were seeded in a 12.5 cm^2 flask precoated with 2 $\mu g/cm^2$ fibronectin (Roche, Milan, Italy) and maintained in endothelial serum free basal medium (Life Technologies, Monza, Italy), supplemented with 20 ng/mL basic Fibroblast Growth Factor, 10 ng/mL Epidermal Growth Factor (Life Technologies), 10% v/v foetal calf serum (FCS) (Life Technologies), and 10% v/v human serum.

DECs were positively selected using Dynabeads M-450 (Life Technologies) coated with Ulex europaeus 1 lectin (Sigma–Aldrich, Milan, Italy), whereas ADMECs were further purified from a subconfluent mixed cell population using CD31-conjugated magnetic beads from Dynabeads (Life Technologies). When the cells were seeded for the experiments, the medium used was the same as described above but without human serum.

4.2. Immunofluorescence

ECs were plated on 8-chamber culture slides (BD Biosciences Discovery Labware, Milan, Italy) coated with 2 $\mu g/cm^2$ fibronectin (Roche, Milan, Italy). When cells grew to confluence, they were fixed and permeabilized with FIX & PERM (Società Italiana Chimici, Rome, Italy). Then cells were incubated with primary monoclonal antibody (mAb) (clone 9) mouse anti-human vimentin (Sigma-Aldrich), (cloneF8/86) mouse anti-human vWF (Dako-Cytomation, Milan, Italy), or mouse anti-human VE-Cadherin (kindly provided by Prof. Dejana from Institute of Molecular Oncology, Milan, Italy) (5 $\mu g/mL$) for 1 h at room temperature (RT) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG for 1 h at RT. Images were acquired with Leica DM3000 microscope (Leica, Wetzlar, Germany) and collated using a Leica DFC320 digital camera (Leica).

4.3. RNA Isolation, cDNA Synthesis and Quantitative Real-Time Polymerase-Chain Reaction (RT-qPCR)

RNA was extracted from cells with euroGOLDtrifast (Euroclone, Milan, Italy) according to the supplier's instructions and reverse transcripted as previously described [13]. qPCR was carried out on a Rotor-Gene 6000 (Corbett, Explera, Ancona, Italy) using iQ SYBR Green Supermix (Bio-Rad, Milan, Italy). Supplementary Table S1 shows the primers used for RT-qPCR. The melting curve was recorded between 55 °C and 99 °C with a hold every 2s. The relative amount of gene expression in each sample was determined by the Comparative Quantification (CQ) method supplied as part of the Rotor Gene 1.7 software (Corbett Research) [35]. The relative amount of each gene was normalized with 18S and expressed as arbitrary units (AU) considering 1 AU obtained from decidual tissue used as a calibrator.

4.4. Microarray

Total RNAs from ECs were validated for integrity and purity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Single-color hybridization microarray experiments for gene expression profile (GEP) were performed with 100 ng total RNA/sample labeled with Cyanine (Cy)-3 dye using the Low Input Quick Amp Labeling Kit (Agilent Technologies). Cy3-labeled RNA was hybridized to the Whole Human Genome (4 × 44 K) oligo microarray platform (Agilent Technologies). Microarray slides were analysed with an Agilent Microarray Scanner (Agilent Technologies). The hybridization signal values for the multiple probes for each gene were obtained with the use of Agilent Feature Extraction Software 10.7.3 (Agilent Technologies). Bioinformatics analyses were performed using GeneSpring 11.5.1 (Agilent Technologies). GEP results were visualized by hierarchical clustering, applying Ward's method with Euclidean distance [36]. GEO number: GSE41946.

4.5. Growth Factors and Chemokines Detection

ECs were grown to confluence in 24 wells plate (BD Falcon) in serum-free medium and stimulated for 24 h with IFN-γ (100 U/mL) or TNF-α (100 ng/mL) (Peprotech, Milan, Italy). The levels of VEGF-A, HGF, IGFBP3 were measured by commercial ELISAs following the manufacturer's instruction (VEGF Human ELISA Kit, Invitrogen Milan Italy; Boster Immunoleader Tema Ricerca, Bologna Italy, for detection of HGF; DRG Products Tema Ricerca, Bologna Italy for detection of IGFBP3). The quantitative determination of IL-8, MCP-1, MIP-1α, RANTES, CXCL9/MIG and CXCL10/IP-10 was performed by a bead-based multiplex immunoassay (Biorad) and a Bioplex 200 system (Biorad Laboratories, Hercules, CA, USA), as previously described [37]. The cells were then lysed for the quantification of the total protein content by Bradford assay [24].

4.6. Migration Assays

LM were isolated as previously described [24]. ECs (2×10^4) were seeded onto 20 μg/mL fibronectin-coated polycarbonate insert of a 24-well TW system (6.5 mm diameter, 8-μm pores; Corning Costar, Milan, Italy) and used 5 days after culture as previously described [33]. LM suspension (2×10^5 cells/100 μL endothelial serum free basal medium with 0.1% *w*/*v* bovine serum albumin) was added to the upper compartment of the TW in the presence or absence of ECs and allowed to migrate through DECs for 1 h at 37 °C. Amounts of 10% FCS, IFN-γ, or conditioned medium (CM) of DECs treated with IFN-γ were added to the lower chamber as a chemoattractant for LM. The number of cells transmigrated was evaluated by Coulter Counter (Coulter Electronics, Luton, UK).

Migrated LM were fixed with FIX & PERM® cell fixation and permeabilization kit (Società Italiana Chimici), according to the manufacturer's instructions. A total number of 5×10^5 cells were incubated on shaking at 800 rpm overnight at 4 °C with PE- or FITC-conjugated mouse monoclonal antibody (mAb) specific to human CD3 (clone MEM-57), CD8 (clone MEM-31), CD45 (clone MEM-28), CD56 (clone MEM-188) (ImmunoTools GmbH) and IgG1 and IgG2a (ImmunoTools GmbH) isotype controls. Cells were fixed with 1% *v*/*v* PFA (Sigma-Aldrich) and analysed for fluorescence with a FACSCalibur flow cytometer (BD Falcon) using CellQuest software (version 5.1, Becton Dickinson European HQ, Erembodegem-Aalst, Belgium).

4.7. Detection of Adhesion Molecules on ECs

ICAM-2 and ICAM-3 were examined via cytofluorimetric analysis [13]. ECs were incubated for 1 h at 37 °C with the phycoerythrin (PE)-conjugated mAb ICAM-2 (clone CBR-IC2/2) or with PE-conjugated mAb ICAM-3 (clone CBR-IC3/1) (Biolegend, Milan, Italy) and their relatives PE-conjugated mouse IgG2a or IgG1 (ImmunoTools GmbH, Friesoythe, Germany) isotype controls. Quantization of ICAM-1, VCAM-1 and E-Selectin was evaluated by an ELISA on the whole cells, in ECs stimulated for 4 h (for ICAM-1 and E-Selectin) or 18 h (for VCAM-1) with TNF-α (100 ng/mL) and then incubated with monoclonal primary antibodies (mAb 6.5B5 anti-ICAM-1, mAb clone 1.4C3

anti-VCAM-1 Sigma-Aldrich or mAb clone 1.2B6 anti-E-selectin; 5 µg/mL) for 1 h at RT, as previously described [38]. The cells were then lysed for the quantification of the total protein concentration via Bradford assay [24].

4.8. Endothelial Leakage

Human endothelial cells (ECs; 2×10^4) were seeded onto polycarbonate inserts of a 24-well TW system (6.5-mm diameter, 3-µm pores; Corning Costar, Milan, Italy) coated with 2 µg/cm^2 of human fibronectin and used after reaching the confluence. Each TW was checked for the formation of intact monolayer on the insert by adding FITC-BSA (1 mg/mL) to the upper chamber and measuring the amount of labelled BSA that passed down to the lower chamber by an Infinite200 (TECAN). The TWs were used only when the intensity of fluorescence in the lower chamber was negligible; in this case, the stimuli were added to the upper chamber together with FITC-BSA and the fluorescence evaluated in the lower chamber at various time points.

4.9. Measurement of Total H_2O_2 Production

Hydrogen peroxide (H_2O_2) production was measured using Ampliflu Red (Sigma-Aldrich) reagent. ECs were seeded on 96-well plate to reach 90% confluence. To assess total H_2O_2, the cell culture medium was substituted with PBS + 2% *w*/*v* BSA + 0.7 mM $MgCl_2$ and 0.7 mM $CaCl_2$ containing 40 µM Ampliflu Red reagent, 1 µg/mL HRP, 5 µg/mL SOD and 100 µM NaN_3 in a final volume of 100 µL. After 5 min of preincubation with TNF-α (100 ng/mL) or Histamine 10^{-5} M, the readings were taken at 576 nm with Infinite200 (TECAN). The cells were then lysed for the quantification of the total protein concentration by Bradford assay [24].

4.10. Statistic Analysis

Data were analysed using Two-way ANOVA, Tukey–Kramer test, and unpaired two-tailed Student's *t*-test or one-way ANOVA with Bonferroni corrections. Results were represented as mean ± SEM. Non-parametric data were assessed by Mann–Whitney U tests and the results were expressed as median and interquartile range. p values < 0.05 were considered significant. All statistical analyses were performed using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

5. Conclusions

DECs differ from ADMECs with respect to the production of several growth factors that play important roles in the control of angiogenesis. DECs also differ from ADMECs in the expression of adhesion molecules and the release of chemokines involved in local leukocyte recruitment, thus contributing to the establishment of the special decidual microenvironment. Our results indicate that DECs display a differential "arsenal" of adhesion molecules and cytokines in response to classical pro-inflammatory stimuli compared to ADMECs and HUVEC: they express constitutively the adhesion molecules ICAM-2 and ICAM-3, but fail to show increased expression of ICAM-1. These cells produce higher levels of the chemokines CXCL9/MIG and CXCL10/IP-10 in response to IFN-γ compared to other ECs, and promote selective migration of FoxP3 positive T cells. These findings are consistent with the local changes that occur during pregnancy which are designed to control the inflammatory response at the foetal-maternal interface. Thus, similar to T-cells, macrophages, dendritic cells and neutrophils, there may be a paradigm shift in the endothelial cells towards an anti-inflammatory phenotype EC2.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/7/1604/s1.

Author Contributions: Conceptualization, R.B., V.G. and M.-P.P.; Methodology, C.A., E.M., F.B. and L.L.; Software, M.D. and A.M.; Validation, G.Z., C.A., E.M., F.B. and L.L.; Formal Analysis, M.D., V.G. and A.M.; Investigation, C.A.; Resources, G.Z. and G.R.; Data Curation, L.L. and R.M.; Writing—Original Draft Preparation, R.B., C.A.,

F.B. and E.M.; Writing—Review and Editing, R.B., C.A., A.M., U.K. and R.M.; Visualization, C.A., F.B. and A.M.; Supervision, R.B., V.G. and M-P.P.; Project Administration, R.B.; Funding Acquisition, G.R.

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Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this article.

Abbreviations

ADMECs	Adult Dermal Microvascular Endothelial cells
AU	Arbitrary Units
BK	Bradykinin
BSA	Bovine Serum Albumin
C1q	Complement component 1q
CCL	Chemokine (C–C motif) Ligand
CD	Cluster of Differentiation
cDNA	Complementary DNA
CM	Conditioned Medium
CQ	Comparative Quantification
CXCL	Chemokine (C–X–C motif) Ligand
DECs	Decidual endothelial cells
ECs	Endothelial cells
ELISA	Enzyme-Linked ImmunoSorbent Assay
E-Selectin	Endothelial–Selectin
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead box P3
GEO	Gene Expression Omnibus
GEP	Gene Expression
HEVs	High Endothelial Venules
HGF	Hepatocyte Growth Factor
HIS	Histamine
HRP	Horseradish Peroxidase
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
IFN-γ	Interferon gamma
IGF-1	Insulin-like Growth Factor-1
IGFBP3	Insulin-like Growth Factor-Binding Protein 3
IgG	Immunoglobulin G
IL	Interleukin
IP-10	Interferon gamma-induced Protein 10
LM	Lympho-Monocytes
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MCP-1	Monocyte Chemoattractant Protein-1
MIG	Monokine Induced by Gamma
MIP-1α	Macrophage Inflammatory Proteins-1α
mRNA	Messenger RNA
NK	Natural Killer
PAF	Platelet-Activating Factor
PE	Phycoerythrin

RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-qPCR	Quantitative real-time polymerase-chain reaction
SOD	Superoxide Dismutase
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor α
Treg	Regulatory T
TW	Transwell
VCAM-1	Vascular Cell Adhesion Protein-1
VE-cadherin	Vascular endothelial cadherin
VEGF-A	Vascular Endothelial Growth Factor A
vWF	von Willebrand Factor

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Decidual Interleukin-22-Producing CD4+ T Cells (Th17/Th0/IL-22+ and Th17/Th2/IL-22+, Th2/IL-22+, Th0/IL-22+), Which Also Produce IL-4, Are Involved in the Success of Pregnancy

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Abstract: Trophoblast expressing paternal HLA-C resembles a semiallograft, and could be rejected by maternal T cells. IL-22 seems to be involved in allograft rejection and thus could be responsible for miscarriages. We examined the role of decidual IL-22-producing CD4+ T on human pregnancy. In those experiencing successful pregnancy and those experiencing unexplained recurrent abortion (URA), the levels of IL-22 produced by decidual CD4+ T cells are higher than those of peripheral blood T cells. We found a correlation of IL-22 and IL-4 produced by decidual CD4+ T cells in those experiencing successful pregnancy, not in those experiencing URA. The correlation of IL-22 and IL-4 was also found in the serum of successful pregnancy. A prevalence of CD4+ T cells producing IL-22 and IL-4 (Th17/Th2/IL-22+, Th17/Th0/IL-22+, Th17/Th2/IL-22+, and Th0/IL-22+ cells) was observed in decidua of those experiencing successful pregnancy, whereas Th17/Th1/IL-22+ cells, which do not produce IL-4, are prevalent in those experiencing URA. Th17/Th2/IL-22+ and Th17/Th0/IL-22+ cells are exclusively present at the embryo implantation site where IL-4, GATA-3, IL-17A, ROR-C, IL-22, and AHR mRNA are expressed. T-bet and IFN-γ mRNA are found away from the implantation site. There is no pathogenic role of IL-22 when IL-4 is also produced by decidual CD4+ cells. Th17/Th2/IL-22+ and Th17/Th0/IL-22+ cells seem to be crucial for embryo implantation.

Keywords: Th22; Th17; pregnancy; IL-22; IL-4; IL-5; IL-17; spontaneous abortion; ectopic pregnancy; implantation

1. Introduction

The conceptus, because of the presence of paternal classical MHC class I antigens (HLA-C) [1], is thought to resemble a semiallograft [2]. The maternal effector CD4+ T helper lymphocytes activated by the paternal antigens expressed by trophoblast, after presentation by maternal APCs, can secrete various cytokines.

On the basis of the cytokines produced, the human effector CD4+ T helper cells have been classified as T helper (Th)1 and Th2, and, more recently, as Th17 cells [3,4] and Th22 cells [5]. Indeed,

CD4+ Th1 cells, which produce interleukin (IL)-2 and interferon (IFN)-γ, are highly protective against infections sustained by intracellular pathogens. CD4+ Th2 cells, which are mainly responsible for host defense against extracellular parasites, including nematodes, produce IL-5, IL-13, and IL-4 [3]. Th17 cells produce IL-17A, IL17F, IL-21, IL-26, and IL-22 [5–7] and express retinoic-acid-related orphan receptor (ROR)-C [8]. They are highly protective against infections by extracellular bacteria by recruiting neutrophils, which contribute to the chronic tissue inflammation. Precursors of Th17 cells can differentiate into Th17/Th1, producing both IL-17 and IFN-γ, and finally into Th1 cells, in response to IL-12 present in the microenvironment [9], whereas an IL-4-rich microenvironment may induce the shifting of memory Th17 cells into Th17/Th2 cells, producing both IL-17 and IL-4 [10].

Th22 cells primarily secrete IL-22 and TNF-alpha and could express granzyme B and IL-13, factors associated with host defense and remodeling of tissue [11]. The expansion of IL-22-producing cells appears to be regulated by the aryl hydrocarbon receptor (AHR) transcription factor, although additional intracellular molecules involved in Th22 differentiation as STAT3, are also required by Th17 cells [12]. Expression of the CCR4 and CCR10 skin-homing receptors on Th22 cells suggests these cells are likely recruited to the skin. Indeed Th22 cells may be involved in the pathogenesis of inflammatory skin disorders such as psoriasis, atopic eczema, and allergic contact dermatitis [5,13,14]. In vitro Th22 cells may develop independently of the Th17 lineage while demonstrating plasticity toward Th1- and Th2-type cells [11]. Under Th1-promoting conditions in vitro, as well as in an IFN-γ–rich inflammatory environment in vivo, Th22 cells display marked plasticity toward the production of IFN-γ, further supporting an important role for T-bet in Th22 cell function. Th22 cells also exhibit plasticity under Th2 culture conditions in vitro, with increased IL-13 expression. Consistent with these results, skin-homing IL-13- and IL-22-producing Th2/IL-22 and Tc2/IL-22 cells were recently found to be elevated in people with atopic dermatitis (AD) [15–17].

Importantly for pregnancy, the Th1 cells (in particular IFN-γ) seem to play a role in acute fetal allograft rejection and thus could be involved in recurrent spontaneous abortion (URA) [18–23], whereas Th2 cells [20] (producing IL-4 and IL-10) and CD4+CD25+Foxp3+ T reg cells (producing IL-10 and TGF beta), inhibiting IFN-γ production, act to enhance fetal allograft tolerance [24] and thus could be responsible for the success of pregnancy. Very recently, we observed a prevalence of Th17/Th2 cells (producing IL-17 and IL-4) in the decidua of those experiencing successful pregnancy, whereas the presence of Th17 (producing IL-17 only) and Th17/Th1 (producing IL-17 and IFN-γ) cells was exclusively found in the decidua of those experiencing unexplained recurrent abortion [25]. More interestingly, Th17/Th2 cells were exclusively present at the embryo implantation site, and IL-4, GATA-3, IL-17A, and ROR-C mRNA levels increased at embryo implantation sites, whereas Th17, Th17/Th1, and Th1 cells were exclusively present apart from implantation sites [25]. Moreover, we found that HLA-G5, a soluble Class I b molecule released by embryo and extravillous trophoblast, could be responsible for the development of Th17/Th2 cells [25].

IL-22 has been shown to be involved in allograft rejection, by increasing IFN-γ production by Th1 and Tc1 cells, known to be responsible for acute allograft rejection, and decreasing IL-10 production by T reg and Th2 cells, known to be responsible for allograft tolerance [26,27]. Because of this, IL-22 produced by CD4+ T cells from decidua could be a factor responsible for miscarriage. Only two discordant publications have described the possible role of IL-22 on URA: one indicates that IL-22 could be responsible for URA because serum IL-22 levels increase compared to cases of successful pregnancy, and the other reports that mRNA for IL-22 decreases in decidua of those experiencing URA compared to successful pregnancy [28,29].

In human early pregnancy decidua, the ILC3 were shown to release IL-22 [30] and could partially differentiate into uterine NK cells. Until now, the role of IL-22-producing CD4+ T cells on human pregnancy has never been documented and has to be clarified.

We generated CD4+ T clones from peripheral blood and decidua of those experiencing successful pregnancy (elective termination pregnancy in the first trimester), URA at the moment of a spontaneous abortion during the first trimester of pregnancy, and ectopic pregnancy (at both embryo implantation

site in the Fallopian tube and distant from that site). By measuring IL-22 but also Th1-type, Th2-type, and Th17-type cytokines, which could be associated with the production of IL-22, we analyzed the different CD4+ Th22-type profiles and the different Th22-like subpopulations at the fetomaternal interface and at the embryo implantation site. Thus, in this study we (i) defined the role of Th cells producing IL-22 in pregnancy, (ii) defined to which Th subpopulation (of Th1-, Th2-, Th0-, Th17-, Th17/Th1-, Th17/Th2, and Th17/Th0 cells) the decidual Th cells producing IL-22 belong, ascertaining the particular profiles preferentially associated with pregnancy failure or successful pregnancy, and (iii) found the particular Th22-type profiles which could preferentially favor embryo implantation.

2. Results

2.1. Associated Production of IL-4, IL-13, and IL-22 by Decidual CD4+ T Cell Clones in Those Experiencing Successful Pregnancy

One hundred twenty-two CD4+ T cell clones were respectively generated from decidual biopsies, and peripheral blood was obtained from nine pregnant women (with successful pregnancy) who voluntarily underwent an elective termination of pregnancy; 125 CD4+ T cell clones were respectively generated from decidual biopsies, and peripheral blood was obtained from four women suffering from unexplained recurrent abortion (Experiment 1 in Section 4.3). IL-4, IL-13, IL-5, IL-17A, IL-17F, IL-22, and IFN-γ were measured in the supernatant of the CD4+ T cell clones by a multiplex bead-based assay.

In those experiencing successful pregnancy, decidua CD4+ T cell clones produce higher levels of IL-4 (p = 0.01), IL-13 (p = 0.0001) (two Th2-type cytokines), IL-22 (p = 0.002) (a Th17/Th22-type cytokine), and IL-17A (p = 0.027) (one of the two Th17-type cytokines), but not higher levels of IL-17F and IL-5 compared to peripheral blood T cell clones (Figure 1). By contrast, IFN-γ production by T cell clones was not statistically different in the decidua compared to peripheral blood (Figure 1).

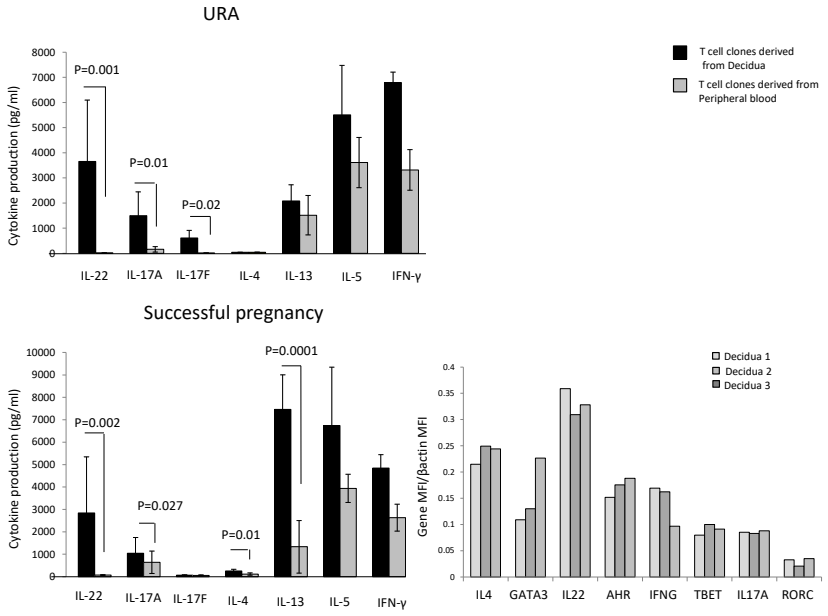

Figure 1. Cytokine production by CD4+ T cell clones derived from decidua of those experiencing successful pregnancy and URA and mRNA expression of cytokines and transcription factors in decidual biopsies of successful pregnancy. CD4+ T cell clones were generated from decidual biopsies, and peripheral blood was obtained from those experiencing successful pregnancy and those experiencing unexplained recurrent abortion (URA) (Experiment 1 in Section 4.3). IL-4, IL-13, IL-5, IL-17A, IL-17F, IL-22, and IFN-γ were measured in the supernatant of the CD4+ T cell clones by a multiplex bead-based assay. The statistical analysis was performed with the Wilcoxon test. The determination of mRNA level for IL-4, GATA-3, IL-17A, ROR-C, IL-22, AHR, T-bet, and IFN-γ in three biopsies of decidua from three pregnant women (with successful pregnancy) was performed by Quantigene 2.0.

In those experiencing URA, decidua CD4+ T cell clones do not produce IL-4, but produce higher levels of IL-22 ($p = 0.001$), IL-17A ($p = 0.01$), and IL-17F ($p = 0.02$) compared to peripheral blood T cell clones (Figure 1). By contrast, IFN-γ, IL-5, and IL-13 production by T cell clones was not statistically different in the decidua compared to peripheral blood (Figure 1).

These results show that there is an accumulation of CD4+ T cells producing IL-17A, IL-17F, and IL-22 in the decidua of those experiencing URA and an accumulation of T helper cells producing IL-17A, IL-22, IL-13, and IL-4 in the decidua of those experiencing successful pregnancy, suggesting an associated production of IL-4, IL-13, and IL-22 by decidual CD4+ T cells in those experiencing successful pregnancy, not found in those experiencing URA.

We also measured the mRNA expression of IL-4 and its associated transcription factor GATA3, IL-17A and its associated transcription factor RORC, and IL-22 and its associated transcription factor AHR directly on decidual biopsies of successful pregnancy. IL-17A, IL-22, IL-4, and their associated transcription factors RORC, AHR, and GATA3 mRNAs are expressed in the decidua of those experiencing successful pregnancy (Figure 1). We confirm the association of IL-22 and IL-4 at the mRNA level in the decidua of those experiencing successful pregnancy.

2.2. In Those Experiencing Successful Pregnancy, IL-22 Is Positively Correlated with the Th2-Type Cytokine IL-4, Whereas, in those Experiencing URA, IL-22 Produced by CD4+ T Cell Clones Derived from the Decidua Is Positively Correlated with Th17-Type Cytokines (IL-17A and IL-17F)

The levels of IL-22 and the levels of IL-4, IL-13, IL-5, IL-17A, IL-17F, and IFN-γ measured in the supernatants of the CD4+ T cell clones derived from deciduae of those experiencing URA and those experiencing successful pregnancy have been correlated.

IL-22 produced by decidual CD4+ T cells of those experiencing successful pregnancy is positively correlated with IL-4 produced by the same cells ($R = 0.680$, $p = 0.0002$) (Figure 2), whereas, in those experiencing URA, IL-22 is positively correlated with IL-17A and IL-17F, but not with IFN-γ, IL-13, or IL-4 (Figure 2). IL-22 is not correlated with IL-13, IL-5, IL-17A, IL-17F, or IFN-γ in those experiencing successful pregnancy (Figure 2).

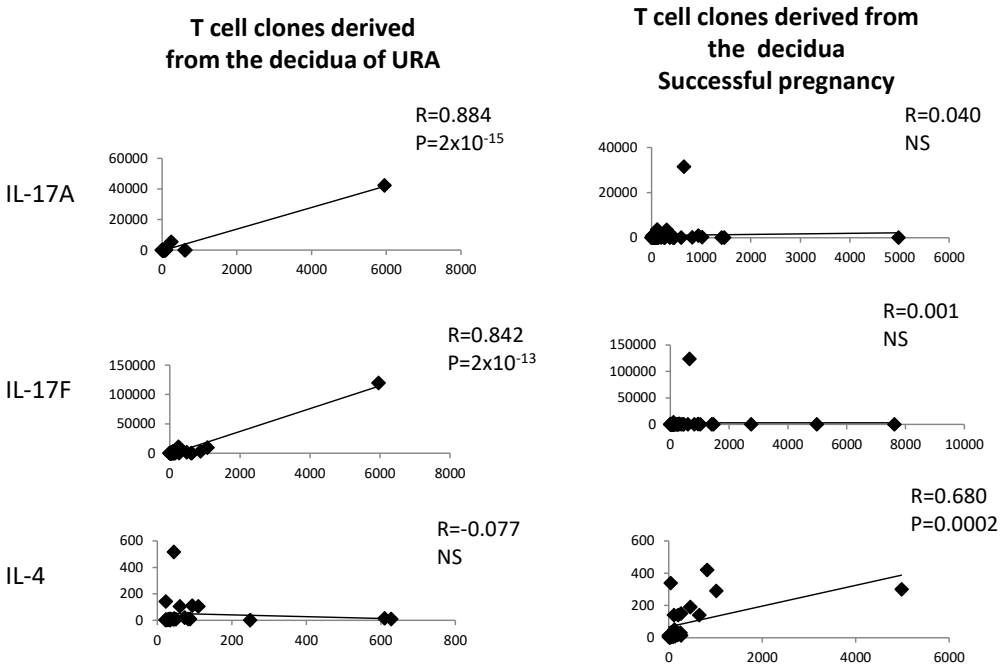

Figure 2. *Cont.*

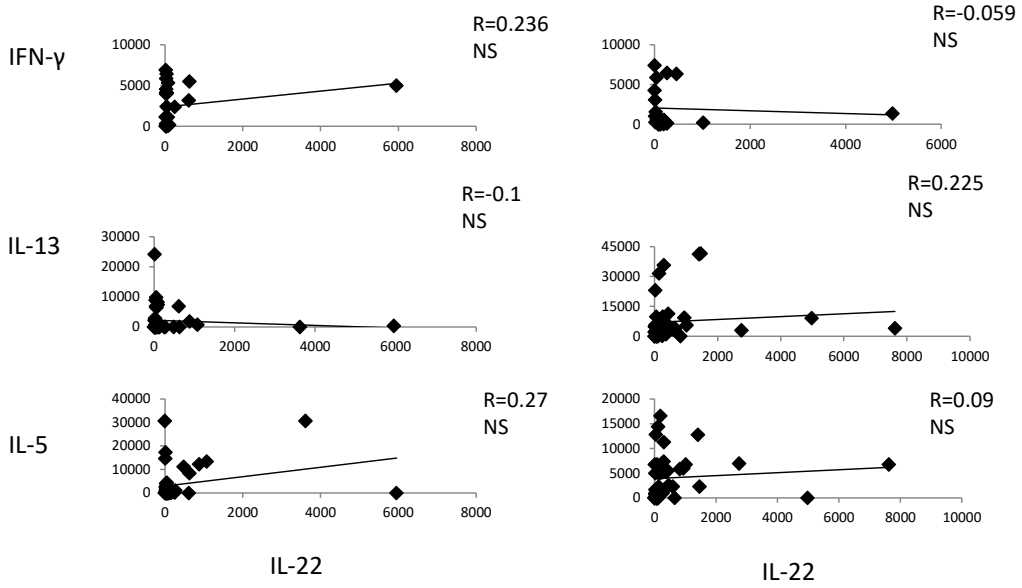

Figure 2. In those experiencing successful pregnancy, IL-22 is positively correlated with IL-4, whereas, in those experiencing URA, IL-22 produced by CD4+ T cell clones derived from the decidua is positively correlated with IL-17A and IL-17F. The levels of IL-22 and the levels of IL-4, IL-13, IL-5, IL-17A, IL-17F, and IFN-γ measured in the supernatants of the CD4+ T cell clones derived from deciduae of those experiencing unexplained recurrent abortion (URA) and those experiencing successful pregnancy (Experiment 1 in Section 4.3) have been correlated. NS means that p is not significant.

These results confirm the associated production of IL-22 and IL-4, but not the associated production of IL-22 and other Th2-type cytokines (IL-13 and IL-5), by decidual CD4+ T cells of successful pregnancy suggested in Figure 1 and show an associated production of IL-22 and Th17-type cytokines by decidual CD4+ T cells in those experiencing URA.

2.3. In Serum of Successful Pregnancy, IL-22 Is Positively Correlated with IL-4

IL-22, IL-4, IFN-γ, IL-5, IL-13, and IL-17A were measured with a multiplex bead-based assay in the serum of 18 women with successful pregnancy and 18 URA patients. IL-22 is positively correlated with IL-4 in the serum of those experiencing successful pregnancy (R = 0.527, p = 0.03) (Figure 3), whereas serum IL-22 is not correlated with serum IL-13, IL-5, IL-17A, and IFN-γ in those experiencing successful pregnancy. Serum IL-22 is not correlated with serum IL-4, IL-13, IL-5, IL-17A, and IFN-γ in those experiencing URA (Figure 3).

These data confirm the associated production of IL-22 and IL-4 in those experiencing successful pregnancy, as suggested by Figures 1 and 2.

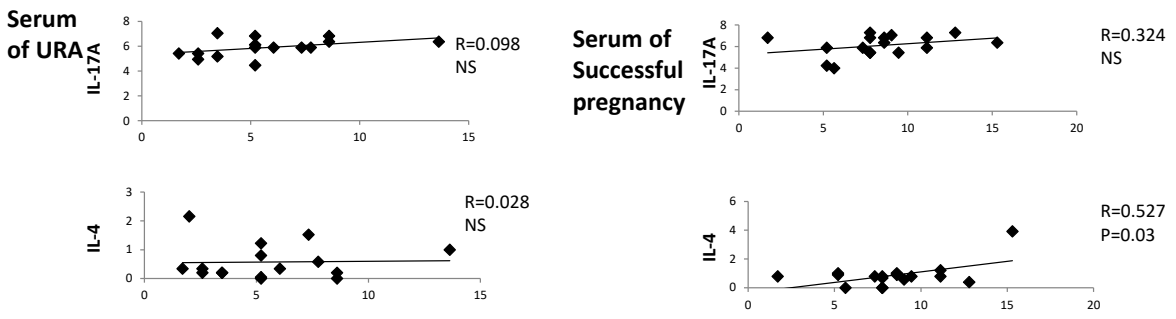

Figure 3. *Cont.*

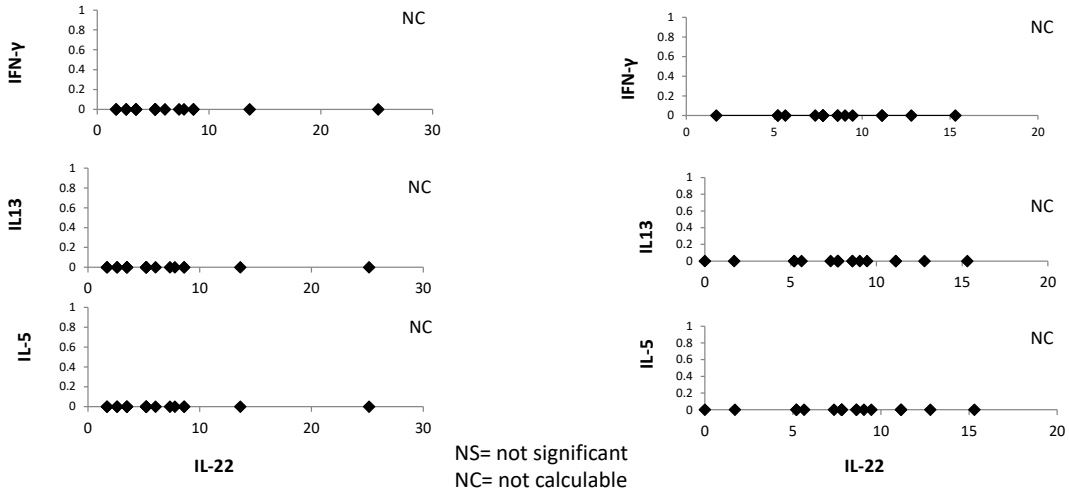

Figure 3. In the serum of successful pregnancy, IL-22 is positively correlated with IL-4. The levels of IL-22, IL-4, IFN-γ, IL-5, IL-13, and IL-17A were measured with a multiplex bead-based assay in the serum of 18 women with successful pregnancy and 18 unexplained recurrent abortion (URA) patients, and these levels were correlated.

2.4. The Prevalence of CD4+ T Helper Cells Producing IL-22 (Th/IL-22+) in the Decidua of Those Experiencing Successful Pregnancy

The role of IL-22 on pregnancy is not clear. It has been reported that IL-22 could be associated with URA because, in the serum of URA, IL-22 levels increase compared to cases of successful pregnancy [29]. However, other authors reported a prevalence of IL-22 in those experiencing successful pregnancy because mRNA for IL-22 decreases in decidua of those experiencing URA compared to those experiencing successful pregnancy [28]. To determine if IL-22 production by decidual CD4+ T cells is associated with successful pregnancy or URA, we derived 92 CD4+ T cell clones respectively from decidua of those experiencing successful pregnancy and URA (as in Experiment 1 in Section 4.3). IL-22 was measured in the supernatant of the CD4+ T cell clones by a multiplex bead-based assay.

The percentage of CD4+ T cells producing IL-22 (Th/IL-22+) in the decidua of those experiencing successful pregnancy (62.9%) is higher than the percentage of T helper cells producing IL-22 in the URA deciduae (21.6%) ($p = 0.002$) (Figure 4), suggesting that there is a prevalence of CD4+ T helper producing IL-22 in the decidua of those experiencing successful pregnancy and that IL-22 could have a positive effect on pregnancy.

2.5. T Helper Subpopulations Producing IL-22 Preferentially Associated with Pregnancy Failure and Successful Pregnancy

The previous figure did not show to which CD4+ T helper subpopulation(s) the decidual Th/IL-22+-cells belong and if particular T helper profiles are preferentially associated with pregnancy failure or successful pregnancy. To investigate the CD4+ cell subsets that produce IL-22, we analyzed the percentages of Th1-, Th2-, Th0-, Th17-, Th17/Th1-, Th17/Th2-, and Th17/Th0 cells, which also produce IL-22 (Th1/IL-22+, Th2/IL-22+, Th0/IL-22+, Th17/IL-22+, Th17/Th1/IL-22+, Th17/Th2/IL-22+, and Th17/Th0/IL-22+). We analyzed these subpopulations not only at decidual level but also in the peripheral blood to investigate if some or all of these IL-22-producing T cell subpopulations could be present in peripheral blood or only at the fetomaternal interface. One hundred twenty-five CD4+ T cell clones were derived respectively from the decidua and peripheral blood of those experiencing successful pregnancy and those experiencing unexplained recurrent abortion (Experiment 1 in Section 4.3) (Figure 5).

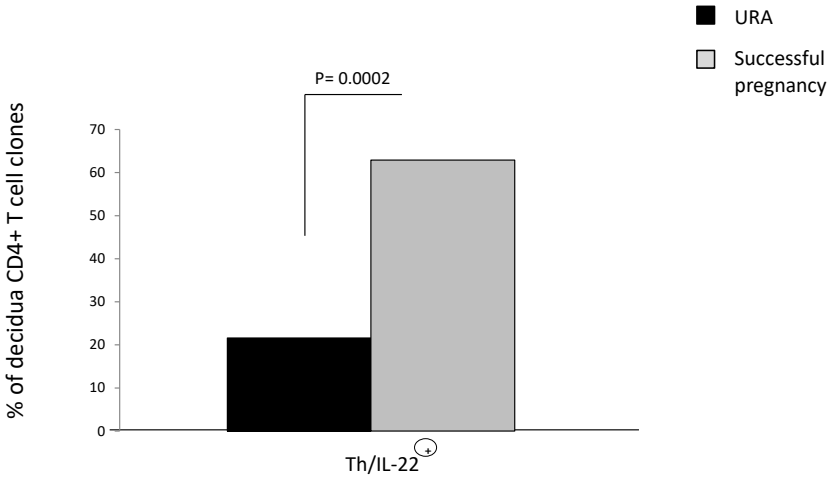

Figure 4. Prevalence of CD4+ T helper cells producing IL-22 (Th/IL-22+) in the decidua of those experiencing successful pregnancy. The levels of IL-22 and the levels of IL-4, IL-13, IL-5, IL-17A, IL-17F, and IFN-γ have been measured in the supernatants of the CD4+ T cell clones derived from deciduae of those experiencing unexplained recurrent abortion (URA) and those experiencing successful pregnancy (Experiment 1 in Section 4.3). The percentage of CD4+ T cells producing IL-22 (Th/IL-22+) in the decidua of those experiencing successful pregnancy and the percentage of T helper cells producing IL-22 in the URA deciduae have been calculated. The statistical analysis was performed with chi-square test.

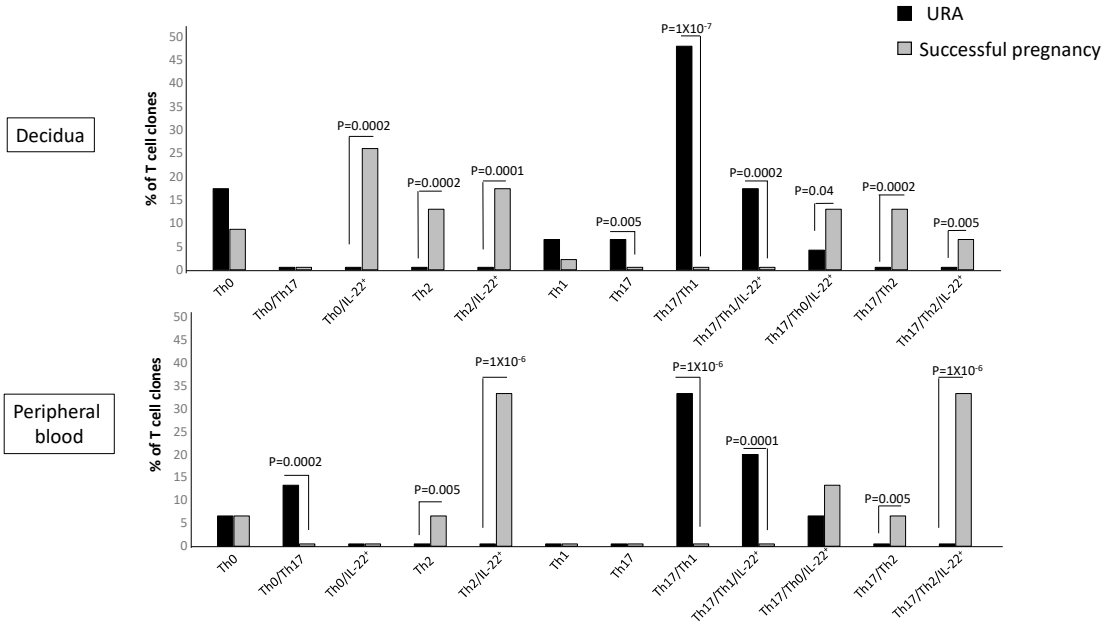

Figure 5. T helper subpopulations present in the decidua and in the peripheral blood of those experiencing unexplained recurrent abortion and those experiencing successful pregnancy. To investigate the CD4+ cell subsets that produce IL-22, the percentages of Th1-, Th2-, Th0-, Th17-, Th17/Th1-, Th17/Th2-, and Th17/Th0 cells, which do not produce IL-22 and which also produce IL-22 (Th1/IL-22+, Th2/IL-22+, Th0/IL-22+, Th17/IL-22+, Th17/Th1/IL-22+, Th17/Th2/IL-22+, and Th17/Th0/IL-22+) were analyzed. Cytokines were measured in the supernatants of the CD4+ T cell clones derived from the decidua and peripheral blood of those experiencing successful pregnancy and those experiencing unexplained recurrent abortion (URA) (Experiment 1 in Section 4.3) by a multiplex bead-based assay. The statistical analysis was performed with the chi-square test.

There are some CD4+ Th subpopulations that do not produce IL-22 but are prevalent in the decidua of those experiencing URA, the Th17 cells, and Th17/Th1, confirming our previous report [25].

The Th17/Th1 cells, but not the Th1 cells, are also prevalent in the URA peripheral blood. There are also some Th subpopulations that do not produce IL-22 but are prevalent in the decidua and in the peripheral blood of those experiencing successful pregnancy, the Th2 cells, and Th17/Th2, confirming our previous reports [25,31].

The CD4+ subpopulations that produce IL-22 in the decidua and in the peripheral blood of those experiencing successful pregnancy or URA are Th0, Th2, Th17/Th1, Th17/Th0, and Th17/Th2 cells.

The percentage of Th0/IL-22+ (producing IFN-γ, IL-4, IL-5, IL-13, and IL-22 (84%), producing IFN-γ, IL-4, IL-5, and IL-22 (8%), and producing IFN-γ, IL-4, IL-13, and IL-22 (8%)) ($p = 0.0002$) (Table 1), of Th2/IL-22+ (all producing IL-4, IL-5, IL-13, and IL-22) ($p = 0.0001$) (Table 1), of Th17/Th0/IL-22+ (producing IFN-γ, IL-4, IL-5, IL-13, and IL-22 (66%), producing IFN-γ, IL-4, IL-5, and IL-22 (17%), and producing IFN-γ, IL-4, IL-13, and IL-22 (17%)) ($p = 0.04$) (Table 1), and of Th17/Th2/IL-22+ (all producing IL-4, IL-17A, IL-17F, IL-5, IL-13, and IL-22) ($p = 0.005$) (Table 1) clones are significantly higher in the decidua of those experiencing successful pregnancy compared to the decidua of women suffering from URA (Figure 5). In contrast, the percentage of Th17/Th1/IL-22+ clones+ (producing IFN-γ, IL-17A, IL-17F, and IL-22, but not IL-4) ($p = 0.005$) (Table 1) is significantly higher in the decidua of those experiencing URA compared to those experiencing successful pregnancy (Figure 5).

Table 1. CD4+ T subpopulations producing IL-22 in the decidua of those experiencing successful pregnancy and those experiencing URA. The percentage of the CD4+ T cell clones that produce IL-22 in the decidua of those experiencing successful pregnancy and those experiencing unexplained recurrent abortion (URA), which also produce Th2-type (IL-4, IL-5, IL-13), and/or Th1-type (IFN-γ) and/or Th17-type (IL-17A) cytokines was determined. All the cytokines were measured by a multiplex bead-based assay in the supernatant of the CD4+ T cell clones.

T Cell Clones from Decidua of URA	%	T cell Clones from Decidua of Normal Pregnancy	%	*P*
Th0/IL-22+	0	Th0/IL-22+	26	0.0002
IFN-γ+/IL-22+ IL-4+	0	IFN-γ+/IL-22+ IL-4+	0	
IFN-γ+/IL-22+ IL-4+ IL-13+ IL-5+	0	IFN-γ+/IL-22+ IL-4+ IL-13+ IL-5+	84	
IFN-γ+/IL-22+ IL-4+ IL-5+	0	IFN-γ+/IL-22+ IL-4+ IL-5+	8	
IFN-γ+/IL-22+ IL-4+ IL-13+	0	IFN-γ+/IL-22+ IL-4+ IL-13+	8	
Th2/IL-22+	0	Th2/IL-22+	17	0.0001
IL-22+ IL-4+	0	IL-22+ IL-4+	0	
IL-22+ IL-4+ IL-13+ IL-5+	0	IL-22+ IL-4+ IL-13+ IL-5+	100	
IL-22+ IL-4+ IL-5+	0	IL-22+ IL-4+ IL-5+	0	
IL-22+ IL-4+ IL-13+	0	IL-22+ IL-4+ IL-13+	0	
Th17/Th2/IL-22+	0	Th17/Th2/IL-22+	6.5	0.0005
IL17+/IL-22+ IL-4+	0	IL17+/IL-22+ IL-4+	0	
IL17+/IL-22+ IL-4+ IL-13+ IL-5+	0	IL17+/IL-22+ IL-4+ IL-13+ IL-5+	100	
IL17+/IL-22+ IL-4+ IL-5+	0	IL17+/IL-22+ IL-4+ IL-5+	0	
IL17+/IL-22+ IL-4+ IL-13+	0	IL17+/IL-22+ IL-4+ IL-13+	0	
Th17/Th0/IL-22+	2	Th17/Th0/IL-22+	13	0.04
IL-17+/IFN-γ+/IL-22+ IL-4+	0	IL-17+/IFN-γ+/IL-22+ IL-4+	0	
IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+ IL-5+	100	IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+ IL-5+	66	
IL-17+/IFN-γ+/IL-22+ IL-4+ IL-5+	0	IL-17+/IFN-γ+/IL-22+ IL-4+ IL-5+	17	
IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+	0	IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+	17	
Th17/Th1/IL-22+	22	Th17/Th1/IL-22+	0	0.005
IL-17+/IFN-γ+ /IL-22+ IL-4+	0	IL-17+/IFN-γ+/IL-22+ IL-4+	0	
IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+ IL-5+	0	IL-17+/IFN-γ+//IL-22+ IL-4+ IL-13+ IL-5+	0	
IL-17+/IFN-γ+/IL-22+ IL-4+ IL-5+	0	IL-17+/IFN-γ+/IL-22+ IL-4+ IL-5+	0	
IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+	0	IL-17+/IFN-γ+/IL-22+ IL-4+ IL-13+	0	
IL-17+/IFN-γ+/IL-22+	100	IL-17+/IFN-γ+/IL-22+	0	

In peripheral blood, contrarily to decidua, the percentage of Th0/IL-22+ cells (producing IFN-γ, IL-4, IL-5, IL-13, and IL-22) and the percentage of Th17/Th0/IL-22+ cells (producing IFN-γ, IL-4, IL-5, IL-13, IL-17A, IL-17F, and IL-22) are not statistically different in those experiencing URA and those experiencing successful pregnancy (Figure 5).

However, as for decidua, the percentage of Th2/IL-22+ (producing IL-4, IL-5, and IL-22 with IL-13 (20%), without IL-13 (80%)) ($p = 0.000001$) and the percentage of Th17/Th2/IL-22+ (producing IL-4,

IL-17A, IL-17F, and IL-22 (20%), with IL-5 and IL-13 (20%) and with IL-5 only (60%)) ($p = 0.000001$) clones in peripheral blood are significantly higher in those experiencing successful pregnancy compared to those experiencing URA (Figure 5). Moreover, as for decidua, the percentage of Th17/Th1/IL-22+ clones+ (producing IFN-γ, IL-17A, IL-17F, and IL-22, without IL-4) ($p = 0.0001$) in peripheral blood of those experiencing URA is significantly higher compared to peripheral blood of those experiencing successful pregnancy (Figure 5).

These results indicate that four subpopulations of CD4+ cells, producing IL-22, which produce IL-4 and/or IL-5 and/or IL-13, named Th0/IL-22+, Th2/IL-22+, Th17/Th0/IL-22+, and Th17/Th2/IL-22+ cells, are associated with the success of pregnancy, whereas the only subpopulation of CD4+ cells associated with URA, which produces IL-22, the Th17/Th1/IL-22+ cells, does not produce IL-4. The associated production of IL-22 and IL-4, as previously shown, seems to be essential for the success of pregnancy. IL-22 produced by decidual CD4+ T cells, if associated with IL-4 (and/or IL-13 and IL-5) production, is not deleterious for pregnancy outcome.

2.6. Th17/Th2/IL-22+ and Th17/Th0/IL-22+ CD4+ T Cells Are Exclusively Present at the Implantation Site of Ectopic Pregnancy

Decidual Th0/IL-22+, Th2/IL-22+, Th17/Th0/IL-22+, and Th17/Th2/IL-22+ cells seem to be important for successful pregnancy development. We wondered whether these cells were present at the implantation site of the embryo and thus could have an important role for embryo implantation. To answer this question, we performed the cytokine analysis in ectopic tubal pregnancies.

We evaluated the percentages of Th0/IL-22+, Th2/IL-22+, Th17/Th0/IL-22+, Th17/Th2/IL-22+, and Th17/Th1/IL-22+ cells among the CD4+ T cell clones ($N = 67$) respectively derived from the implantation site of the embryo and distant from the implantation site in the same Fallopian tube of four women suffering from ectopic pregnancy (Experiment 2 according to Section 4.3).

There is no significant difference in the percentage of Th0/IL-22+, Th2/IL-22+, and Th17/Th1/IL-22+ CD4+ T cell clones generated from the implantation site and distant from the implantation site (Figure 6A) (although the last subpopulation seems to be prevalent apart from the implantation site and the first subpopulation seems to be prevalent at the implantation site). At the implantation site, the percentage of Th17/Th0/+IL-22+ (producing IFN-γ, IL-4, IL-5, IL-13, IL-17A, IL-17F, and IL-22 (86%), producing IFN-γ, IL-4, IL-17A, IL-17F, and IL-22 (7%), producing IFN-γ, IL-4, IL-13, IL-17A, IL-17F, and IL-22 (7%)) ($p = 0.00001$) and the percentage of Th17/Th2/IL-22+ (producing IL-4, IL-17A, IL-17F, and IL-22 with IL-5 and IL-13 (67%) and with IL-13 only (33%)) ($p = 0.0001$) CD4+ T cell clones are higher than that of those clones derived distant from the implantation site (Figure 6A).

We confirmed these results by determining the mRNA level of IL-4, GATA-3, IL-17A, ROR-C, IL-22, AHR, T-bet, and IFN−γ in Fallopian tube tissues taken at the embryo implantation site and tissue sampled distant from the implantation site of an additional woman suffering from ectopic pregnancy (Figure 6B). At the implantation site, the levels of mRNA for IL-22 and its associated transcription factor AHR, for Th2-type molecules (IL-4 and GATA3) and for Th17-type molecules (IL-17A and RORC), are increased compared to the mRNA levels for these molecules apart from the implantation site. In contrast, away from the implantation site, the mRNA production level of IFN-γ and its associated transcription factor T-bet is increased compared to those expressed at the embryo implantation site (Figure 6B).

Interestingly, IL-4, GATA-3, IL-17A, ROR-C, IL-22, and AHR mRNA levels seem to be higher at the implantation site than those found in the decidual biopsies of successful pregnancy, indicating that the production of IL-22, IL-4, and IL-17A is concentrated at the embryo implantation site (Figure 7). By contrast, the levels of IFN-γ and T-bet were higher away from the implantation site compared to those found in the decidual biopsies of successful pregnancy and at the embryo implantation site of the tubal biopsy (Figure 7). These results indicate that IFN-γ increases when implantation fails or does not occur.

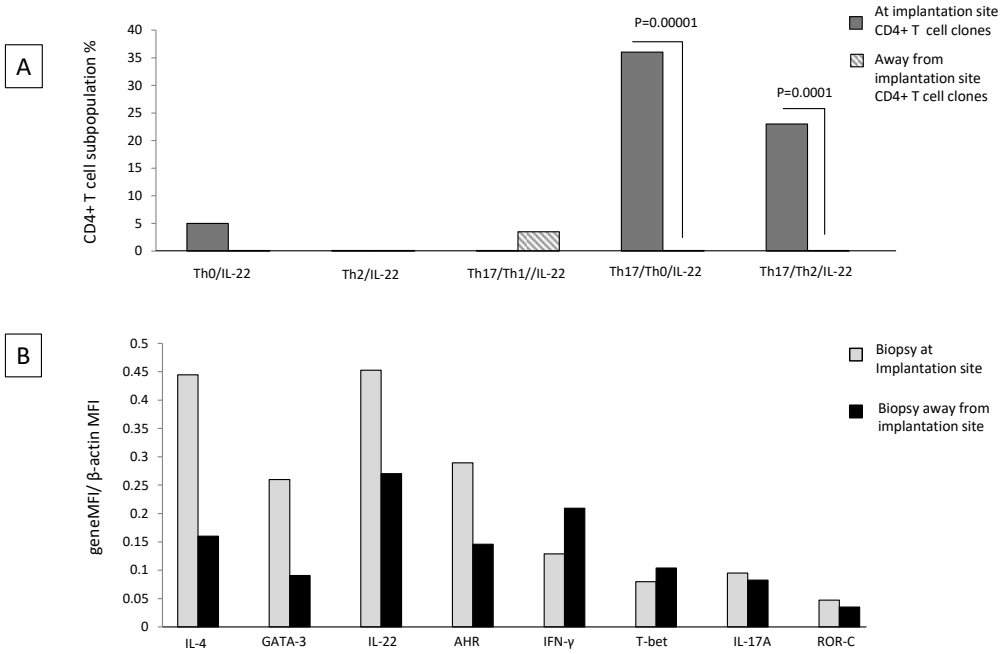

Figure 6. Th17/Th2/IL-22+ and Th17/Th0/IL-22+ CD4+ T cells are exclusively present at the implantation site of ectopic pregnancy. The percentages of Th0/IL-22+, Th2/IL-22+, Th17/Th0/IL-22+, Th17/Th2/IL-22+, and Th17/Th1/IL-22+ cells among the CD4+ T cell clones respectively derived from the implantation site of the embryo and distant from the implantation site in the same Fallopian tube of four women suffering from ectopic pregnancy were evaluated (Experiment 2 according to Section 4.3). The statistical analysis was performed with the chi-square test. The determination of mRNA level for IL-4, GATA-3, IL-17A, ROR-C, IL-22, AHR, T-bet, and IFN -γ in Fallopian tube tissue taken at the embryo implantation site and tissue sampled distant from the implantation site of an additional woman suffering from ectopic pregnancy was performed by Quantigene 2.0.

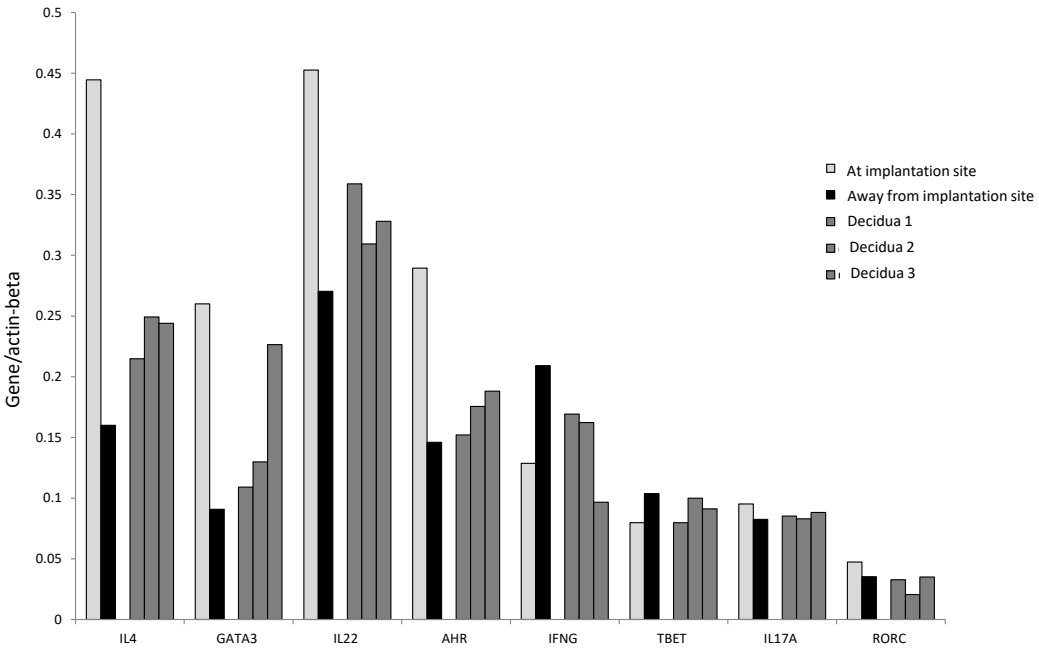

Figure 7. The determination of mRNA level for IL-4, GATA-3, IL-17A, ROR-C, IL-22, AHR, T-bet, and IFN -γ in Fallopian tube tissue taken at the embryo implantation site and distant from the implantation site of a woman suffering from ectopic pregnancy and in decidual biopsies from three women with successful pregnancy was performed by Quantigene 2.0.

3. Discussion

Trophoblast expressing paternal Class I antigens thought to resemble a semiallograft, and could be rejected by maternal CD4+ T cells. IL-22, which could be produced by CD4+ Th17 and Th22 cells, has been shown to be involved in allograft rejection, by increasing IFN-γ production by Th1 and Tc1 cells, known to be responsible for acute allograft rejection, and decreasing IL-10 production by T reg and Th2 cells, known to be responsible for allograft tolerance [26,27]. For this, it has been suggested that IL-22 at the fetal maternal interface could be responsible for spontaneous abortion. Some authors confirmed this hypothesis by reporting that, in the serum of women suffering from URA, there are increased levels of IL-22 compared to the levels of IL-22 present in the serum of women with successful pregnancy [29]. However, Roomandeh et al. (2018) [29] measured IL-22 only in the serum and did not investigate IL-22 production at fetal maternal interface, where the factors of uterine microenvironment could control the cytokine profile of decidual cells producing IL-22.

We investigated the effect on pregnancy outcome of IL-22 produced by T helper cells in the peripheral blood and, more importantly, in the decidua of those suffering from URA (during a spontaneous abortion) and of women experiencing successful pregnancy. Our results show that the percentage of CD4+ T cells producing IL-22 (Th/IL-22+) in the decidua of those experiencing successful pregnancy (62.9%) is higher than the percentage of T helper cells in the deciduae of those experiencing URA (21.6%) ($p = 0.002$), suggesting that there is a prevalence of T helper cells producing IL-22 in the decidua of those experiencing successful pregnancy compared to those experiencing URA.

Our results, in agreement with O'Hern Perfetto et al. (2015) [28], who reported that mRNA for IL-22 increases in decidua of those experiencing successful pregnancy compared to those experiencing URA, suggest that IL-22 produced by CD4+ T cells could have a positive effect on pregnancy and that, although IL-22 produced by CD4+ T cells could be responsible for fetoallograft rejection, IL-22 could also be beneficial for pregnancy at least in some conditions. O'Hern Perfetto et al. (2015) [28] suggested by immunohistochemistry that IL-22 could be produced by decidual NK cells, but did not investigate if these cells could be the only source of IL-22 and if IL-22 could be produced by decidual CD4+ T cells at the fetomaternal interface.

Thus, our previous results suggested that IL-22 could also be beneficial for pregnancy at least in some conditions. The results, we will discuss below, will show the conditions under which IL-22 could be beneficial for pregnancy. In fact, we found an associated production of IL-4 and IL-22 by decidual CD4+ T cells in those experiencing successful pregnancy, not found in those experiencing URA, which was confirmed at the mRNA level in the decidua of those experiencing successful pregnancy, with the associated expression of mRNA for IL-22, IL-4, and their transcriptional factors, AHR and GATA3, respectively. In agreement with these results, we found i) that IL-22 produced by decidual CD4+ T cells of those experiencing successful pregnancy is positively correlated with IL-4 produced by the same cells, whereas, in those experiencing URA, IL-22 is positively correlated with IL-17A and IL-17F, but not with IL-4, and ii) that IL-22 is positively correlated with IL-4 in the serum of those experiencing successful pregnancy.

Furthermore, we investigated to which CD4+ T helper subpopulation(s) the decidual Th/IL-22+ cells belong and if particular T helper profiles, characterized by the associated production of IL-22 and IL-4, are preferentially associated with pregnancy failure or successful pregnancy. We analyzed the percentages of Th1-, Th2-, Th0-, Th17-, Th17/Th1-, and Th17/Th2-cells, which also produce IL-22 (Th1/IL-22+, Th2/IL-22+, Th0/IL-22+, Th17/IL-22+, Th17/Th1/IL-22+, and Th17/Th2/IL-22+). We analyzed these subpopulations not only at the decidual level but also the peripheral blood. We found that four subpopulations of CD4+ cells producing IL-22 and IL-4 (Th0/IL-22+, Th2/IL-22+, Th17/Th0/IL-22+, and Th17/Th2/IL-22+) are associated with the success of pregnancy, whereas the only subpopulation of CD4+ cells producing IL-22 and associated with URA does not produce IL-4. The associated production of IL-22 and IL-4 seems to be essential for the success of pregnancy. IL-22 produced by decidual CD4+ T cells, if associated with IL-4 production, is not deleterious for pregnancy.

Thus, our results suggest that the condition under which IL-22 produced by CD4+ T cells could be beneficial for pregnancy when IL-22 production is associated with IL-4 production.

Decidual Th0/IL-22+, Th2/IL-22+, Th17/Th0/IL-22+, and Th17/Th2/IL-22+ cells seem to be important for successful pregnancy development. We wondered whether these cells were present at the implantation site of the embryo and thus could play an important role in embryo implantation. To answer this question, we performed the same kind of cytokine analysis in ectopic tubal pregnancies. We evaluated the percentages of Th0/IL-22+, Th2/IL-22+, and Th0/IL-22+ of Th2/IL-22+, Th17/Th0/IL-22+, Th17/Th2/IL-22+, and Th17/Th1/IL-22+ cells among the CD4+ T cells respectively derived from the implantation site of the embryo and distant from the implantation site in the same Fallopian tube of patients suffering from ectopic pregnancy.

We found that, at the implantation site, the percentage of Th17/Th0/+IL-22 and of Th17/Th2/IL-22+CD4+ T cells is higher than those of T cells derived distant from the implantation site. We also confirmed the associated and increased expression of mRNA for IL-4 and IL-22 and their respective transcriptional factors, GATA3 and AHR, in the tissue derived from the embryo implantation site compared to the tissue distant from the implantation site in the Fallopian tube with ectopic pregnancy.

We are not surprised by the associated production of IL-22 and a Th2-type cytokine (IL-4) by CD4+ T cells, because Plank et al. (2017) [11] reported that in vitro Th22 cells may demonstrate a plasticity toward Th2-type cells. Under Th2-promoting conditions in vitro, as well as in vivo, Th22 cells display marked plasticity toward the production of IL-13 (another Th2-type cytokine). Consistent with these results, skin-homing IL-13- and IL-22-producing Th2/22 and Tc2/22 cells were recently found to be elevated Th2-type pathologies as atopic dermatitis (AD) [15]. A Th2/Th22 inflammatory pathway has also been reported in acute canine AD skin lesions [16,17]. We demonstrated for the first time the possible association of IL-22 and another Th2-type cytokine different from IL-13, IL-4 produced by CD4+ T cells.

We could wonder what could be the beneficial role of IL-22 in pregnancy. The receptor for IL-22 (IL-22R1) is present on trophoblast cells and human villi [32]. This receptor is known to be present only on epithelial cells, and trophoblast cells are epithelial cells of fetal origin. As IL-22 is important for epithelial regeneration and wound repair [33,34], IL-22 at the fetal maternal interface could have a positive effect on pregnancy by repairing damage of the trophoblast cells. IL-22 could also be beneficial for pregnancy because it induces the proliferation and survival and decreases the apoptosis of trophoblast cells [32]. In addition, IL-22 could be beneficial for pregnancy by protecting trophoblast from pathogens, which cause a fivefold increase in the number of miscarriages in virtue of its ability to induce the secretion of antimicrobial peptides locally [35]. Very recently, in late pregnancy, in particular in preterm birth, it has been shown that IL-22 could contribute to defense against inflammatory responses at the fetal maternal interface in response to intrauterine infection [36]. In fact, the authors demonstrated that IL-22 is upregulated in uterine tissue in response to bacterial endotoxin and prevents apoptosis of placental cells. Importantly, supplementation with recombinant IL-22 significantly improved pregnancy outcomes in mice, which were challenged with intrauterine LPS (expressed by Gram-pathogens) treatment [36] (Figure 8).

Th17/Th0/IL-22+ and Th17/Th2/IL-22+ CD4+ T cells, which are present at the embryo implantation site, produce IL-22, which could have a positive effect on pregnancy when these CD4+ T cells produce it in association with IL-4, as does IL-17 [25], which can induce trophoblast proliferation and invasion and a protection against extracellular pathogens by recruiting neutrophils. IL-22 could act positively on pregnancy by inducing epithelial regeneration [33,34] and could, at the fetal maternal interface, repair damage of the trophoblast cells. IL-22 could also be beneficial for pregnancy because it induces the proliferation and survival and decreases the apoptosis of trophoblast cells [32] and could be beneficial for pregnancy by protecting trophoblast from pathogens, responsible for miscarriages, by its ability to induce the secretion of antimicrobial peptides locally [35,36].

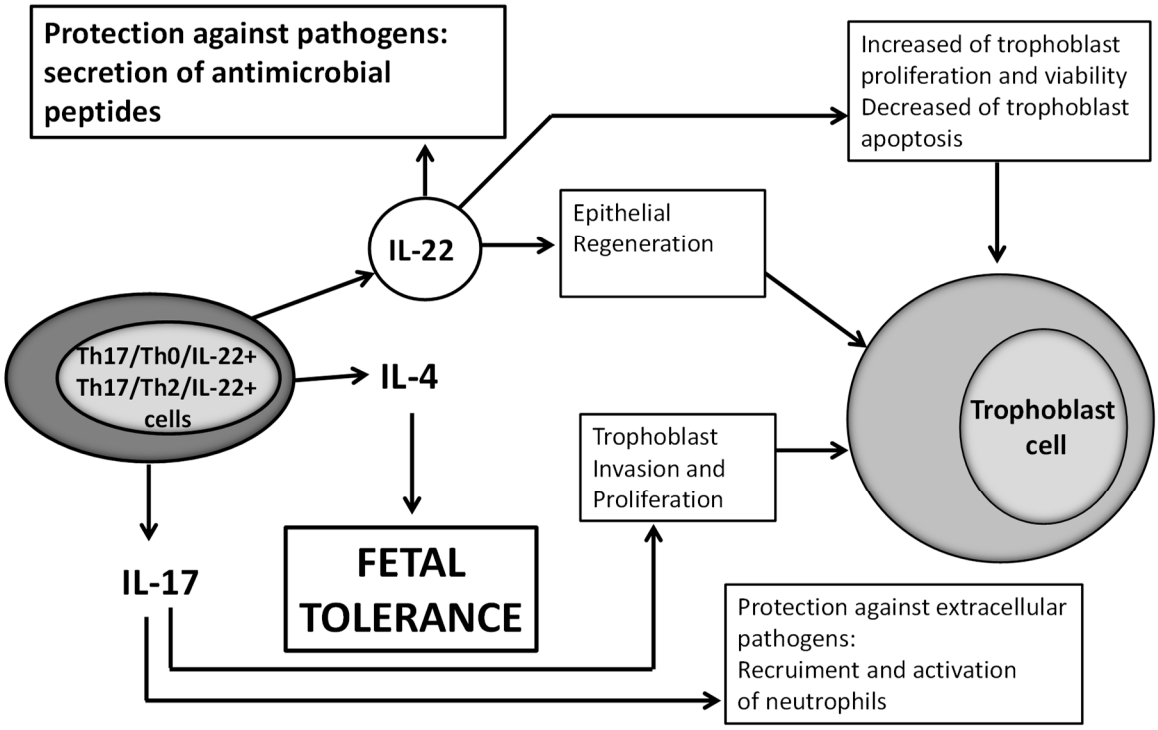

Figure 8. Possible positive roles of Th22/Th2/IL-22+ and Th17/Th0/IL-22+ cells at fetal maternal interface.

4. Material and Methods

4.1. Reagents

PHA was purchased from GIBCO Laboratories (Grand Island, NE, USA) and phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical Co. (St. Louis, MO, USA). OKT3 (anti-CD3) mAb was purchased from Ortho Pharmaceuticals (Raritan, NJ, USA). Anti-CD4 and anti-CD8 were obtained from Becton-Dickinson (Mountain View, CA, USA). Human recombinant IL-2 was a generous gift from Eurocetus (Milano, Italy). FCS was from HyClone Lab Inc. (Logan, UT, USA).

4.2. Subjects

Thirty pregnant women with normal gestation and no spontaneous abortion in their past history had requested elective termination were enrolled in the study with 27 women, who had histories of at least 7 $\pm$ 3 (range, 4–10) prior first-trimester spontaneous abortions, which could not be explained on the basis of conventional criteria (normal parental chromosomes, hysterosalpengography and hysteroscopy, endometrial biopsy, hormonal analysis including FSH, LH, estradiol, testosterone, cervical cultures for the presence of ureaplasma, mycoplasma and chlamydia, lupus anticoagulant, anti-phospholipid antibodies, and thyroid function tests). Specimens of deciduae and peripheral blood were obtained at the time of spontaneous abortion (at 8–11 weeks of pregnancy with normal karyotype of trophoblast), at which time all women were in excellent health, had no history of atopy or allergy, and were taking no medication. Trophoblast-invaded tubal mucosa at the implantation site and tubal mucosa distant from the implantation site were obtained from 6 women (with no history of spontaneous abortion) whose ectopic pregnancies were terminated by surgical removal as a result of threatened tubal rupture. The women agreed to participate in the study at the Hospital of Rijeka, Croatia. All subjects received verbal and written information about the aim and the design of the research, and all women provided written informed consent. The study was approved by local ethics committees of the Medical Faculty of Rijeka of the Clinical Hospital Center of Rijeka (N. 2170-29.02/1-06-1 and 2170-24-09-7-06-02). The mean age and the gestational age values of the

three groups of patients (successful pregnancy, unexplained recurrent abortion, and ectopic pregnancy) were not statistically different.

4.3. Generation of CD4+ T-Cell Clones from Peripheral Blood, from Decidual Biopsies of Those Experiencing Successful Pregnancy and Those Experiencing URA, and from Fallopian Tube Biopsies of Ectopic Pregnancy

Specimens of deciduae (separated from villus with normal karyotype) and of Fallopian tubes were washed twice in PBS (pH 7.2) and then disrupted in small fragments (2–3 mm in diameter). Short-term T-cell lines were generated by culturing single fragments for one week in 24-well plates (Costar, Cambridge, Massachusetts) in 2 mL of RPMI 1640 supplemented with 2 mM L-glutamine, 20 M-mercaptoethanol, 10% FCS (complete medium) (Hyclone Laboratories, Logan, Utah), and IL-2 (Eurocitus, Milan, Italy) (20 U/mL). T-cell clones were then generated from short-term cultures of decidual and tubal T cells derived in the presence of IL-2, as well as from PBMC obtained from the same donors, using a method described elsewhere [31]. The phenotype of CD3+CD4+ of T-cell clones was assessed by flow cytometer analysis.

4.4. Induction of Cytokine Production by T-Cell Clones

To induce the cytokine production, 10^6 T-cell blasts from each T-cell clone were cultured in the presence of PMA (20 ng/mL; Sigma, St. Louis, MO, USA) plus monoclonal antibody against CD3 (100 ng/mL; Ortho Pharmaceuticals, Raritan, New Jersey). After 36 h, culture supernatants were collected, filtered, and stored in aliquots at −80 °C.

4.5. Determination of Cytokine Concentrations in Supernatants with Bead-Based Multiplex Immunoassays

The quantitative determination of IL-4, IL-5, IL-13, IL-17A, and IFN-γ was performed by a bead-based multiplex immunoassay (Biorad Laboratories, Hercules, CA, USA) and the determination of IL-17F and IL-22 by another bead-based multiplex immunoassay (Millipore, Billerica, Massachusetts) using a Bioplex 200 system (Biorad Laboratories, Hercules, CA, USA), as we have previously described elsewhere [37]. In brief, supernatant was added to antibody-conjugated beads directed against the cytokines listed above in a 96-well filter plate. After a 30 min incubation, the plate was washed and biotinylated anti-cytokine antibody solution was added before another 30 min incubation. The plate was then washed and streptavidin-conjugated PE was added. After a final wash, each well was suspended with assay buffer and analyzed with the Bioplex 200 system. Standard curves were derived from various concentrations of the different cytokine standards and followed the same protocol as the supernatant samples. The concentration of each cytokine (pg/mL) in each T cell clone supernatant was calculated thanks to the Bioplex200 software.

4.6. Quantification by a Multiplex Gene Assay (Quantigene 2.0) of IL-4, IL-17A, IL-17F, IL-23R, IFN-γ, RORC, GATA3, AHR, and IL-22 mRNA

The mRNA quantization for the genes was performed with a Multiplex Gene Assay (Quantigene 2.0, Thermo Fisher, Waltham, MA, USA), as we previously described elsewhere [38,39]. Briefly, the mRNA expression of IL-4, IL-17A, IL-17F, IL-23R, IFN-γ, RORC, GATA3, AHR, IL-22, and Actb (high expression housekeeping gene) was measured using the QuantiGene multiplex assay (Thermo Fisher, Waltham, MA, USA). Samples (biopsies of decidua from those experiencing successful pregnancy and tubal biopsies of those experiencing ectopic pregnancies at the implantation site and away from the implantation site) were lysed after treatment in a lysis mixture; mRNA expression in lysates was detected and measured according to the manufacturer's instructions. Samples were frozen in RNA later (Qiagen, Germany). Each sample was weighed, and the appropriate lysis solution was added to a final volume of 150 µL containing 50% Lysis Mixture (Thermo Fisher, Waltham, MA, USA) and 1 g/L Mixture (Thermo Fisher, Waltham, MA, USA) and 1 g/L proteinase K. The mixture was shaken at 65 °C for 30 min to lyse the cells. The lysate was stored at −80 °C for later use.

A panel of oligonucleotide capture probes, each with a unique sequence of 15 bases, was covalently linked to carboxylated fluorescently encoded beads (Luminex, Bio-rad, Massachusetts, USA). We mixed each sample lysate diluted at 1:1 and 1:2 with the pooled capture beads in a round-bottom assay well and hybridized for 16 h at 54 °C (final volume in each well was 100 µL). The assay mixture was transferred to a MultiScreen filter plate (Millipore, Billerica, MA, USA), and unbound material was filter-washed from the wells by rinsing 3 times with wash buffer. The plate was then hybridized at 54 °C for 1 h with 100 µL/well of a bDNA amplifier in Amplifier Diluent (Thermo Fisher, Waltham, MA, USA). Afterward, the plate was filter-washed twice with a wash buffer and incubated at 50 °C for 1 h with 100 µL/well of 5′-dT (Biotin)-conjugated label probe (Thermo Fisher, Waltham, MA, USA) diluted in Label Probe Diluent (Thermo Fisher, Waltham, MA, USA). After 2 washes, streptavidin-conjugated R-phycoerythrin diluted in SA-PE diluent (20 mmol/L Tris-HCl, 400 mmol/L lithium chloride, 1 mL/L Tween 20, 1 mL/L bovine serum albumin, and 5 mL/L Micr-O-protect) was added, and the plate was shaken and incubated at room temperature for 30 min. We washed the beads to remove unbound SA-PE and then analyzed them with Bio-Plex 200 system (Bio-Rad). The SA-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads. Expression of target-specific RNA molecules was calculated as mean values from triplicate cultures and normalized against the Actin gene (high expression housekeeping gene).

4.7. Serum from Peripheral Blood of Those Experiencing Successful Pregnancy and URA

We analyzed the serum of 18 first-trimester women experiencing successful pregnancy who had requested elective termination and the serum of 18 women with URA, who underwent a spontaneous abortion. In all sera, isolated from 5 mL of peripheral blood and then frozen at −80 °C, the quantitative determination of IL-4, IL-13, IL-17A, IFN-γ, and IL-22 by bead-based multiplex immunoassays as we have previously described above.

4.8. Statistics

Statistical analyses were performed using SSPS software (SPSS, Inc, Evanston, IL, USA). Due to non-parametric distribution, all comparisons between cytokine concentrations in controls and those experiencing URA were performed by the Wilcoxon test. The subpopulations percentages were analyzed by the chi-square test. A p-value <0.05 was considered statistically significant.

5. Conclusions

As IL-17, IL-22 when produced by CD4+ T cells in association with IL-4 could be another cytokine essential for the maintenance of pregnancy. IL-22 could be essential at certain stages of pregnancy or deleterious at other stages. The chronology of action of IL-22 should be further investigated

Author Contributions: M.-P.P. conceived the study and designed all the experiments, analyzed all the data, and wrote the manuscript. D.R. organized handling and shipment of samples, and participated in the design of the ectopic pregnancy experiments. L.L., F.L. and O.K. performed the multiplex bead-based assays, the T cell clone cultures, and the decidual and Fallopian tube T cell clones cultures, and operated Quantigene 2.0. H.H. collected pregnancy specimens. E.M. approved and authorized all the processes. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Crohn's Colitis: Development of a multiplex gene expression assay comparing mRNA levels of susceptibility genes

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Summary Crohn's disease (CD) is a multifactorial immunologically mediated disease. In this study we explored, for the first time, the efficacy of the Multiplex Gene Assay technology for detecting mRNA expression profile of 24 selected CD related genes in endoscopic biopsies and surgical specimens from CD patients with colonic localization of the disease. The polymorphisms of genes most frequently associated with CD were also analysed in DNA samples from the same patients. The analysis of endoscopic samples showed increased expression of 7 genes in inflamed mucosa compared to non-inflamed mucosa and suggests the activation of the autophagy process and of a Th17 adaptive response. The analysis of surgical specimens showed increased expression of 16 genes in inflamed tissue compared to non-inflamed internal controls and revealed the activation of immune-adaptive Th17 response in association with a Th1 response.

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Furthermore, an increased expression of genes involved in ionic transport and signal transduction was found in inflamed mucosa compared to non-inflamed internal controls. This study confirms the activation of Th17 and Th1 adaptive-immune response also in colonic CD. It should be stressed that these responses have been disclosed in biopsy tissue, while only Th17 differentiation is revealed in endoscopic tissue. Interestingly, the polymorphisms analysis revealed that a homozygous genotype is associated to a more complicated clinical course.

Introduction

Crohn's disease (CD) is a chronic relapsing condition characterized by a transmural inflammation that can potentially affect any segment of the gastrointestinal tract. It predominantly involves the terminal ileum and colon, and often results in complications such as strictures, perforations and fistulae [1]. The disease affects any age groups, but typically occurs in young adults. Based on predominant characteristics, CD expressions can be assigned to three general phenotypes: stricturing, perforating or inflammatory [2]. Although genetic, immunological and environmental factors are involved in the pathogenesis of CD, aetiology is still unknown and no causal treatment is available. Studies performed on twins and members of the same family confirmed a significant correlation between genetic factors and CD occurrence. Approximately half of monozygotic twins and one third of children of two affected parents develop this disease [3].

International collaborative research, focusing on an unbiased appraisal of the human genome, has been particularly successful in identifying genes and genetic loci that contribute to CD susceptibility [4]. Analyses of the genes and genetic loci involved in CD show several pathways that have a key role in intestinal homeostasis, including barrier function, epithelial antimicrobial defence, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, endoplasmic reticulum stress and main metabolic pathways [5].

Multiplex Gene Assay (QuantiGene Plex 2.0) based on Luminex xMAP Technology is a sandwich branched-chain DNA assay that utilizes cooperative hybridization. The analysis has been chosen because the quantification of mRNA from tissue is direct to specifically capture target mRNA transcripts onto labeled xMAP beads [6]; the resolving power of this technique is large, allowing detection up to 32 genes in simultaneous analysis, thus leading to a single and very detailed phenotypic description for each patient [7].

In this paper, we used Multiplex Gene Assay to analyze differences in gene expression in tissue samples from endoscopies and surgical biopsies of colon mucosa from CD patients. A panel of genes, directly or indirectly related with CD, as suggested by Genome-Wide Association (GWA) studies for Immune Bowel Disease (IBD) and some susceptibility genes shared with different diseases were studied [8].

In particular, we studied:

- the expression of *HAMP, SLC40A1* which are involved in iron metabolism;
- the expression of *IL23R, CCR6, IL17A, IL17F, IFNG, MYD88, TNSF15* which regulate the differentiation of T helper (TH)-1 or TH-17 lymphocytes, *MYD88 CARD15, ATG16L1, CARD14* involved in innate recognition of pathogens and in autophagy;
- *JAK2, STAT3, LRRK2* which modulate cell signalling;
- *BMP2 ESR1* which are involved in bone metabolism and osteoporotic processes;
- *JAK2, HAMP* which are possible target genes for future biological therapies;
- *SLC22A4, SLC22A5* which regulate metabolism of organic cations;
- *ABCB1* which protect the intestinal epithelium against xenobiotics;
- *MICA* which is involved in the interaction with gamma delta T-cells of the intestinal immune system;
- *DEFB4, CAMP, HAMP* defensins which are involved in innate immune response against pathogens;
- *DLG5* which maintains the structure of intestinal epithelium, informing about the status of integrity of epithelial cells.

Materials and methods

Patients

Seven patients affected by colonic CD were recruited at the Gastroenterology and Endoscopy Units of the IRCCS-CSS Hospital at S. Giovanni Rotondo (Italy) (Table 1a). Diagnosis of colonic CD was performed by ileocolonoscopy Seven patients, affected by colonic CD and programmed for surgery, were recruited at the Unit of Surgery, AOUC, University of Florence (Table 1b). For these patients, CD diagnosis was performed by clinical/endoscopic criteria and confirmed by histological analysis. Table 1 shows clinical characteristics of the 14 patients.

Tissue samples from inflamed and non-inflamed colonic areas were obtained from each patient either in endoscopic procedures either in the course of surgery; non-inflamed colonic samples were used as internal controls.

All samples were stored in RNA later (Qiagen, Germany) before homogenization. Peripheral blood was collected in EDTA, and subjected to genomic DNA extraction (QIA amp DNA Blood Maxi Kit, Qiagen GmbH, Hilden, Germany) and genotyping to analyze polymorphisms of relevant genes: the three principal polymorphisms of the gene *CARD15/NOD2* (R702W, G908R, and 1007fs) and some polymorphisms of genes *ATG16L1, IL23R, LRRK2*. We developed a microarray

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Table 1 a: clinical characteristics of colonic CD patients undergone endoscopy (endoscopies).

Patient	Pt1a	Pt2a	Pt3a	Pt4a	Pt5a	Pt6a	Pt7a
Localization of CD[a]	L2	L2—L4	L2	L1—L2	L3	L1—L2	L3—L2
Age of CD onset (years)	22	27	55	35	18	42	22
I° Surgery/relapse	I° Surgery	Relapse	I° Surgery	No	I°Surgery	No	No
Disease behavior[b]	B1	B3	B2—B3	B1	B2	B3	B1
Therapy[c]	M, C	M, C	B	B	M, B	M	I
Smoking status[d]	No	Ex-S	No	No	10 cig/day	No	5 cig/day

b: clinical characteristics of colonic CD patients undergone surgery (surgical specimens)

Patient	Pt8b	Pt9b	Pt10b	Pt11b	Pt12b	Pt13b	Pt14b
Localization of CD[a]	L2	L3	L1—L2	L3	L2	L3	L2
Age of CD onset (years)	16	27	24	34	18	28	36
I° Surgery/relapse	I° Surgery	Relapse	I° Surgery	I° Surgery	I° Surgery	I° Surgery	Relapse
Disease behavior[b]	B2	B2, B3	B2, B4	B2	B2, B4	B2, B3	B2
Therapy[c]	B, Ab, C	C, M, I, Ab	I, M	C, B	Ab, M	C	I, Ab
Smoking status[d]	18 cig/day	No	15 cig/day	No	No	Ex-S	No

[a] Localization of CD. L1: terminal ileum, L2: colon, L3: ileum colon, L4: upper G (gastrointestinal).
[b] Disease behavior. B1: nonstricturing/nonpenetrating, B2: stricturing, B3: penetrating, B4: perianal disease.
[c] Therapy. M: mesalazine, I: immunosuppressant, B: biologics, C: corticosteroids, Ab: antibiotics.
[d] Smoking status. Current smoke: no. cigs-day/no. year. Ex-S: ex-smoker.

panel of 24 genes involved in CD pathogenesis and evaluated the co-expression of these genes in samples of inflamed and non-inflamed colon mucosa obtained from endoscopical and surgical specimens of colonic CD patients. Table 2 reports the panel of the investigated genes, the accession number, the number of Mendelian Inheritance in Man (MIM) (used as a reference) and their respective encoded product and function. To facilitate the interpretation of the results, we designed a panel of 26 genes (24 genes which values of expression is normalized by two housekeeping genes) divided in four categories according to their biological function:

- transport across epithelia: SLC40A1, ABCB1, SLC22A5, SLC22A4, HAMP;
- immune response: CCR6, IL-17A, IL-17F, STAT3, MICA, MYD88, IL-23R, JAK2, IFNG, NOD2;
- antimicrobial activity: *CAMP, DEFB4, HAMP, LRRK2*;
- physiological activities: STAT3, LRRK2, TNFSF15, CARD14, ATG16L1, ESR1, BMP2, DLG5.

Multiplex Gene Assay

The mRNA quantization for the genes listed in Table 2 was performed with a Multiplex Gene Assay (Quantigene 2.0, Affymetrix, CA, USA), as previously described [7]. The mRNA expression for *Ccr6, Tnfsf15, Il-17A, Stat3, Card14, Slc40a1, Abcb1, Slc22a5, Atg16l1, Esr1, Il-17F, Bmp2, Card15, Mica, Myd88, Il-23r, Slc22a4, Defb4, Jak2, Dlg5, Lrrk2, Hamp, Camp, Ifn-γ, Actb* (high expression housekeeping gene), *Hptr1* (low expression housekeeping gene), was measured using the QuantiGene multiplex assay (Panomics, Fremont, CA, USA). Samples were lysed after treatment in lysis mixture; mRNA expression in lysates was detected and measured according to manufacturer's instructions. Briefly, samples were frozen in RNA later (Qiagen, Germany). Each sample was weighed and the appropriate lysis solution was added to a final volume of 150 μL containing 50% Lysis Mixture (Panomics) and 1 g/L Mixture (Panomics) and 1 g/L proteinase K. The mixture was shaken at 65 °C for 30 min to lyse the cells. The lysate was stored at −80 °C for later use.

A panel of oligonucleotide capture probes, each with a unique sequence of 15 bases, was covalently linked to carboxylated fluorescently encoded beads (Luminex, Bio-rad, Massachusetts, USA). We mixed each sample lysate diluted at 1:1 and 1:2 with the pooled capture beads in a round-bottom assay well and hybridized for 16 hours at 54 °C (final volume in each well was 100 μL). The assay mixture was transferred to a MultiScreen filter plate (Millipore, Billerica, MA, USA), and unbound material was filter-washed from the wells by rinsing 3 times with wash buffer. The plate was then hybridized at 54 °C for 1 h with 100 μL/well of bDNA amplifier in Amplifier Diluent (Panomics). After the plate was filter-washed twice with wash buffer and incubated at 50 °C for 1 h with 100 μL/well of 5'-dT (Biotin)-conjugated label probe (Panomics) diluted in Label Probe Diluent (Panomics). After 2 washes, streptavidin-conjugated R-phycoerythrin diluted in SA-PE diluent (20 mmol/L Tris-HCl, 400 mmol/L lithium chloride, 1 mL/L Tween 20, 1 mL/L bovine serum albumin, and 5 mL/L Micr-O-protect) was added, and the plate was shaken and incubated at room temperature for 30 min. We washed the beads to remove unbound SA-PE and then analysed them with Bio-Plex 200 system (Bio-Rad). The SA-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads. Expression of target-specific RNA molecules was calculated as the mean values from triplicate cultures and normalized against Actin gene (high expression housekeeping gene).

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Table 2 Panel of the selected genes investigated in our study.

Symbol	Complete name	Group	Accession number	MIM	Gene product function/s	Reference
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Low expression housekeeping gene	M26434	308000	It plays a central role in the generation of purine nucleotides, chosen as a low expression housekeeping gene	Fedrigo et al., 2010
ACTB	Actin beta provided	High expression housekeeping gene	M28424	102630	Is involved in the cell motility, structure, and integrity	Vandesompele et al., 2002
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member1	1	AF215636	604653	Exports iron from duodenal epithelial cells	Benyamin et al., 2015
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1	M14758	171050	Transports various molecules across extra- and intra-cellular membranes. It belongs to a protein sub-family involved in multidrug resistance	Brant et al., 2004
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5	1	AF057164	603377	Transports several small organic cations in the liver, kidney, intestine. It is involved in elimination of drugs and environmental toxins	Peltekova 2004
SLC22A4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	1	AB007448	604190	Polyspecific transporter of organic cations in the liver, kidney, intestine, and involved in the elimination of these molecules	Girardin et al., 2012
CCR6	Chemokine (C-C motif) receptor6	2	U68030	601835	Induces B-lineage maturation and antigen-driven B-cell differentiation	Fransen et al., 2014
IL17A	Interleukin 17A	2	U32659	603149	Produced by Th17-type CD4+ cells. Regulates the activities of NF-kB and mitogen-activated protein kinases	Pelletier 2010
IL17F	Interleukin 17F	2	AF384857	606496	Produced by Th17-type CD4+ cells Stimulates the production of other cytokines, including IL6, IL8. It also inhibits angiogenesis by endothelial cells	Ueno et al., 2015
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	2—4	BC014482	102582	Activates transcription of cell growth and apoptosis' genes as responses to inflammation	Noble et al., 2010; Nguyen et al., 2015
MICA	MHC class I polypeptide-related sequence A	2	L14848	600169	Acts as a stress-induced antigen broadly recognized by intestinal intra-epithelial gamma delta T cells	Allez et al., 2007; Muro et al., 2014
MYD88	Myeloid differentiation primary response gene (88)	2	U84408	602170	Acts as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways	Cosmi et al., 2014
IL23R	Interleukin 23 receptor	2	AF461422	607562	Expressed on Th17 cells. Involved in the IL23A signaling pathways with the receptor molecule IL12RB1/IL12Rbeta1	Naser et al., 2012

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Table 2 (*Continued*)

Symbol	Complete name	Group	Accession number	MIM	Gene product function/s	Reference
JAK2	Janus kinase 2	2	3717	147796	Is involved in cytokine receptor signaling pathways and is required for responses to gamma interferon	Danese et al., 2016
IFNG	Interferon, gamma	2	3458	147570	It encodes a cytokine with antiviral, immunoregulatory and anti-tumor properties and activates macrophages	Tuller et al., 2013
CAMP	Cathelicidin antimicrobial peptide	3	BC055089	600474	It is an antimicrobial protein (defensin)	Koczulla and Bals, 2003; Koczulla et al., 2003
CARD15	Nucleotide-binding oligomerization domain containing 2	2	AF178930	605956	Induces immune response to intracellular bacterial by recognizing the muramyl dipeptide (MDP)	
DEFB4	Defensin, beta 4A	3	AJ314835	602215	Acts as an antibiotic peptide locally regulated by inflammation	Wehkamp et al., 2009; Gersemann et al., 2008
HAMP	Hepcidin antimicrobial peptide	1/2/3	AF309489	606464	It is involved in iron transport, antimicrobial, defence and inflammatory responses	Verga Falzacappa and Muckenthaler, 2005; Mleczko-Sanecka et al., 2010
LRRK2	Leucine-rich repeat kinase 2	3/4	AK026776	609007	It is involved in autophagy and implicated in clearance of intracellular bacteria	Gardet et al., 2010; Schapansky et al., 2014
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	4	AF039390	604052	Induces apoptosis in endothelial cells	Bamias et al., 2003
CARD14	Caspase recruitment domain family, member 14	4	AF322642	607211	Regulates the molecular scaffolding process and activates NF-kappa B	Tsoi et al., 2012; Bertin et al., 2001
ATG16L1	ATG16 autophagy related 16-like 1	4	AK000897	610767	Induces autophagy processes involved in degradation of cell organelles	Parkes, 2012
ESR1	Estrogen receptor 1	4	X03635	133430	Involved in the metabolic pathway of the hormones and in several diseases including osteoporosis	Aguirre et al., 2007
BMP2	Bone morphogenetic protein 2	4	650	112261	Induces bone and cartilage formation	Cejalvo et al., 2007
DLG5	Discs, large homolog 5	4	U61843	604090	It encodes for scaffolding molecules involved in cell-cell contact and in the maintenance of epithelial cell integrity. Its products are also involved in the transmission of extracellular signals	Stoll et al., 2004

MIM: Mendelian inheritance in man; 1: transport across epithelia; 2: immune response; 3: antimicrobial activity; 4: different physiological activities.

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Polymorphism analysis

Genomic DNA was extracted from peripheral blood leucocytes of all CD patients and healthy controls by a standard non-enzymatic method, using the QIAamp DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany). In addition, DNA samples from 70 healthy Caucasian subjects (140 alleles) were analysed as controls. Three coding exons of the *CARD15/NOD2* gene, of the associated with the three main single-nucleotide polymorphisms (SNPs) are as follows:

- exon 4, R702 W (C2104T);
- exon 8, G908R (G2722C);
- exon 11, 1007fs (3020insC).

These coding exons were amplified by PCR using pairs of primers derived from the published sequence of the gene (available upon request). These three main variants associated with susceptibility to CD, represented 32, 18, and 31%, respectively, of the total CD mutations.

Direct sequencing of PCR amplified products (SNPs rs87950, rs127951 and rs137955) of the *CARD15/NOD2* gene was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The samples were analysed in an ABI Prism 310 (Applied Biosystems) Genetic Analyzer and sequence variations were confirmed analysing newly amplified fragments and sequencing both DNA strands. Samples were genotyped for the SNPs of genes *ATG16L1, LRRK2, IL-23R* (in particular we considered the rs7517847 and rs11209026 for *IL-23R* rs2241880 for *ATG16L1*, rs11564258 and rs3761863 for LRRK2) using 5′exonuclease TaqMan genotyping assays on an ABI Prism 7900 Real-Time polymerase chain reaction (PCR) System, according to manufacturer's instructions (Applied Biosystems, Foster City, CA).

Statistical analysis

Statistical analysis was performed using SSPS software (SPSS, Inc, Evanston, IL). All comparisons of genes mRNA quantization in tissues (inflamed and non-inflamed areas) were performed by Wilcoxon test. Data are reported as mean and ranges unless otherwise stated. A P value of < 0.05 was accepted as statistically significant.

Results

Expression of CD susceptibility genes in the inflamed colon

The simultaneous expression of 24 genes involved in the CD pathogenesis was studied in colonic endoscopic biopsies ($n = 7$) and surgical specimens ($n = 7$) from patients with Crohn's colitis. Gene expression in inflamed colonic mucosa was compared to that of non-inflamed mucosa collected from the same patient.

Gene expression in endoscopic samples

The analysis of data obtained from endoscopic samples revealed a significant increase in mRNA level of 7/24 genes compared to control tissues (Fig. 1, panel A). In particular significant differences between inflamed and non-inflamed mucosa were found in the expression of molecules and receptors related to TH17 adaptive response as interleukin 17 A (*Il-17A*, $P = 0.018$), Interleukin 17 F (*Il-17F*, $P = 0.028$), Interleukin 23 Receptor (*Il-23r*, $P = 0.043$) and defensin beta 4 (*Defb4*) ($P = 0.018$). Significant differences were also found in the expression of Autophagy Related 16-like1 (*Atg16l1*, $P = 0.028$) Janus Kinase 2; (*Jak2*, $P = 0.028$) and Leucine-Rich Repeat Kinase 2 (*Lrrk2* $P = 0.018$). The level of mRNA for the Defensin Beta4 (*Defb4*) showed the greatest absolute value of expression, both in inflamed and non-inflamed areas.

Gene expression analysis in surgical specimens

The gene expression analysis in surgical specimens obtained from patients with Crohn's colitis revealed an increased expression of 15/24 genes in inflamed tissues compared to non-inflamed tissues. (Fig. 1, panel B). In particular the compared analysis of gene expression in inflamed and non inflamed mucosa revealed higher expression of genes involved in TH17 response as *Ccr6* ($P = 0.018$), *Il-17A* ($P = 0.018$), *Il-17F* ($P = 0.018$), *Il-23r* ($P = 0.018$) as well as in autophagy *Atg16l1* ($P = 0.018$), thus confirming the results obtained with endoscopic samples. Beside genes involved in TH17 response, we also found higher expression of *Ifnγ* ($P = 0.018$) suggesting the simultaneous activation of TH1 response and/or the presence of IFN-γ producing cells of innate immunity. According with the high inflammatory status of colonic mucosa from patients subjected to surgery we found increased expression of genes involved in iron transport/antimicrobial defense *Hamp* ($P = 0.028$), iron export from duodenal epithelial cells *Slc40a1*, ($P = 0.043$), signal transduction *Stat3*, ($P = 0.018$) in cytokine receptor signaling pathways *Jak2* ($P = 0.043$), multidrug resistance *Abcb1*, ($P = 0.018$), in elimination of drugs and environmental toxins *Slc22a5* ($P = 0.018$) and finally in cell—cell contact and in the maintenance of epithelial cell integrity *Dlg5* ($P = 0.018$).

In addition, the products of these 15 genes are also involved in the transmission of extracellular signals *Dlg5* ($P = 0.018$).

The remaining genes (*SLC22A4, MYD88, MICA, CAMP, TNFSF15, CARD14, ESR1*) exhibited no significant differences of mRNA levels, but their values might be taken into account to assess which metabolic pathway is active at that time of the investigation.

Detection CARD15, ATG16L1, LRRK2, IL-23R polymorphism

To investigate the influence of genetic factors [7] on the quantitative expression of the immune-related genes, we sequenced DNA samples obtained from peripheral blood of all patients for the presence of polymorphic genes, frequently associated to CD as *CARD15* [9], *ATG16L1* [10], *LRRK2* [11,12], *IL-23R* [13,14].

All CD patients included in this study, either donors of endoscopic biopsies or of surgical specimens, showed of at least one polymorphism in heterozygosis, suggesting the involvement of genetic factors in the dysregulated expression of the reported genes. Table 3 shows the complete list

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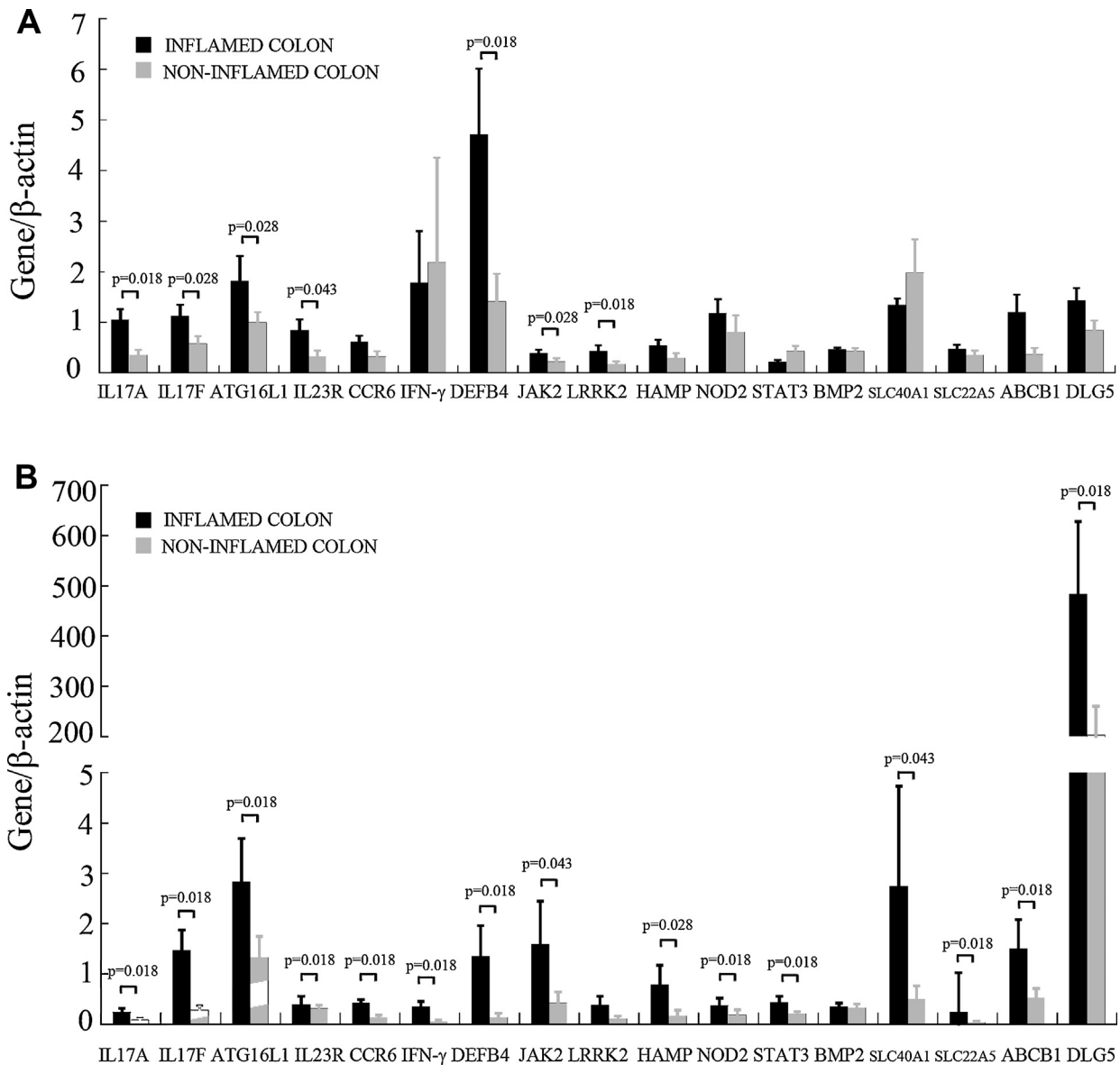

Figure 1 Quantitative evaluation of gene expression using Multiplex gene Assay (Quantigene 2.0) in endoscopic tissues (Panel A) and surgical specimens (Panel B) of CD patients. The abscissas shows mRNA levels of the genes compared to controls; the axis of the ordinates shows the value of expression of the gene normalized to the housekeeping actin (gene / β actin ratio).The first seven genes of both panels (A and B) are more closely related to immunity. P value is reported only when statistically significant ($P < 0.05$).

of polymorphisms found in our CD cohort as well as the details of the analysed SNPs. Interestingly, matching clinical features of patients (Table 1) with SNPs status (Table 3), we observed that patients with a homozygous and double-heterozygous genotype underwent to a more complicated clinical course of CD, since they were affected with a surgical recurrence of CD.

Discussion

Current hypothesis for CD pathogenesis emphasizes the loss of immunological tolerance and the altered permeability of epithelial mucosa as responsible for an abnormal inflammatory immune response against antigens commonly found in the intestinal lumen. In this way, a chronic inflammatory process is established [15]. These observations, combined with the presence of family history for the disease [16], leads to assume that the pathogenesis of CD is the result of the interaction of at least three factors:

- genetic susceptibility [17];
- immunological priming by the enteric microflora [18];
- tissue damage mediated by the immune system [19].

The loss of tolerance and the consequent adaptive-immune response are amplified in the presence of defects in mechanisms of early removal of invading bacteria. In particular, dysfunction of autophagy caused by genetic/phenotypic variations within CD susceptibility genes, such as *ATG16L1* and *NOD2/CARD15* functions have

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Table 3 List of SNPs in patients undergone endoscopy (a) and surgery (b).

a. Endoscopic tissue

	SNP	Pt1a	Pt2a	Pt3a	Pt4a	Pt5a	Pt6a	Pt7a
CARD15	rs 87950	wt	wt	wt	wt	wt	wt	wt
	rs 127951	wt	wt	wt	wt	hz	wt	wt
	rs 137955	wt	wt	wt	wt	hz	wt	wt
IL23R	rs 7517847	wt	hz	wt	hz	wt	wt	wt
	rs 11209026	wt	hz	wt	wt	wt	wt	wt
ATG16L1	rs 2241880	wt	wt	hz	wt	hz	hz	hz
LRRK2	rs 11564258	wt	hz	wt	wt	wt	wt	wt
	rs 3761863	hz	homo	hz	wt	hz	wt	hz

b. Surgical specimen

	SNP	Pt8b	Pt9b	Pt10b	Pt11b	Pt12b	Pt13b	Pt14b
CARD15	rs 87950	wt	wt	wt	wt	wt	wt	wt
	rs 127951	hz	homo	wt	wt	wt	wt	wt
	rs 137955	hz	wt	wt	wt	wt	wt	wt
IL23R	rs 7517847	wt	wt	wt	hz	wt	wt	hz
	rs 11209026	wt	wt	wt	wt	wt	wt	wt
ATG16L1	rs 2241880	hz	homo	hz	wt	hz	hz	wt
LRRK2	rs 11564258	wt	wt	wt	wt	wt	hz	wt
	rs 3761863	hz	hz	wt	wt	hz	hz	hz

wt: wild type; hz: heterozygous; homo: homozygous; Pt: patient.

been involved [20,21]. Genome-Wide Associations studies [22] are very useful for the identification of susceptibility genes; however, only studies of co-expression which potentially capture the inflammatory process in its complexity, can show homeostasis alteration relevant in the CD pathogenesis.

In this study, we have investigated through Multiplex Gene Assay the expression profile of selected genes involved in immune-inflammatory response in endoscopic biopsies and surgical specimens from patients with colonic CD. In order to achieve a complete assessment of immune homeostasis in inflamed tissue, we collected also non-inflamed colonic mucosa samples for each patient. (Fig. 1A, B) The use of Multiplex Gene Assay allowed us to obtain a detailed analysis of immune-inflammatory events arising in the course of the disease. In particular, the involvement of a TH-17 response was strongly suggested by the dysregulated expression of *CCR6, IL-17A, IL-17F,* IL-23R found in all samples either from endoscopy either from surgery. The involvement of TH-1 mediated cellular response is suggested by the higher expression of *INF-γ* in inflamed mucosa from patients subjected to surgery (Fig. 1B).

The nature of T helper cell development in CD has been an area of discussion for several years. The predominating hypothesis has been that the adaptive-immune response in CD is dominated by Th1 cells [23]. However in the last years, the identification of Th17 as a unique subset of T helper cells has challenged this view, and today some authors argue that the adaptive-immune response in CD is dominated by Th1 and Th17 cells [24].

The use of Multiplex Gene Assay allow us to detect significant higher expression of *Il-23r, Il17-A*, and *Il-17F* genes compared to the respective controls (not inflamed tissue), strongly supporting the involvement of Th17-mediated adaptive response in the pathogenesis of Crohn disease [25]. As Th17 cells produce *Il17A* and *Il17F*, whose genes are co-expressed on the same chromosomal region [26,27], the presence of Th17 in CD gut tissues examined is suggested not only by expression of both *Il-17A, Il-17F* mRNA, but also by the expression of *Il-23r* mRNA, a membrane receptor expressed by CD4+ cells which drives Th17 differentiation [28,29].

As further proof of a TH17-mediated response, the Defensin beta 4 gene was strongly increased compared to non-inflamed tissues in both type of samples. Defensin beta 4 is a typical antimicrobial peptide, whose production by epithelial cells is strongly induced by Th17 effector T cells through the combined production of Il-17 and Il-22. Colonic *Hbd2* was dysregulated at mRNA and protein level in CD [38]. A higher expression of the Hepcidin gene (*Hamp*) and of solute carrier family 40 member 1 (*Slc40a1)* gene was also found in inflamed mucosa compared to non-inflamed tissues but it was limited to surgical samples. These genes code for antimicrobial peptides typically produced in the context of a Th17 response [30] strongly involved in regulating iron availability at mucosal levels and in preventing pathogen colonization [31]. The high expression of molecules involved in iron transport as hepcidin and *Slc40a1* might be responsible for the down-regulation of iron cell release, leading to hyposideremia (the so-called ''anaemia by inflammation'') [32] so that the use of drugs anti-hepcidin could be helpful in the treatment of *anemia* in patients with chronic inflammation. In this regard, it has been determined that hepcidin binding to ferroportin leads to the binding and activation of the protein Janus Kinase2 [33], therefore also in this way *JAK2* appears to be a possible target for CD pharmacological treatment [34]. Numerous clinical trials have recently demonstrated that inhibitors of *JAK2* are

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safe and efficacious, as they inhibit the entire metabolic pathway [35].

Despite the numerous evidences for the role of Th17 cells, these cells are unexpectedly rare in the tissues and biological samples of patients with chronic inflammatory disorders [36]. One possible explanation may be that Th17 cells show a transient phenotype, resulting from their tendency to shift into Th1 cells in the context of inflammatory microenvironments. Th17 cells rapidly shift into the Th1 phenotype in the presence of IL-12 and/or TNF-α as well as by the fact that they possess self-regulatory mechanisms limiting their own expansion [37,38]. In our case, it is a great expression of the Ccr6 gene, a surface determinant expressed by TH17 lymphocytes [39] was found only in inflamed mucosa from surgical specimens of CD patients. In the same specimens, we also evidenced high expression of Ifn- γ gene, a finding which may suggest the differentiation of TH17 lymphocytes [30] to TH1 cells, that underscores the progression toward a more aggressive and more pathogenic phenotype compared to the TH17 unshifted cells [37]. These Th17-derived Th1 cells, named non-classic Th1 cells, were proposed as a possible target for the therapy of inflammatory disorders.

The differences that we have highlighted between the gene expression in biopsies from endoscopies and intraoperative tissue, reveal that the answer to the insult by pathogens is divided into a succession of phases that are represented by elements of the innate response in endoscopic tissue of the colon and the acquired immunity response in the deeper tissues such as intraoperative biopsies.

From this study, we expect that these genetic insights will transform the landscape of common-complex diseases; we might characterize them better for therapeutic purposes if we can ''isolate'' the set of genes specific for each disease [8].

Conclusions

In conclusion, the use of advanced Multiplex Gene Assay technologies allowed to confirm the relevance of the immune-inflammatory response mediated by Th17 related molecules in the pathogenesis of colonic CD. Data obtained in surgical specimens also suggest that TH-17 cells may progress toward a more aggressive TH-1 phenotype in advanced stages of the disease. Overall, our findings provide a further rationale for the use of antibody treatment against Il-23 with anti-Il-12Rbeta1 and anti-Il-23R antibodies in CD patients [40,41].

Ethics approval and consent to participate

This study was approved by EC of AOUC of Florence, on May 2nd 2011, no. protocol 2011/0016888, rif. 95/10, authorization Gen Dir 17/572011 no. protocol 2011/0018055 and written informed consent was obtained from all study subjects.

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Disclosure of interest

The authors declare that they have no competing interest.

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