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Efficiency of nanovaccines compared with antibiotic therapy in the prevention and immunomodulation of listeriosis

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ABSTRACT

Although the main antimicrobial therapy for adult and neonatal listeriosis is ampicillin, prophylactic treatments are currently lacking. We compared the efficiency of nanovaccines with prenatal or postnatal ampicillin treatments in pregnant female mice during the development of neonatal listeriosis. Nanovaccines prepared with gold nanoparticles and listeriolysin O (LLO) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) peptides and inoculated into pregnant females, prevented neonatal listeriosis and the appearance of comorbidities, better than pre or postnatal ampicillin treatment. Evaluation of the ability of nanovaccines as immunomodulators of blood cells in patients with listeriosis, revealed the strong and specific immunogenic capacity of the use of GAPDH nanovaccines as putative immunotherapies in listeriosis.

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KEYWORDS Gold glyconanoparticles; glyceraldehyde-3-phosphate-dehydrogenase; adjuvants; nanovaccines; antibiotics

Introduction

Human listeriosis is a food-borne invasive infectious disease caused by the bacterial pathogen *Listeria monocytogenes* (LM) that can cause serious clinical syndromes such as bacteremia, sepsis, meningoencephalitis, or neonatal meningitis. Listeriosis has become an increasingly common health problem in Spain, where the number of cases per year has a notoriously increase between 2012 and 2016 according to the European Union health authorities, with the highest proportion of hospitalized cases of all zoonoses under surveillance by European agencies (96.5%) [1,2]. More complete studies of listeriosis in Spain covering from 2000 to 2021, which included the large outbreak in 2019 in Andalusia, concluded that the overall in-hospital mortality rate in adults with listeriosis, increased twofold because of the growing proportion of elderly and immunosuppressed patients [3]. Analysis of pregnancy-related listeriosis in Spain during the same period also revealed significant increases in obstetric listeriosis, neonatal listeriosis and adverse fetal-neonatal outcomes [4]. Antibiotic treatment is the only measure used to fight against listeriosis, which requires long-term treatment of hospitalized patients for approximately one month. In this context, live stock and processed foods appear to be sources of antibiotic resistance, especially to amoxicillin-clavulanate and chloramphenicol but not to ampicillin [3–7].

Several listeriosis outbreaks have occurred in the last 15 years in the north of Spain [8–10], but the most severe outbreak occurred in the summer of 2019 in Andalusia, southern Spain, and was caused by stuffed pork due to a failure in the production process [11]. Two groups of individuals were especially affected by listeriosis. Adults with solid or blood-related tumours in response to chemotherapeutic treatment and neonates born to women infected during the pregnancy. Treatment for listeriosis

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involves a combination of high doses of ampicillin, with or without intravenous gentamicin or penicillin G, for at least 21 days. Pregnant women with fever and confirmation of *L. monocytogenes*-positive blood culture results are treated intravenously with high doses of ampicillin for at least 14 days, to avoid miscarriage or severe fetal infection [12,13]. In elderly individuals, listeriosis is associated with a case fatality rate of 17% [10,13] despite treatment with antibiotics, which can be explained by the bacteria invading the central nervous system (CNS). We conceived two types of immunotherapies to decrease these high mortality rates in listeriosis, prophylactic and therapeutic vaccines. For this purpose, we have prepared gold glyconanoparticles coupled to *L. monocytogenes* peptides (here named as nanovaccines) [14]. First, we compared the efficiency of ampicillin with that of nanovaccine therapies in experimental neonatal listeriosis. Second, to design putative immunotherapies for patients with listeriosis with antibiotic therapies, we must examine the ability of nanovaccines to induce innate and specific immune responses under these conditions. Therefore, we isolated blood monocyte-derived dendritic cells (MoDC) from selected patients with listeriosis in our two hospitals under antibiotic treatment or not and evaluated the capacity of the nanovaccines to function as immunomodulators of MoDC.

Materials and methods

Mice, peptides, bacteria, and evaluation of ampicillin susceptibility in vitro and in vivo

We used female C57BL/6, CBA/J and BALB/c mice at 8–10 weeks of age from Charles-River as resistant, intermediate and highly susceptible mouse strains to *Listeria monocytogenes* [15,16]. Glyceraldehyde-3-phosphate dehydrogenase from L. monocytogenes, here GAPDH in the whole manuscript, ($GAPDH_{1\text{-}22}$) or listeriolysin O ($LLO_{91\text{-}99}$) peptides were synthesized by GeneScript (Piscatawya, NJ, USA) at a peptide purity ≥ 98% by HPLC. The *L. monocytogenes* clinical isolate HUMV05 (S. Microbiology, Hospital U. Marqués de Valdecilla, Santander, Spain) was used for our experiments (serotype 4b, sequence type CC1, hypervirulent) [10,17]. To evaluate ampicillin susceptibility, we defined two concentrations – the noninhibitory concentration (NIC) as the highest ampicillin concentration not affecting bacterial growth at 24 h of incubation, and the minimal inhibitory concentration (MIC) of ampicillin as the lowest concentration of ampicillin that completely prevented bacterial growth of the clinical *L. monocytogenes* isolate HUMV05. We used the broth microdilution method and *Streptococcus pneumoniae* ATCC 49619 as the quality control strain according to a previously described method [12]. In brief, a bacterial culture of HUMV05 on BHI agar was cultured for 24 h at 37°C. This suspension was adjusted by turbidometry to 0.5 McFarland followed by a 1/10 dilution to obtain an inoculum of 1×10^7 CFU/mL. The final volume of the microtitre plates was 100 µL per well, and the ampicillin log2 dilutions ranged from 0.0016 to 4 mg/L. Next, 100 µL of the bacterial inoculum was added to the wells to obtain a final concentration of 5×10^5 CFU/mL, followed by incubation at 37°C for 24 h. Optical density of each well at 600 nm was measured using a plate reader. The MIC was determined as the lowest concentration of ampicillin that completely inhibited the visible growth of *L. monocytogenes* (*in vitro* analysis). Similarly, the NIC and MIC were determined in triplicate in mice after log2 dilutions of ampicillin in BHI broth, which ranged from 0.0016 to 4 mg/mL, followed by the addition of 5×10^3 CFU/mL of HUMV05 *L. monocytogenes* in a total volume of 100 µL and inoculation of the mice via the lateral tail vein (*i.v.*). The mice were sacrificed 24 h postinoculation, and the spleens were collected, homogenized, and plated on BHI agar plates. CFU/mL were counted and compared to those of controls without any antibiotics (*in vivo* analysis). All experiments were performed in triplicates ± SD.

Patients and listeriosis cases

Listeriosis was confirmed in fetuses and stillborn or newborn infants according to the Commission Decision of 28/IV/2008 and classified as listeriosis (S. Microbiology, Hospital U. Donostia, San Sebastian, Spain), and ampicillin susceptibility was also evaluated as described previously [12]. Adult listeriosis was diagnosed by bacteria recovered from blood, ascitic fluid, stool or urine and confirmed as *L. monocytogenes* by serotyping and MLST genotyping (S. Microbiology, Hospital U. Marqués de Valdecilla, Santander, Spain and S. Microbiology, Hospital U. Donostia, San Sebastian, Spain) [18]. Sera and PBMCs from pregnant mothers with listeriosis and adult listeriosis and healthy controls were stored at the IDIVAL biobank.

Nanovaccine synthesis, formulations and toxicities

To obtain gold nanoparticles (GNP) carrying the $GAPDH_{1\text{-}15}$ peptide (GNP-GAPDH) or $LLO_{91\text{-}99}$ peptide (GNP-LLO), we performed a previously reported procedure [19]. Chemical preparation of GNP conjugated to peptides is performed by reduction in situ of Au(III) salt with sodium borohydride as previously described and the lack of toxicity was evaluated in dendritic cells (DC) by a 100% cell viability and 1-2% promotion of apoptosis, while in mice evaluating the amount of IL-1 in serum less than 16 pg/mL

[19]. The amount of $GAPDH_{1-15}$ or LLO_{91-99} peptides on the GNP was estimated to be ~50 µg/mg GNP. DIO-1 (2 µg/mouse), a Toll-like receptor 2 and 4 (TLR2/4)-targeted molecule [20], was used as an adjuvant for the nanovaccine formulations (DIO-1 is the brand name given by the company to this adjuvant).

Neonatal listeriosis and clinical tests for vaccination and ampicillin therapies

On day 9 of gestation (E9), two groups of pregnant C57BL/6 female mice (n = 5) were vaccinated intravenously (*i.v.*) via the lateral tail vein with 5 µg of GNP-LLO or GNP-GAPDH/mice formulated with DIO-1 as described previously [17]. All groups of pregnant mice, except controls (NI), were *i.v.* inoculated at the embryonic stage (E16) via the lateral tail vein with 5×10^3 CFU/mouse of *L. monocytogenes* HMVU05 in 100 µL of saline (LM-NT). At embryonic stage E17, a group of pregnant mice (n = 5) was *i.v.* inoculated with ampicillin (10.2 mg/kg/day) every 12 h until the first day of birth (P1). At P1, a group of pregnant mice (n = 5) was *i.v.* inoculated with ampicillin (10.2 mg/kg/day) every 12 h until sacrifice at P4. All the animals were examined every day. At P4, all born neonates were killed to obtain the brains and other organs. The brains were weighed, homogenized, and plated for CFU examination on blood agar plates. Mothers were also sacrificed at P4, and serum was obtained to examine cytokines and anti-LLO and anti-GAPDH antibodies.

Clinical tests were performed at P4 on all pups born to mothers infected or not infected. Metric tests of health conditions were as follows: Weight (g), length (mm), movement assays in which pups were placed on a 10-cm metric paper and movements were recorded after 5 min (cm) and results express as % of movement, and skin appearance as wrinkled or flattered.

Immunochemistry

Neonates were sacrificed on P4 and immersed in 4% formalin for cryopreservation of complete bodies. Organs commonly affected by listeriosis, such as the brain, lungs, and blood vessels commonly [16,17], were resected, sectioned, and fixed by immersion in 4% formaldehyde for 24 h. Organs were embedded in paraffin and cut into 3 µm thick sections for histological analysis. Sections of each organ were stained with hematoxylin and eosin (HE) and analyzed independently by two pathologists. For immunohistochemical analysis, EnVision technology (Dako) was used, and samples were treated by staining the endothelial vascular cells with primary monoclonal anti-CD31 antibody biotinylated followed by streptavidin conjugated with horseradish peroxidase (EnVision Mouse HRP, Dako) as previously described [17], quantification values are expressed as percentages of CD31 cells. Blood vessels were observed in whole brains with magnifying lens and classified as few (70-90% than controls) or normal (≤ 5% of controls). Diaminobenzidine (Dako) was used as the chromogen. Immunochemical analysis of necrotic-apoptotic cells was performed by TUNNEL staining and performed according to manufacturer instructions (Roche) and presented as percentages of apoptotic cells.

MoDC from healthy donors and patients with listeriosis and cytokine analyses

Leukocytes from whole blood cells were collected from the interphase of Ficoll gradients to obtain PBMCs, analyzed by flow cytometry to calculate percentages of neutrophils (PMN), lymphocytes and monocytes (Mo) and stored in liquid nitrogen at 10×10^6/mL in complete medium supplemented with 10% human AB serum (Corning, Thermo Fisher Scientific) as previously described [10]. To obtain monocyte-derived dendritic cells (MoDC), PBMCs were thawed and monocytes (Mo) were positively selected as $CD14^+$ positive cells by passing through MACS^{TM} columns (Miltenyi, Bergisch Gladbach, Germany) and differentiated into MoDC for 7 days with GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) by standard procedures as previously reported [10,17], which were confirmed as nonactivated MoDCs by flow cytometry as 98% $CD45^+ CD11c^+ HLA\text{-}DR^{+/-} CD86^- CD14^-$ cells (antibodies used were: anti-HLA-DR-FITC, anti-CD45-PerCP, anti-CD11c-APC, anti-CD14-PE and anti-CD86-V450). MoDC from healthy donors (CONT) or patients with listeriosis were incubated with GNP-LLO or GNP-GAPDH nanovaccine formulations (50 µg/mL in the presence of 2 µg/mL of DIO-1) for 16 h and next infected with LM-HUMV05 strain. MoDC activation was verified by flow cytometry, as 99% of the $CD45^+ CD11c^+ HLA\text{-}DR^+ CD86^+ CD14^-$ cells and supernatants were collected, filtered to remove bacteria and stored at −80°C until use. Cytokines were quantified in mouse serum using a Luminex 200 platform with a magnetic system (Milliplex MAP Mouse High Sensitivity T cell magnetic bead panel, EMD Millipore Corporation, Billerica, MA, USA) following the manufacturer´s instructions. The cytokine concentrations are expressed as the means of three replicates in pg/mL ± SD. Human cytokines IL-10 and IL-12 released into MoDC supernatants were quantified using a multiparametric Luminex kit (Milliplex human HSTCMAG-28SK) following the manufacturer´s instructions. Results are expressed as the mean cytokine concentration (pg/mL) ± SD.

In vitro assays of MoDC infected with clinical L. monocytogenes isolates

A set of MoDC from healthy donors were prepared as nonactivated MoDC (phenotype of 98% $CD45^+$ $CD11c^+$ $HLA\text{-}DR^{+/-}CD86^-CD14^-$ cells) or activated MoDC with GNP-GAPDH nanovaccine formulations (50 μg/mL in the presence of 2 μg/mL of DIO-1) (phenotype of 99% of the $CD45^+CD11c^+HLA\text{-}DR^+$ $CD86^+CD14^-$ cells). Next, nonactivated or activated MoDC were infected with clinical *L. monocytogenes* isolates of each patient at a multiplicity of infection, MOI, of 20:1 (bacteria: cells) for 16 h as described previously [10] and control patients (CONT) were infected with LM-HUMV05 clones. The results are expressed as CFU/mL ± SD ($^*P \leq 0.05$).

ELISA for anti-LLO and anti-GAPDH antibodies

$GAPDH_{1\text{-}22}$ and $LLO_{189\text{-}201}$ peptides (50 μg/mL) that contained MHC class II epitopes, were coated onto 96-well plates in carbonate buffer (pH 8.0) overnight at 4°C and used to evaluate antibodies in the serum of pregnant mice or in listeriosis patients after a 1/10 dilution following a previously reported procedure [10,17,19]. Reactions were developed with HRP-conjugated goat anti-human IgG or goat anti-mouse IgG, and the absorbance was analyzed at 450 nm. The results are presented as optical units (OD) and mean values ± SD of triplicate experiments ($p < 0.05$).

Statistical analysis and limitations of the study

For statistical analysis, Student's t-test was applied to all mice assays infected with *L. monocytogenes* HUMV05 and for the *in vitro* assays of MoDC infected with LM since the sample sizes were > 4. Two groups of 5 mice per condition were inoculated, and a *P* value <0.05 was considered to indicate statistical significance. ELISA analyses were performed in triplicate. GraphPad Prism software was used to generate all the graphs presented. The limitations of this study implied a short cohort of listeriosis patients (only 8 patients); however, this selection corresponds to half of the patients during 2013–2015 in HUMV and HUD institutions and includes all groups at high risk of listeriosis. A greater cohort should be used in the future to verify all the analyses and statements performed here with human samples. Such a putative study might collect patients from at least 5 different health institutions to assure a minimal of 50 listeriosis patients.

Ethics statement

This study was carried out in accordance with the Guide of Care and Use of Laboratory Animals for the Spanish Ministry of Science and Innovation. The Committee on the Ethics of Animal Experiments of the University of Cantabria approved the protocol (Permit Number: PI-01-17), which follows the Spanish legislation (RD1201/2005). All animal culling was performed by cervical dislocation, and all efforts were made to minimize suffering. The use of human data and bacterial clinical isolates and the preparation of preclinical vaccines was approved by the Committee of Clinical Ethics of Cantabria (CEm), including Informed Consent and General Project Information documents to patients (Permit Acta Number18/2020, internal code: 2020.259). The study also has the approved by the Research Ethics Committee of UNIR (code PI:003_2025).

Results and discussion

Vaccines serve as prophylactic or therapeutic tools against pathogens, preventing bacterial dissemination or inducing immune responses that help destroy them. Protein-based nanoparticles are vaccine platforms with features such as versatility, low toxicity, directionality, long half-lives, low cost and large-scale production and storage under refrigeration or room temperature [21,22]. Our group has designed prophylactic vaccines for experimental listeriosis using gold nanoparticles (GNP) conjugated with the LLO peptide $LLO_{91\text{-}99}$ or the GAPDH peptide $GAPDH_{1\text{-}15}$, named here GNP-LLO or GNP-GAPDH nanovaccines, for simplicity. These nanovaccines confer protection against mouse listeriosis [17,19] but also have other therapeutic effects on solid tumours [23].

Comparison of ampicillin and vaccination therapies to block neonatal listeriosis

The normal dose of ampicillin for pregnant women 100–300 mg/kg/day [5,7,10,22,24] should be adjusted to 1–2 mg/kg/day for mice as mice weight is at least 1000-fold lower. The *L. monocytogenes* (LM) HUMV05 clinical isolate presented good ampicillin susceptibility, with a NIC of 0.09 mg/L and an MIC of 0.185 mg/L (Figure 1(A)), a range similar to the MIC ranges of clinical isolates detected in the north of Spain from 2010 to 2020 (≤ 0.12–1 mg/L) [12]. Therefore, the use of two doses of 10.2 mg/kg/mouse ampicillin, one every 12 h, seemed adequate to mimic human ampicillin therapies for a neonatal listeriosis model in pregnant female mice (Figure 1(B)). Our experiments analyzed 6 different conditions: (1) no bacterial infection and no treatment (NI), (2) LM inoculation and no treatment (LM-NT), (3) LLO nanovaccines and LM inoculation (GNP-LLO/LM), (4) GAPDH nanovaccines and LM inoculation (GNP-GAPDH/LM), (5) prenatal ampicillin treatment and LM inoculation (LM/Antb prenatal)

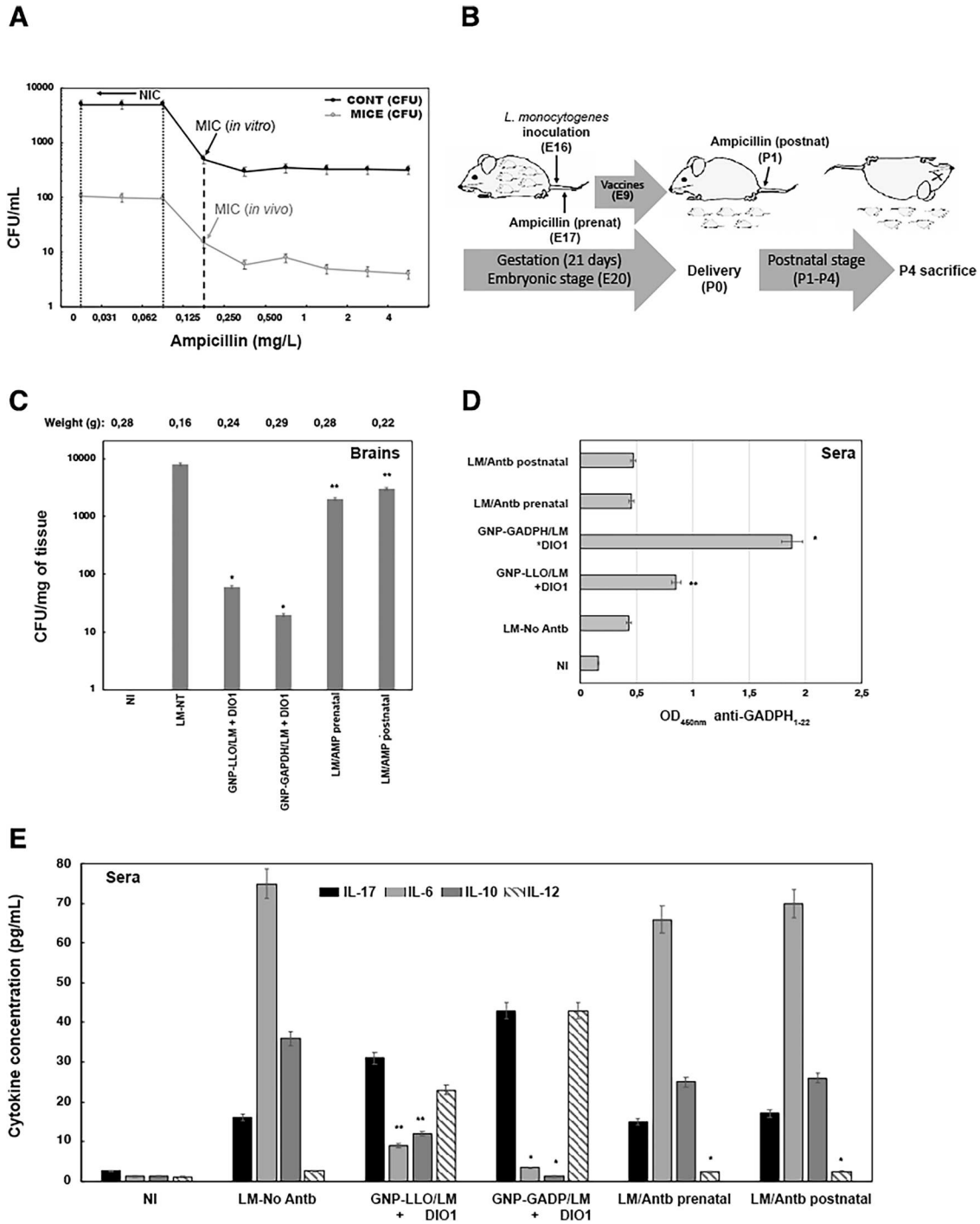


Figure 1. Effects of nanovaccines and ampicillin therapies on bacterial dissemination in neonates and on the immunocompetence of pregnant females. (A) Ampicillin susceptibility of *L. monocytogenes* HUMV05 (LM) *in vitro* (black graph) and *in vivo* using female mice ($n = 10$) (gray graph) *i.v.* inoculated into the tail vein for 24 h with LM in saline supplemented with log2 dilutions of ampicillin, and the number of CFUs was determined in homogenized spleens after they were plated on BHI agar plates. The NIC and MIC for *in vitro* and *in vivo* analyses were 0,009 mg/L and 0,0185 mg/L, respectively, and the average weight of females was 0,018 kg. (B) Model graphic of planned experiments at different embryonic stages: Vaccination with GNP-LLO or GNP-GAPDH nanovaccines was performed at E9, prenatal ampicillin treatment was administered at E17 and postnatal ampicillin treatment of neonates at P1. Infection with LM occurred at E16, and finally, all neonates and pregnant females were sacrificed at P4. (C) Bacterial burden in P4 neonates born to noninfected and nontreated females (NI), nontreated but LM-infected females (LM-NT), vaccinated with GNP-LLO and LM infected (GNP-LLO/LM), vaccinated with GNP-GAPDH and LM infected (GNP-GAPDH/LM), prenatally treated with ampicillin and LM infected (LM/AMP prenat) or postnatally treated with ampicillin and LM-infected (LM/AMP postnat) was measured in weighted brains (upper numbers in graphic) after homogenization and plating on BHI agar plates. Weight values of mice are the following: NI (0,28 g); LM-NT (0,16 g); GNP-LLO (0,24 g); GNP-GAPDH (0,29 g); LM/AMP prenatal (0,28 g); LM/AMP postnatal (0,22 g). The results are expressed as the mean ± SD CFU/mL (*$P \leq 0.05$, **$P \leq 0.01$). (D) Anti-GAPH1-22 antibodies in the serum of pregnant females treated as described in (C) were measured by peptide ELISA. The results are presented as the mean ± SD of the values of OD450 nm units from triplicate experiments. (*$P \leq 0.05$, **$P \leq 0.01$). (E) Levels of cytokines in the serum of pregnant females as described in (C) and (D). Cytokines were measured with a multiparametric Luminex 200 platform (EMD Millipore Corporation). The results are expressed as pg/mL of each cytokine ± SD of triplicate samples (*$P \leq 0.05$, $P \leq 0.01$).

and (6) LM inoculation at E16 and postnatal ampicillin treatment at P1 (LM/Antb postnatal) (Figure 1(C–E)). CFU recovery in the brains of the pups born to GNP-LLO- and GNP-GAPDH-vaccinated pregnant mice was 80-90% lower percentages than that in the control (LM-NT). However, CFU recovery in the brains of pups born to mothers treated with ampicillin (pre-natal treatment) or after born (post-natal treatment), was only 6-10% lower percentages than that in the control (LM-NT) (Figure 1(C)). We concluded that GNP-GAPDH nanovaccines were the most effective therapy and that compared to any ampicillin treatment, both nanovaccines were better at preventing LM access to the brains of neonates.

Effective vaccines during pregnancy should implement the immunocompetence of pregnant mothers to avoid or decrease the possibility of infection with *L. monocytogenes*. For this purpose, several immune biomarkers relevant to listeriosis have been reported to be easily detected in the serum of pregnant mothers, with high titres of anti-GAPDH IgG antibodies indicating a good ability to elicit LM-specific immune responses and high Th1/Th2 ratios of cytokines, which are relevant to prevent bacterial dissemination [10,17,19,23,25,26]. Since IL-12 and IL-17 are Th1 cytokines involved in bacterial clearance while IL-6 and IL-10 are Th2 cytokines responsible for LM propagation [25], we evaluated the levels of these four cytokines in our experiments. Vaccination with GNP-GAPDH nanovaccines resulted in very high titres of anti-GAPDH antibodies, high levels of IL-12 and IL-17 and strong reductions in serum IL-6 and IL-10 levels in mothers (Figure 1(D,E)). In contrast, the results of the ampicillin treatments revealed low levels of anti-GAPDH antibodies and Th1 cytokines but high levels of Th2 cytokines.

Next, we evaluated the effect of nanovaccines and ampicillin therapies in listeriosis co-morbidities, after histological examinations of P4 neonates, and immunostaining of endothelial vascular cells with anti-CD31 antibodies. Neonates born to LM infected mothers presented high bacterial dissemination into the brains as detailed in Figure 1(C) (bars expressing CFU/mg of tissue) and a reduction in brain weight (upper values in Figure 1(C)), verified by low cellularity after HE staining of the brain sections (compared NI-NT, GNP-LLO/LM and GNP-GAPDH/LM with LM-NT purple staining in Figure 2(A)), significant necrosis (70% of apoptotic-necrotic cells in LM-NT images compared to NI-NT in Figure 2(A)) and very low vascularization (10% of CD31 positive cells in LM-NT images of the brains compared to 65-75% of CD31 cells in those of noninfected controls, NI-NT, as shown in Figure 2(A)). Neonates also showed a lack of movement (2% of movement in LM-NT mice compared to 95-100% movement in NI-NT) with difficulties breathing, which correlated with retarded fetal development, collapsed lungs lacking alveoli and a reduction in blood vessels (2-5% of CD31 cells in lungs of LM-NT images compared to NI images in Figure 2(B)). Other comorbidities included sickness, gray and wrinkled skin, a nondistended stomach, and bones that were not consistent and mostly cartilaginous (data not shown). Pups born to mothers vaccinated with GNP-GAPDH showed normal morphologies in their brains and lungs (0,9%) of apoptotic cells, similar to those of noninfected mice (NI-NT), with normal vascularization of both organs (70% of CD31 cells in GNP-GAPDH/LM images in Figure 2(A,B)). Pregnancy vaccination with GNP-LLO resulted in only a small decreased in pup brain cellularity (1,5% of apoptotic cells in GNP-LLO/LM images in Figure 2(A)), normal lungs and normal numbers of blood vessels in the brains and lungs (55% of CD31 positive cells in GNP-LLO/LM images in Figure 2(B)). Prenatal ampicillin treatment resulted in some necrosis in the brain with an inflammatory infiltrate (40% of apoptotic cells in LM/Antb prenat images in Figure 2(A), the black arrow indicates the infiltrate in the brain), and postnatal ampicillin treatment resulted in prominent necrosis of the brain (68% of apoptotic cells in LM/Antb postnat images in Figure 2(A), red arrows). Vascularization of the brain was reduced by both ampicillin treatments (3-5% of CD31 positive cells in Figure 2(A), CD31 immunostaining), and the lungs of the neonates were immature (HE staining in Figure 2(B)). Other comorbidities were also observed in response to postnatal ampicillin treatment, such as the slow movement of pups (3% of movement), a decrease in the number of blood veins (3% of CD31 positive cells), and a decrease in the weight of the brain (Figure 1(C), 0,22 g *vs*. 0,28 g in upper weight values). Only vaccination with GNP-GAPDH and GNP-LLO nanovaccines prevented severe comorbidities in neonatal listeriosis. Prenatal ampicillin treatment seemed better than postnatal therapy, although severe effects on the brain were not completely avoided.

Effects of nanovaccines as immunomodulators in human samples

Listeriosis affected not only neonates but also immunocompromised adults, that might not respond well to antigens in vaccine formulations [27], thus, we envision that therapeutic vaccines might serve as immunomodulators for listeriosis patients, as they show good results for other acute and chronic bacterial infections [28,29]. Therefore, we examined the efficiency of GNP-LLO and GNP-GAPDH nanovaccines as immunomodulators in terms of their ability to activate MoDCs and examined with two parameters: (i) MoDC production of IL-12 and IL-10 after LM infection, which are proinflammatory and

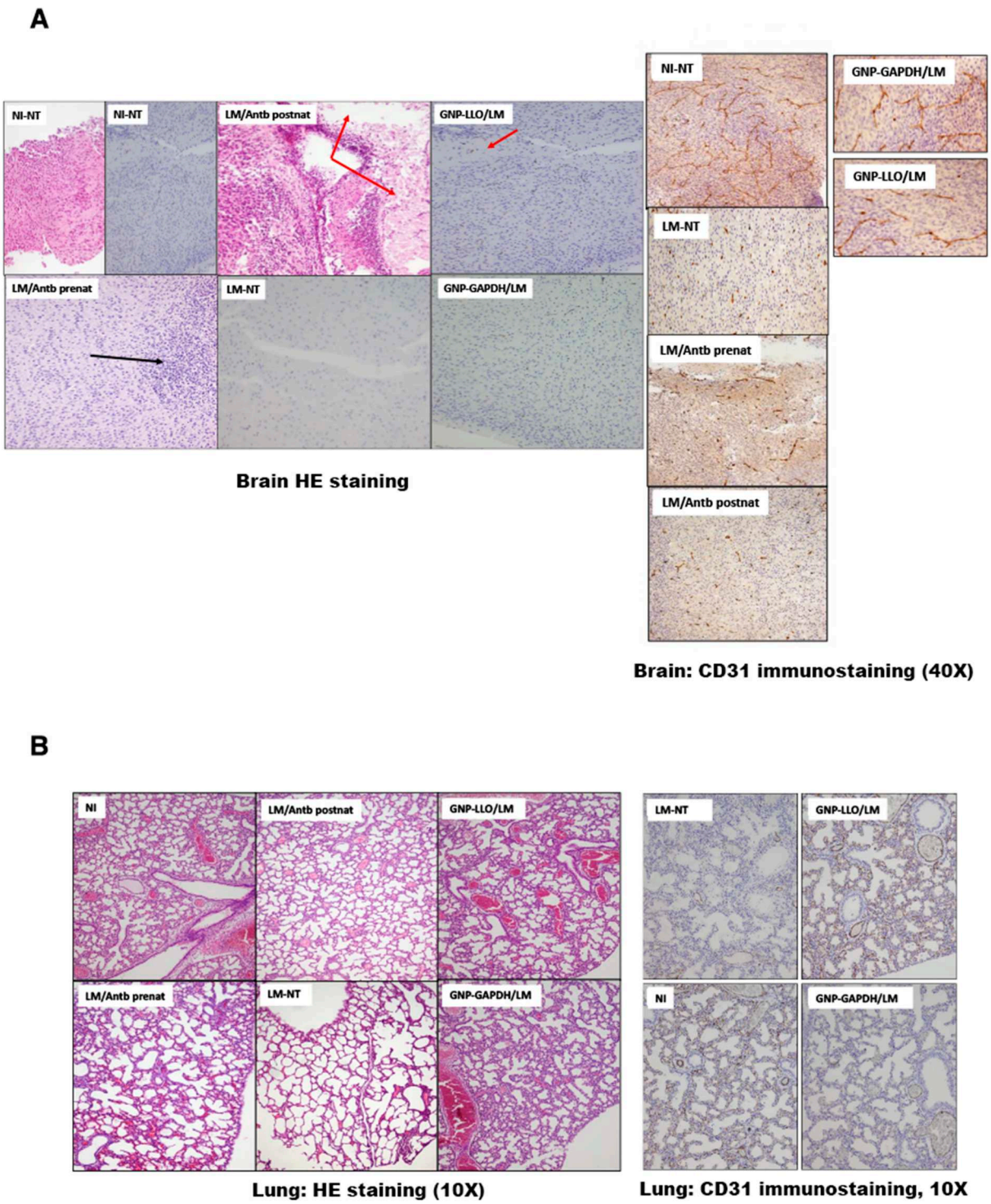


Figure 2. Effects of nanovaccine and ampicillin therapies on listeriosis comorbidities. (A) Left images show HE staining of brain sections from P4 neonates born to NI-NT, LM infected mothers without any treatment (LM-NT), GNP-LLO/LM or GNP-GAPDH/LM-vaccinated and LM infected, LM/Antb prenat LM-infected and ampicillin treated pregnant females or LM/Antb postnat LM-infected mothers and ampicillin-treated P1 neonates. Right images correspond to immunohistochemical staining of the CD31 marker in brain sections from mice subjected to some treatments NI-NT, LM/AMP prenat or LM/Antb postnat. (B) Left images show the HE staining of lung sections from P4 neonates born to pregnant females as described in (A). Right images correspond to immunohistochemical staining of CD31 marker in lung sections of the NI-NT, LM-NT, GNP-LLO/LM and GNP-GAPDH/LM samples.

anti-inflammatory cytokines, respectively, and (ii) the capacity of MoDC to clear an LM infection after 16 h. For this purpose, we selected eight adult patients with listeriosis from a 2013–2015 retrospective study and requested permission to use serum and PBMCs to differentiate them from MoDC before analysis PBMC and serum samples were stored at the IDIVAL biobank (Permit Acta Number18/2020, internal code: 2020.259). This selection involved eight listeriosis patients, five patients from HUD and three from

HUMV, and eight healthy controls from the IDIVAL biobank (CONT, referred to as the mean ± SD of eight different samples) (Figure 3(A)). Four listeriosis patients were not treated with antibiotics-three pregnant mothers of HUD and a patient with severe prostate cancer of HUMV. The other four patients with listeriosis were all oncologic patients with squamous cell glottis carcinoma (HUD001), multiple myeloma (HUD006), lung and bladder carcinoma (HUMV09) and glioblastoma multiforme (HUMV13) who were treated with different antibiotics as follows: Ampicillin (AMP), ampicillin combined with gentamicin (AMP + GENT), levofloxacin (LEVO) or amoxicillin combined with clavulanic acid (AMOX + CLAV), respectively.

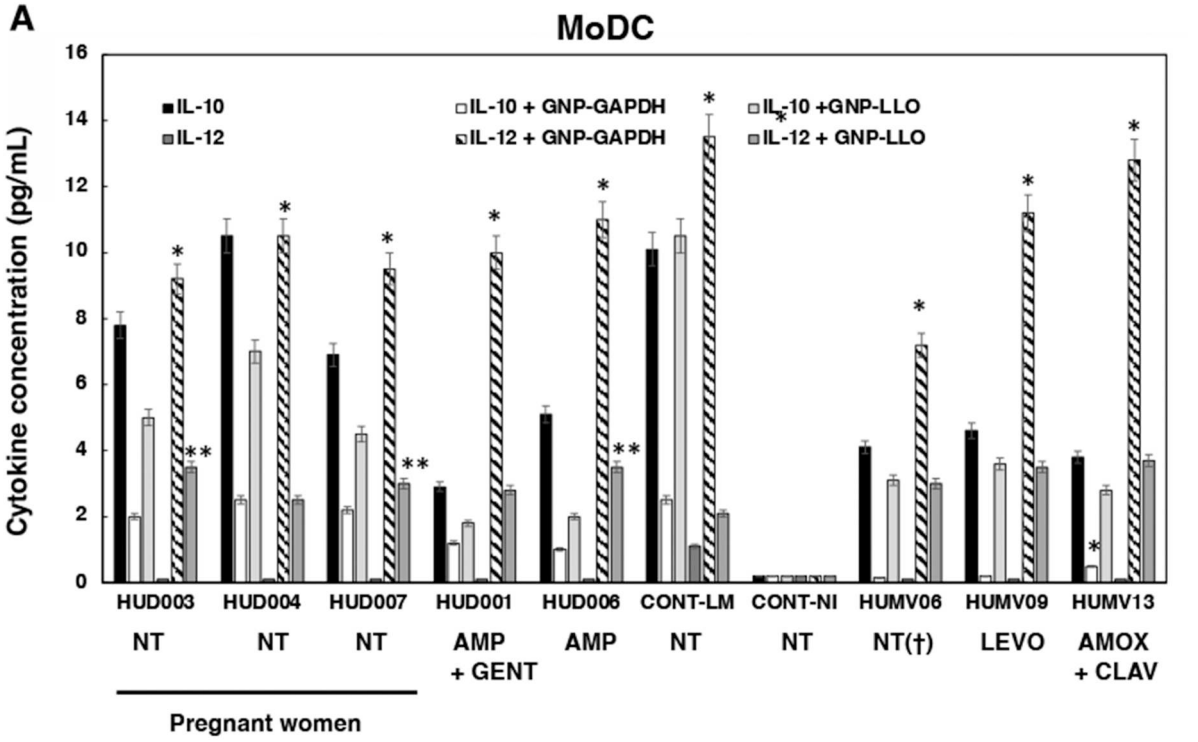
First, we explored the ability of GNP nanovaccines to serve as immunomodulators for MoDC, evaluating the production of IL-12 (proinflammatory) versus that of IL-10 (anti-inflammatory) after LM infection (HUMV05 hypervirulent strain). LM infection is characterized by high levels of IL-10 and low levels of IL-12 in MoDC that are not activated (compare black and dark gray bars in CONT-LM and NT of Figure 3(A)) [10,17,19,26]. MoDC activated by different immunomodulators in the presence or absence of adjuvants such as DIO-1 are known to shift the cytokine pattern toward low levels of IL-10 and high levels of IL-12, which is correlated with significant killing of LM [10,17]. The results shown in Figure 3(A) indicated that GNP-GAPDH nanovaccines in the presence of DIO-1 served as good modulators of MoDC, independent of the pathology or the antibiotic therapy (CONT-LM compared to different patients with listeriosis, Figure 3(A)) (DIO-1 alone cause no activation: CONT-NI samples). We observed that GNP-GAPDH nanovaccines caused a 4-fold reduction in IL-10 levels (black and white bars in Figure 3(A)) and a 9.5-fold increase in IL-12 levels (dark gray and striped bars in Figure 3(A)) in most listeriosis and control MoDC samples. GNP-LLO nanovaccines caused medium levels of MoDC activation, with a 2-fold reduction in IL-10 levels and a 2-fold increase in IL-12 levels. These results strongly suggest that GNP-GAPDH nanovaccines are excellent pro-inflammatory immunomodulators of MoDC for all adults who are healthy or at high risk of listeriosis, cancer patients and pregnant women.

Next, we explored the ability of MoDC of healthy volunteers preincubated with or without GNP-GAPDH, as shown in Figure 3(A), to clear infection with each LM clinical isolate. MoDC of the controls were infected with HUMV5 hypervirulent strain of LM (#CONT file in Figure 3(B)). We first observed that LM clinical isolates of oncologic patients, presented 200–1000 higher CFU/mL values after MoDC infection (ranging from 3.9×10^5 to 1×10^6 CFUs/mL) than clinical isolates of pregnant mothers (ranging from 1×10^2 to 1.8×10^3) (CFU/mL MoDC column in Figure 3(B)), suggesting that LM clinical isolates from oncologic patients were more virulent than that of pregnant mothers ($1{,}5 \times 10^2 \pm 16$ in the #CONT file of Figure 3(B)). MoDC pre-incubated with GNP-GAPDH and DIO-1 reduced the CFU/mL values to almost no bacteria at all in all MoDC samples, with values ranging from 10 to 50 CFU/mL (CFU/mL MoDC + GNP-GAPDH column in Figure 3(B)). These results confirmed the activity of GNP-GAPDH nanovaccines as versatile MoDC immunomodulators, that seems to induce MoDC microbicidal capacity and pro-inflammatory cytokines.

DCs participate in both innate and specific immunity in listeriosis [30]; since serum levels of anti-GAPDH LM antibodies are reported to correlate with the induction of specific cellular immune responses [10], we evaluated the levels of anti-LLO and anti-GAPDH antibodies in the serum of the patients with listeriosis selected for this study. We observed that the serum of oncologic patients with listeriosis had worse serum anti-GAPDH antibodies levels (OD values $\leq$0.8) than pregnant and LM-infected mothers did (OD values $\geq$1.0), while the serum anti-LLO antibody levels were not significantly different in any of the patients with listeriosis. Innate immune responses in listeriosis depend on the function of phagocytes, both neutrophils (PMN) and monocytes (Mo) [10,30,31]. Blood tests of our selection of listeriosis patients (Table 1) indicated that pregnant women presented PMN and Mo numbers (HUD003, HUD004, HUD007) and HUD007 in Table 1 only slightly higher than controls (CONT in Table 1), and normal values of lymphocytes. However, oncologic patients showed very high PMN numbers, with some patients presenting immature granulocytes (HUD007 labelled as * in Table 1), lower values of Mo and very low numbers of lymphocytes. Therefore, it seems that innate phagocyte-dependent immune responses are more deficient in cancer patients than in pregnant women. We concluded that compared with pregnant women, cancer patients with listeriosis presented a much worse immunological picture, as innate immunity evaluated by functional PMN and Mo, and cellular immunity, as determined by serum levels of anti-GAPDH-LM and total number of lymphocytes, decreased.

Our group has performed two epidemiological analyses of listeriosis in Spain during the past two decades, focusing on two groups: Pregnant women and hospitalized patients who were immunosuppressed [3]. Our results revealed an increased fatality rate of patients hospitalized with LM among immunosuppressed patients, including patients with solid tumours [32]. In the case of neonatal listeriosis, our results indicated that the number of cases has increased in the last 20 years in Spain, with a

A



B

Patient Code	Admission & discharge date	Type of infection	Complex clones-serotypes	Patient type/other treatment (evolution)	Listeriosis treatment*	CFU/mL (MoDC)	CFU/mL (MoDC + GNP-GAPDH)**	Anti-LLO antibodies	Anti-GAPDH antibodies
HUD003	18/02/2014-27/08/2014	Bacteremia	CC87-1/2b	Pregnancy-abortion at 13th week/NT	NT	$1.0 \times 10^2 \pm 10$	$0.1 \times 10^2 \pm 10$	0.43 ± 0.1	2.20 ± 0.2
HUD004	16/11/2013-27/08/2014	Bacteremia	CC1-4b	Pregnancy-Cesarean-2nd twin lost/NT	NT	$1.2 \times 10^2 \pm 12$	$0.1 \times 10^2 \pm 12$	0.32 ± 0.1	2.00 ± 0.1
HUD007	22/08/2015-27/08/2015	Corioamnionitis	CC1-4b	Pregnancy-Cesarean/NT with premature neonate	NT	$1.8 \times 10^3 \pm 11$	$0.4 \times 10^2 \pm 11$	0.15 ± 0.1	1.51 ± 0.1
HUD001	09/11/2013-20/08/2014	Bacteremia	CC4-4b	Squamous cell glotis carcinoma/IMRT + cisplatine	Ampicilin	$1.0 \times 10^6 \pm 39$	$0.5 \times 10^2 \pm 39$	0.14 ± 0.1	0.74 ± 0.1
HUD006	10/11/2014-14/11/2014	Bacteremia, meningitis	CC4-4b	Multiple myelome IgGλ /Lenalidomide, velcade, dexametaxone	Ampicilin + gentamicin	$0.8 \times 10^6 \pm 28$	$0.3 \times 10^2 \pm 28$	0.25 ± 0.1	0.38 ± 0.2
HUMV06	25/88/2014 - 27/08/2014	Bacteremia, Exitus	CC213-4b	Prostate adenocarcinoma with metastasis in bones and liver	NT*	$3.9 \times 10^5 \pm 26$	$0.1 \times 10^2 \pm 26$	0.13 ± 0.1	0.04 ± 0.1
HUMV09	23/08/2015-24/08/2015	Bacteremia	CC87-1/2b	Lung and bladder carcinoma	Levofloxacin	$8.3 \times 10^5 \pm 25$	$0.2 \times 10^2 \pm 25$	0.11 ± 0.1	0.72 ± 0.2
HUMV13	21/03/2015-22/03/2015	Bacteremia	CC26/1/2a	Glioblastoma multiforme	Amoxicillin + clavulanic acid	$8.5 \times 10^5 \pm 25$	$0.2 \times 10^2 \pm 25$	0.10 ± 0.1	0.23 ± 0.1
#CONT	-	NONE	-	HEALTHY	-	$1.5 \times 10^2 \pm 16$	$0.1 \times 10^2 \pm 20$	0.10 ± 0.1	0.10 ± 0.1

*No antibiotic treatment but treatment with taxocel
**Virulence calculated in control MoDC at 16 hours post-infection with each patient clinical LM isolate. CONT patients were infected with LM-HUMV05, an hypervirulent strain.
***CONT refers to mean values of eight control and healthy fellows obtained from IDIVAL biobank from the same period of time (2013-2015), non-hospitalized and pre-treated or not (#) with GNP-GAPDH for 16 hours before infection.

Figure 3. Effects of nanovaccines as immunomodulators on the MoDC of patients with listeriosis. (A) MoDC from patients with listeriosis (HUD003, HUD004, HUD007, HUD001, HUD006, HUMV06, HUMV09, HUMV13) or healthy donors (CONT) (phenotype of 98% $CD45^+ CD11c^+ HLA\text{-}DR^{+/-} CD86^- CD14^-$ cells) were incubated with GNP-LLO or GNP-GAPDH nanovaccines (50 ng/mL + 2 µg/mL of DIO-1) for 16 hours. Next, MoDC were infected at a MOI of 20:1 (bacteria: cells) with LM-HUMV05 for 6 hours as previously described, except for the noninfected control (CONT-NI) [5]. The concentrations of cytokines in the filtered MoDC supernatants are expressed as the mean cytokine concentration (pg/mL) ± SD. (*$P \leq 0.05$). (B) Clinical parameters of eight selected cases of listeriosis with permission to obtain serum and PBMCs. HUMV codes corresponded to Hospital U. Marques de Valdecilla cases, and HUD codes corresponded to Hospital U. Donostia-San Sebastian. In a different experiment, MoDCs from healthy volunteers were infected with each LM clinical isolate from listeriosis patients (clones and serotypes are shown in Figure 3(B)) for 16 hours as described previously [5] and control patients were infected with LM-HUMV05 clones. The results are expressed as CFU/mL ± SD (*$P \leq 0.05$). Anti-LLO and anti-GAPDH antibody titres are expressed as OD_{450} nm units ± SD (*$P \leq 0.01$).

Table 1. Innate parameters of listeriosis patients.

Patient code	Patient type	Antibiotic	*PMN (42-75)	Lymph (20-51)	Mo (1-13)
CONT	Healthy volunteers	NT	42 ± 0,4	46 ± 0,5	12 ± 0,2
HUD003	Pregnancy-abortion	NT	52,5% ± 0,3	14 ± 0,3	8 ± 0,2
HUD004	Pregnancy-cesarean	NT	66,6 ± 0,5	23,3 ± 0,4	6,6 ± 0,1
HUD007	Pregnancy-cesarean	NT	67,5 ± 0,4	18,1 ± 0,3	8,6 ± 0,2
HUMV06	Prostate Adenocarcinome	NT	*55 ± 0,3	9 ± 0,2	5,1 ± 0,1
HUD001	Squamous cell glottis carcinoma	Ampicillin	76,1 ± 0,4	10 ± 0,3	6 ± 0,3
HUD006	Multiple myeloma IgG	Ampicillin + gentamicin	87 ± 0,5	5,8 ± 0,2	4,6 ± 0,2
HUMV09	Lung and bladder carcinoma	Levofloxacin	95 ± 0,6	3 ± 0,1	1,1 ± 0,1
HUMV13	Gliobastoma multiforme	Amoxicillin + clavulanic acid	89 ± 0,3	5,2 ± 0,2	4,7 ± 0,2

Results are expressed as percentages of cells ± SD. Blood tests correspond to routine assays indicating the percentages of neutrophils (PMN), lymphocytes (Lymph) and monocytes (Mo). Controls are healthy donors. Values in parentheses refer to ranges of normal values. *Patient with immature neutrophils.

substantial burden on fetal and neonatal outcomes, including miscarriages, fetal loss, stillbirths, neonatal deaths and prematurity [4]. Therefore, surveillance and prevention of human listeriosis are urgently needed in the growing population of patients with immunosuppression, especially cancer patients, and pregnant women, as these two populations are at the highest risk of listeriosis. In this context, our study here of listeriosis biomarkers that are easy to follow in blood tests of patients at risk, such as the number of functional phagocytes, the levels of anti-GAPDH antibodies and the Th1/Th2 cytokine ratios (IL-12/IL-10, respectively), might aid in the early diagnosis of listeriosis. Moreover, our finding that GNP-GAPDH nanovaccines efficiently activated the blood MoDC of these patients, validated these nanovaccines as novel immunodulators, opening a field where nanovaccines might serve not only for listeriosis prevention [31] but also as alternative therapies for patients with listeriosis, even in combination with antibiotic therapies.

Conclusions

Compared with the GNP-LLO nanovaccines, the GNP-GAPDH nanovaccines avoided bacterial dissemination and the appearance of comorbidities in neonates and provided benefits for the pregnant mothers through the implementation of proinflammatory Th1-Th17 cytokines while reducing the levels of anti-inflammatory Th2 cytokines. Prenatal or postnatal ampicillin treatments were much less effective at preventing bacterial dissemination or the development of severe comorbidities and did not benefit the immunocompetence of pregnant females. GNP-GADPH nanovaccines were also validated as efficient immunomodulators to activate the MoDCs of immunocompromised individuals who had listeriosis and who were receiving antibiotic treatment.

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CAD designed, helped with the execution of the experiments and analyses, coordinated, and directed the entire study, and wrote the paper. RCG and DSC performed the experiments with mice to collect organs for further analyses of serum and lymphocyte populations and analyzed the data. MM synthesized the gold nanoparticles coupled to *L. monocytogenes* peptides and NM performed analyses of the toxicity and pharmacokinetics of the nanovaccines in different cell types. MU helped with immunogenicity analyses in the mice. JF and JGR performed all the anatomopathological analyses of mouse organs, including the histochemical staining. JGOV and EGL performed all cytokine analyses of mouse and human sera and MoDC supernatants. JCM and JMM provided blood samples and clinical histories of the patients with listeriosis they had the custody of informed consent and helped with the writing and discussions. VS, EV and VMT helped with the writing and discussions. CAD thanks Prof. M. Fresno (CBMSO, Madrid, Spain) for the kind gift of DIO-1 adjuvant and Dr. J.M. Marimón (Servicio Microbiología, Hospital Universitario de Donostia, 20014 Donostia-San Sebastian, Gipuzkoa, Spain) for providing listeriosis samples. J. Navas (School of Medicine, Universidad de Cantabria, Santander, Spain) is acknowledged for critical review of the manuscript and suggestions.

Author contributions

CRediT: **Carmen Alvarez-Dominguez:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing; **Ricardo Calderón-Gonzalez:** Data curation, Formal analysis, Investigation, Methodology, Validation; **David Salcines-Cuevas:** Investigation, Validation; **Vicente Soriano:** Data curation, Formal analysis; **María Ubeda:** Data curation, Formal analysis; **Noemi Montoya:** Data curation, Formal analysis; **Jorge Calvo-Montes:** Data curation, Formal analysis; **Marco Marradi:** Data curation, Formal analysis; **Javier Freire:** Data curation, Formal analysis; **Javier Gomez-Roman:** Data curation, Formal analysis; **Elena Gonzalez-Lopez:** Data curation, Formal analysis; **Javier Gonzalo Ocejo-Vinyals:** Data curation, Formal analysis; **Jose Maria Marimon:** Data curation, Formal analysis, Methodology, Validation; **Elena Vazquez:** Data curation, Formal analysis; **Víctor Moreno-Torres:** Data curation, Formal analysis.

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