CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE GENÉTICA Y BIOLOGÍA MOLECULAR

"Maternal and fetal inflammatory responses associated with preterm birth and adverse neonatal outcomes"

Tesis que presenta

M. EN C. VALERIA GARCÍA FLORES

Para obtener el grado de

Doctora en Ciencias

en la Especialidad de

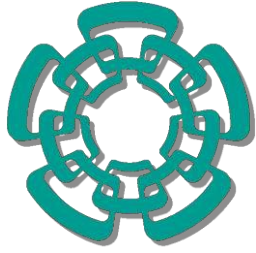**Genética y Biología Molecular**

Directores de la Tesis

Dr. Luis Marat Alvarez Salas
Dr. Nardhy Gomez-Lopez

Ciudad de México   Junio 2017

CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE GENÉTICA Y BIOLOGÍA MOLECULAR

"Respuesta inflammatoria materna y fetal asociada con el embarazo pretérmino y complicaciones neonatales"

Tesis que presenta

M. EN C. VALERIA GARCÍA FLORES

Para obtener el grado de

Doctora en Ciencias

en la Especialidad de

Genética y Biología Molecular

Directores de la Tesis

Dr. Luis Marat Alvarez Salas
Dr. Nardhy Gomez-Lopez

Ciudad de México   Junio 2017

OFFICIAL ACKNOWLEDGEMENTS

FUNDING

Index

RESUMEN

La inflamación materna y fetal produce efectos adversos en el embarazo y los neonatos. Por lo tanto, es crítico encontrar un tratamiento que pueda atenuar la respuesta inflammatoria materna y fetal. Mediante el uso de un modelo animal de inflamación materna sistémica (inyección intraperitoneal de lipopolisacárido o LPS) o de inflamación fetal (administración intra-amniótica de LPS), se encontró lo siguiente: 1) la inflamación sistémica induce resultados adversos en el embarazo y neonatos, debido a una respuesta severa de citocinas maternas y una respuesta leve de citocinas fetales; 2) la inflamación fetal induce resultados adversos en el embarazo y en neonatos debido a una respuesta leve de citocinas maternas y una respuesta severa de citocinas fetales; 3) exendin-4 mejoró modestamente la tasa de embarazo pretérmino en hembras con inflamación sistémica o fetal ; 4) el tratamiento con exendin-4 mejoró la tasa de sobrevivencia de neonatos nacidos de hembras con inflamación sistémica, pero no con inflamación fetal; 5) la inflamación sistémica facilitó la difusion de exendin-4 en el útero y la interface materno-fetal; 6) los neonatos nacidos de hembras con inflamación sistémica y tratadas con exendin-4, mostraron un perfil de citocinas similar al de los neonatos del grupo control; y 7) el tratamiento con exendin-4 en hembras con inflamación sistémica, tuvo efectos inmunomodulatorios al inducir una polarización e incremento de neutrófilos anti-inflammatorios, así como la supresión de la expansion de células T reguladoras CD8+ en los neonatos. En resumen, estos resultados proveen evidencia de que atenuando la inflammación sistémica materna a través de una novedosa intervención como es la administración de exendin-4, puede mejorar la calidad de vida en neonatos nacidos de mujeres con esta condición clínica.

ABSTRACT

Maternal and fetal inflammation leads to adverse pregnancy and neonatal outcomes. Therefore, finding an intervention that ameliorates maternal and fetal inflammatory responses is critical. Using animal models of systemic maternal inflammation (intraperitoneal injection of lipopolysaccharide or LPS) and fetal inflammation (intra-amniotic administration of LPS), it was found that: 1) systemic inflammation induced adverse pregnancy and neonatal outcomes by causing a severe maternal cytokine response and a mild fetal cytokine response; 2) fetal inflammation induced adverse pregnancy and neonatal outcomes by causing a mild maternal cytokine response and a severe fetal cytokine response; 3) exendin-4 treatment of dams with systemic inflammation or fetal inflammation improved adverse pregnancy outcomes by modestly reducing the rate of preterm birth; 4) exendin-4 treatment of dams with systemic, but not local inflammation, considerably improved neonatal outcomes, and such neonates continued to thrive; 5) systemic inflammation facilitated the diffusion of exendin-4 through the uterus and the maternal-fetal interface; 6) neonates born to exendin-4 treated dams with systemic inflammation displayed a similar cytokine profile to healthy control neonates; and 7) treatment with exendin-4 had immunomodulatory effects by inducing an M2 macrophage polarization and increasing anti-inflammatory neutrophils, as well as suppressing the expansion of CD8+ regulatory T cells, in neonates born to dams with systemic inflammation. Collectively, these results provide evidence that dampening maternal systemic inflammation through novel interventions, such as exendin-4, can improve the quality of life for neonates born to women with this clinical condition.

1. Introduction

1.1 Preterm birth

Preterm birth is one of the most common, yet harmful, obstetrical syndromes [1-5] and is the leading cause of perinatal morbidity and mortality worldwide [6-9]. Up to 70% of all preterm births are preceded by spontaneous preterm labor [1, 5, 10, 11], a syndrome comprised of multiple pathological processes [12]. While many putative causes are associated with spontaneous preterm labor, the only one that is causally linked to preterm birth is inflammation/infection [13-36]. Inflammation can be due to microorganisms (i.e. intra-amniotic infection) or danger signals derived from necrosis and cellular stress (i.e. sterile intra-amniotic inflammation) [37-50]. Systemically, intra-amniotic infection can be manifested as clinical chorioamnionitis, which refers to the presence of maternal fever associated with clinical signs (foul-smelling discharge and uterine tenderness as well as maternal and fetal tachycardia) and laboratory abnormalities such as leukocytosis [45, 51-54]. Locally, intra-amniotic infection is characterized by an increased white blood cell (WBC) count [55-60] and elevated concentrations of cytokines [43, 61] and lipid mediators (e.g. prostaglandins) [62-74] in the amniotic cavity. This local inflammatory response can indicate a systemic activation of the fetal innate immune system, a phenomenon referred to as fetal inflammatory response syndrome (FIRS) [75, 76].

1.2 Fetal inflammatory response syndrome

Clinically, FIRS is defined by elevated cytokines in the fetal plasma, such as IL-6 [77], and by the presence of the fetus-related histopathological lesions funisitis and chorionic vasculitis [78-80]. Fetuses with FIRS are often born to mothers with subclinical microbial invasion of the amniotic cavity [75]. If the infection reaches the fetus, it may result in a systemic fetal infection that can progress toward multiple organ dysfunction, septic shock, and death [81]. Finding a treatment that can target both the maternal and fetal inflammatory responses is critical for the prevention of inflammation-induced adverse pregnancy outcomes.

3

Previously, our research group provided evidence that using an inhibitor of inflammation such as Rosiglitazone, a selective peroxisome proliferator–activated receptor γ (PPARγ) agonist, resulted in reduced systemic and local inflammation and improved fetal outcomes in both microbial product- and sterile inflammation-induced models of preterm labor [82-85]. However, the use of Rosiglitazone is not approved by the FDA for administration to pregnant women [86]. Therefore, searching for anti-inflammatory compounds could potentially result in therapeutic approaches to prevent preterm birth and adverse neonatal outcomes.

1.3 Exendin-4

Exendin-4 is a synthetic 39-amino-acid peptide amide with the sequence His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2. Exendin-4 is a glucagon-like peptide-1 receptor (GLP1R) agonist which is commonly used to treat diabetes mellitus type 2 [87]. GLP1R is expressed in pancreatic beta cells and activation of this receptor stimulates the adenylyl cyclase pathway which results in increased insulin synthesis and release of insulin [88]. In addition to the pancreas, GLP1R is expressed in several other organs including the intestine, lung, kidney, breast, and brain [89].

1.4 Properties of Exendin-4

The widespread distribution of GLP1R receptor in organs besides the pancreas has resulted in multiple studies examining this receptor as a target for the treatment of various diseases. While large comparative studies between different GLP-1R agonists have not been performed, comparisons between studies utilizing individual GLP-1 analogues suggest that these compounds can block protein kinase C and NF-κB signaling, reduce the subsequent expression of cytokines (e.g. TNF, IL-6, IFNγ, MCP-1), downregulate inflammasome components (e.g. NLRP3, IL-1β) and adhesion molecules such as ICAM-1, and activate the cAMP/Ca^{2+}, CAMKKβ, and pAMPK pathways which induce anti-inflammatory effects [90]. These promising results, combined with the established

safety and effectiveness of GLP-1R agonists, highlight these compounds as useful inhibitors of chronic inflammation.

Exendin-4 has been established to have potent immunomodulatory effects in both mice and humans. It was found that LPS lead to an increased migration of RAW264 cells and mouse peritoneal macrophages, and this effect was reversed by exendin-4 treatment; moreover, Exendin-4 decreased the concentration of iNOS, TNFα, IL-6, and IL-1β produced by RAW264 cells and mouse peritoneal macrophages [91]. Knockdown of the GLP-1R prevented the inhibitory effect of Exendin-4 on macrophage migration [91]. Exendin-4 was administered in a murine model of liver inflammation and the authors observed a decrease in the number of monocytes per lesion in the endothelium wall of the artery as well as a decrease in the number of macrophages in the liver together with a reduction in the expression of TNFα, IL-1β, and IL-6 [92]. It was found that Exendin-4 treatment significantly improved neutropenia in a rat model of systemic inflammation with LPS and decreased the systemic levels of pro-inflammatory cytokines such as IL-1α, IL-1β, and IL-6 [93]. In humans, it has been demonstrated that Exendin-4 can decrease the RNA expression of iNOS, reduce the concentrations of nitrite, IL-1β, MCP-1, and TNFα, and reduce ROS production in macrophages stimulated with LPS [94, 95]. PBMCs isolated from type 2 diabetic patients were cultured and higher concentrations of TNFα, IL-1β, and IL-6 were detected in the supernatants which were reduced after treatment with Exendin-4. Moreover, Exendin-4 suppressed the release of CCL5/RANTES and CXCL10/IP-10 in T cells from patients with type 2 diabetes. Finally, it was shown that Exendin-4 suppressed the phosphorylation of ERK and p38 MAPK in CD4+ T lymphocytes and monocytes from these patients [96]."

Therefore, exendin-4 may be a plausible treatment for systemic maternal inflammation and FIRS for the prevention of adverse maternal and fetal pregnancy outcomes.

1.5 Neonatal Immunology

Neonates are highly susceptible to infection, which can result in long-term developmental disorders and even death [97, 98] . This susceptibility is partially due to the rapid transition from the sheltered environment *in utero* to the outside world, which results in exposure of the neonatal immune system to commensal organisms and pathogens [97]. The critical nature of this period is exacerbated by Th2-skewed adaptive immunity [99, 100] and a reliance on transferred maternal antibodies [101], resulting in a dependence on innate immune mechanisms for protection [101, 102]. However, neonatal innate immune cells such as neutrophils [103-106], monocytes [107, 108], and dendritic cells [109] are also limited in their responses compared to adult cells. This immunosuppressed state has disadvantages such as predisposing newborns to severe infection and weakening their response to vaccination [98, 110]. Particularly, preterm neonates are at a higher risk for infection than term neonates since a severe state of immunosuppression is observed at earlier gestation [111].

In mice, CD4+CD25+ Treg cells have been identified in peripheral lymph nodes within several days of birth. CD4+CD25+ Treg cells have also been implicated in the development of neonatal tolerance to transplantation antigens. It has been demonstrated that CD4+CD25+ Treg cells from neonatal tolerized mice suppressed the development of donor-specific CD8+ T-cell responses *in vitro*. In addition to CD4+ Treg cells, recent reports implicate key roles for CD8+ Treg cells in neonatal life [112]. CD8+ regulatory T cells express Foxp3 and therefore seem to share phenotypical and functional characteristics with classical CD4+ regulatory T cells [113]. These regulatory cells inhibit T-cell responses (e.g. Th17 cells) *in vivo* [114] and seem to participate in the mechanisms of maternal-fetal tolerance during normal pregnancy [115]. In late pregnancy, however, maternal CD8+ regulatory T cells in the decidua regulate the timing of term parturition by IL6-mediated mechanisms [116]. In addition, maternal CD8+ regulatory T cells have been implicated in the systemic and local (i.e. the maternal-fetal interface) immune mechanisms that lead to spontaneous preterm labor [117, 118]. CD8+ regulatory T

cells are induced *ex thymo* through antigen presentation in the neonatal period in mice [119]; however, their role in humans is unknown.

2. Justification

Preterm birth (PTB) is the leading cause of neonatal morbidity and mortality worldwide. Inflammation is the only process which has a well-established causal link to PTB.

Maternal and fetal inflammation leads to adverse pregnancy and neonatal outcomes. Therefore, finding an intervention that ameliorates maternal and fetal inflammatory responses is critical.

3. Hypothesis

Administration of exendin-4 will prevent endotoxin-induced preterm birth by dampening inflammation and fetal inflammatory syndrome for the prevention of adverse maternal and fetal pregnancy outcomes

4. General objective

To evaluate the protective role of Exendin-4 in preventing adverse pregnancy and neonatal outcomes

5. Specific aims

5.1 Evaluate the maternal and fetal cytokine responses in systemic and local models of inflammation-induced preterm birth and adverse neonatal outcomes

5.2 Determine whether an anti-inflammatory peptide, Exendin-4, can dampen the inflammation in order to prevent adverse pregnancy and neonatal outcomes

5.3 Localize Exendin-4 in the maternal and fetal tissues

5.4 Investigate the anti-inflammatory properties of Exendin-4 on the neonatal immune response by measuring the plasma cytokine response, inflammation-related gene expression, M1-M2 macrophage polarization, pro- and anti-inflammatory neutrophil phenotypes, and CD4+ and CD8+ regulatory T cell subsets.

6. MATERIALS AND METHODS

6.1 Observational experiments

Animals

C57BL/6 (B6) mice were purchased from The Jackson Laboratory in Bar Harbor, ME, USA, and bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University, Detroit, MI, USA. All mice were housed under a circadian cycle (12h light/12h dark). Females 8–12 weeks old were mated with males of the same background and proven fertility. Female mice were checked daily between 8:00 a.m. and 9:00 a.m. for the appearance of a vaginal plug, which indicated 0.5 days *post coitum* (dpc). Females were then placed into new cages and their weights were monitored daily. A gain of two or more grams by 12.5 dpc confirmed pregnancy. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University (Protocol No. A-07-03-15).

Animal models of preterm birth/fetal inflammatory response

Intraperitoneal administration of lipopolysaccharide (LPS) [120]: Pregnant B6 mice were intraperitoneally injected on 16.5 dpc with 10 µg of LPS (*Escherichia coli* 055:B5; Sigma-Aldrich) (n=10) in 200 µL of PBS using a 26-gauge needle. Controls were injected with 200 µL of sterile 1X PBS (n=8). Mice were placed to be video monitored.

Intra-amniotic administration of LPS [120]: Pregnant B6 mice were anesthetized on 16.5 dpc by inhalation of 2–3% Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) (Aerrane, Baxter Healthcare Corporation, Deerfield, IL, USA) and 1–2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.5–2% Isoflurane and 1.5–2 L/min of oxygen. Mice were positioned on a heating pad and stabilized with adhesive tape. Fur removal from the abdomen and thorax was achieved by applying Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to those areas. Body temperature was maintained in the range of 37 ± 1 ºC and detected with a rectal probe

(VisualSonics, Toronto, Ontario, Canada), and respiratory and heart rates were monitored by electrodes embedded in the heating pad. An ultrasound probe was fixed and mobilized with a mechanical holder, and the transducer was slowly moved towards the abdomen. Ultrasound-guided intra-amniotic injection (n=8) of 100 ng LPS (Escherichia coli O111:B4; Sigma-Aldrich) dissolved in 25 µL of sterile 1X phosphate-buffered saline (PBS; Fisher Scientific Bioreagents, Fair Lawn, NJ, USA) was performed in each amniotic sac using a 30-gauge needle (BD PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ, USA). Controls were injected with 25 µL of sterile 1X PBS (n=8). The syringe was stabilized by a mechanical holder (VisualSonics Inc., Toronto, Ontario, Canada). Following the ultrasound, mice were placed under a heat lamp for recovery (defined as when the mouse resumes normal activity, such as walking and responding), which typically occurred 10–20 min after removal from anesthesia. After recovery, mice were video monitored.

Exendin-4 treatment

Pregnant B6 mice were intraperitoneally injected with 10 µg/kg (n=10), 20 µg/kg (n=8) and 30 µg/kg (n=10) of Exendin-4 (Enzo Life Sciences, Ann Arbor, MI) diluted in sterile 1X PBS six hours after intraperitoneal administration of LPS. A second of pregnant B6 mice were intraperitoneally injected with 30 µg/kg of exendin-4 six hours after intra-amniotic administration of LPS (n=8).

Control pregnant mice were only injected with 10 µg/kg (n=10), 20 µg/kg (n=5) and 30 µg/kg (n=5) of Exendin-4 at 16.5 dpc or 1X PBS (7-8).

Video monitoring

Pregnancy parameters including rate of preterm birth and pup mortality were recorded via video camera (Sony Corporation, Tokyo, Japan). Preterm birth was defined as delivery occurring before 18.0 dpc, and its rate was represented by the percentage of females delivering preterm among the total number of mice injected. The rate of pup mortality for each litter was defined as the proportion of

delivered pups found dead among the total litter size. Neonatal survival was recorded 1 week postpartum.

6.2 Cellular and Molecular experiments

Tissue collection from dams and fetuses

Pregnant B6 mice were injected with LPS (both intraperitoneal and intra-amniotic) (n=10 per group) and a control pregnant mice group was only injected with 1X PBS (n=10 each) on 16.5 dpc, as described previously. On 17.5 dpc, mice were euthanized with CO_2 followed by cervical dislocation for the assurance, and peripheral blood was collected by cardiac puncture and placed into a 1.5 ml safe-lock microtube (Fisher Scientific, Hanover Park, IL, USA). Animal dissection to obtain the uterus was performed, and the amniotic fluid was collected from each amniotic sac with a 26-gauge needle and placed into a 0.5 ml safe-lock microtube (Fisher Scientific). The fetal lungs were also collected.

Fluorescent *in vivo* imaging to detect exendin-4

Pregnant B6 mice were injected intraperitoneally with 30 µg/kg of Fluorescein-TRP[25]-exendin-4 (FLEX) (cat # AS-63899, Anaspec Inc., Fremont, CA, USA) six hours after i.p. administration of LPS (n= 3).

Control pregnant mice were only injected with the same concentrations of LPS, FLEX or 200 µL of PBS at 16.5 dpc (n=3 each) and the uterus, placenta, decidua, fetal membranes, and whole fetus were collected to perform imaging with an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA, USA) in epifluorescence mode. Whole fetuses with placenta and fetal membranes from the same mice were immediately frozen in Tissue-Tek O.C.T Compound (Sakura Finetek USA, Torrance, CA). Ten-µm-thick cryosections were cut, placed on Fisherbrand Superfrost Plus microscope slides (Thermo Scientific, Waltham, MA, USA), fixed with 4% paraformaldehyde (Electron Microscopy Sciences USA Hatfield, PA) and washed with 1X PBS. Slides were mounted with ProLong diamond antifade

mountant with DAPI (Life Technologies, Grand Island, NY, USA). Immunofluorescence was visualized using an Olympus BX 60 fluorescence microscope (Olympus, Tokyo, Japan). The pictures were taken using an Olympus DP71 camera and DP Controller Software (Olympus).

Tissue collection from neonates

Pregnant B6 mice were injected intraperitoneally with 30 µg/kg Exendin-4 six hours after intraperitoneal administration of LPS (n=3). Control pregnant mice were only injected with the same concentration of exendin-4 at 16.5 dpc (n=3) or 200 µL of PBS. Thriving neonates were euthanized at 15 days of age and peripheral blood, brain, thymus, lung, spleen, liver, and small and large intestine were collected. For RNA studies, the neonatal brain, lung, liver, and small intestine were placed into RNAlater Stabilization Solution (Invitrogen by Thermo Fisher Scientific, Baltics UAB, Lithuania) according to the manufacturer's instructions. For leukocyte isolation, the neonatal thymus, lung, spleen, liver, and large intestine was utilized.

Leukocyte isolation

Neonatal lung, liver, and large intestine were cut into small pieces using fine scissors and enzymatically digested with StemPro Cell Dissociation Reagent (Accutase, Life Technologies) for 10 min at 37°C. The spleen and thymus were gently dissociated using two glass slides to prepare a leukocyte suspension as previously described [121]. Leukocyte suspensions were filtered using a 35µm cell strainer (Falcon, Tamaulipas, Mexico) and washed with 1X PBS.

Immunophenotyping

Leukocyte suspensions from the neonatal tissues were stained using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies) prior to incubation with extracellular and intracellular mAbs. Leukocyte suspensions were centrifuged at 1250 X g for 7 min at 4°C and cell pellets were incubated for 10 min

with the CD16/CD32 mAb (FcgIII/II Receptor; BD Biosciences, San Jose, CA) and subsequently incubated with specific extracellular and intracellular fluorochrome-conjugated anti-mouse mAbs (Supplementary Table 1) for 30 min. After extracellular staining, the cells were washed with fluorescence-activated cell sorting (FACS) buffer (bovine serum albumin 0.1%, sodium azide 0.05%, 1X PBS; BD Biosciences, San Jose, CA, USA) to remove excess Ab. For immunophenotyping of macrophages and neutrophils, the cells from the neonatal lung, liver, and large intestine were fixed following the extracellular staining and permeabilized using the BD Cytofix/Cytoperm fixation and permeabilization solution (BD Biosciences). For immunophenotyping of regulatory T cells, following the extracellular staining, the cells from the neonatal thymus and spleen were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) prior to intranuclear Foxp3 staining.

Leukocyte subsets were gated within the viability gate. Immunophenotyping included identification of 1) macrophages (CD11b+F4/80+) and their M1/M2 phenotypes by the expression of IL-10 and iNOS; 2) neutrophils (CD11b+Ly6G+) and their anti- and pro-inflammatory phenotypes by the expression of IL-10 and iNOS; and 3) CD4+ and CD8+ regulatory T cells (CD3+CD4+Foxp3+CD25+ and CD3+CD8+Foxp3+CD25+ cells, respectively).

The total number of specific leukocytes was determined using Count Bright absolute counting beads (Molecular Probes, Eugene, OR, USA). As a control for cellular auto-fluorescence, unstained cells were treated in the same manner. Stained and unstained cell suspensions were re-suspended in 0.5 mL of FACS buffer and acquired using an LSRFortessa flow cytometer and FACSDiva 8.0 software (BD Biosciences). Data was analyzed using FlowJo software version 10 (TreeStar, Ashland, OR, USA).

RNA isolation, cDNA synthesis and reverse transcription quantitative polymerase chain reaction (RTqPCR) analysis

Total RNA was isolated from 20 to 30 mg of fetal (17.5 dpc) and neonatal (15 days of age) tissues using QIAshredders, RNase-Free DNase Sets, and RNeasy Mini Kits (all from Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentrations and purity were assessed with the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA integrity was evaluated with the Bioanalyzer 2100 (Agilent technologies,Wilmington, DE). Complementary DNA (cDNA) was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA). Gene expression profiling was performed on the BioMark™ System for high-throughput qRT-PCR (Fluidigm, San Francisco, CA, USA) with the TaqMan® gene expression assays (Applied Biosystems, Life Technologies Corporation, Foster City, CA, USA) listed in Supplementary Table 2.

Chemokine/cytokine concentrations

Neonatal peripheral blood samples were centrifuged at 800 X g for 10 min at 4°C and plasma was separated and stored at -20°C until analysis. Amniotic fluid samples were centrifuged at 1300 X g for 10 min at 4°C and the supernatant was separated and stored at -20°C until analysis. The ProcartaPlex Mouse Cytokine & Chemokine Panel 1A 36-plex (Invitrogen by Thermo Fisher Scientific, Vienna, Austria) was used to measure the concentrations of IFNɣ, IL12p70, IL1B, TNFα, GM-CSF, IL-18, IL-17α, IL-22, IL-23, IL-27, IL-9, IL15/IL15R, IL-13, IL-4, IL-5, IL-6. IL-10, Eotaxin,IL-28, IL-3, LIF, IL1a, IL-31, GRO-α, MIP1α, IP10, MCP-1, MCP-3, MIP1β, MIP2, RANTES, G-CSF, M-CSF, and ENA-78 in the serum, plasma, and amniotic fluid samples, according to the manufacturer's instructions. Plates were read using the Luminex 100 SystemFill (Luminex, Austin, TX, USA), and analyte concentrations were calculated with ProcartaPlex Analyst 1.0 Software from Affymetrix, San Diego, CA. The sensitivities of the assays were: 0.09 pg/ml (IFNy), 0.21 pg/ml (IL-12p70), 0.14 pg/ml (IL-1B), 0.39 pg/ml (TNFα), 0.19 pg/ml (GM-

CSF), 9.95 pg/ml (IL-18), 0.08 pg/ml (IL-17a), 0.24 pg/ml (IL-22), 2.21 pg/ml (IL-23), 0.34 pg/ml (IL-27), 0.28 pg/ml (IL-9), 0.42 pg/ml (IL-15/IL-15R), 0.16 pg/ml (IL-13), 0.03 pg/ml (IL-4), 0.32 pg/ml (IL-5), 0.21 pg/ml (IL-6). 0.69 pg/ml (IL-10), 0.01 pg/ml (Eotaxin), 20.31 pg/ml (IL-28), 0.11pg/ml (IL-3), 0.28 pg/ml (LIF), 0.32 pg/ml (IL-1a), 0.45 pg/ml (IL-31), 0.05 pg/ml (GRO-α), 0.13 pg/ml (MIP-1α), 0.26 pg/ml (IP-10), 3.43 pg/ml (MCP-1), 0.15 pg/ml (MCP-3), 1.16 pg/ml (MIP-1B), 0.37 pg/ml (MIP-2), 0.35 pg/ml (RANTES), 0.19 pg/ml (G-CSF), 0.02 pg/ml (M-CSF), and 5.67 pg/ml (ENA-78). Inter-assay and intra-assay coefficients of variation were less than 10%.

Statistical Analysis

Observational mouse data were analyzed using IBM SPSS, version 19.0, and all other analyses were performed with GraphPad Prism version 5. For rate of preterm birth and rate of pup mortality, the statistical significance of group comparisons was assessed using Mann-Whitney U test. For flow cytometry data, the statistical significance of group comparisons was assessed using Mann-Whitney U tests. For qRT-PCR arrays, negative ΔCt values were determined using multiple reference genes (*Gusb, Hsp90ab1, Gapdh, and Actb*) averaged within each sample to determine gene expression levels. A heat map was created for the group mean expression matrix (gene x group mean), with each gene expression level being standardized first. The heat map represents the Z-scores of the mean ($-\Delta$Ct) and the hierarchical clustering using correlation distance. A p-value ≤ 0.05 was considered significant.

6. Results

Models of inflammation-induced preterm birth and adverse neonatal outcomes

Two previously established models of inflammation-induced preterm birth were compared: systemic administration of LPS via intra-peritoneal injection (maternal inflammatory response model, MIR) and local administration of LPS via intra-amniotic injection (fetal inflammatory response model, FIR) [120]. Mice were injected intra-amniotically (100ng/25μL) or intraperitoneally (10μg/200μL) with LPS (or PBS controls) and observed pregnancy outcomes (Figure 1A). Both the MIR and FIR models resulted in a high rate of preterm birth (80 and 87.5%, respectively) while all of the controls injected with PBS delivered at term (Figure 1B&1E).
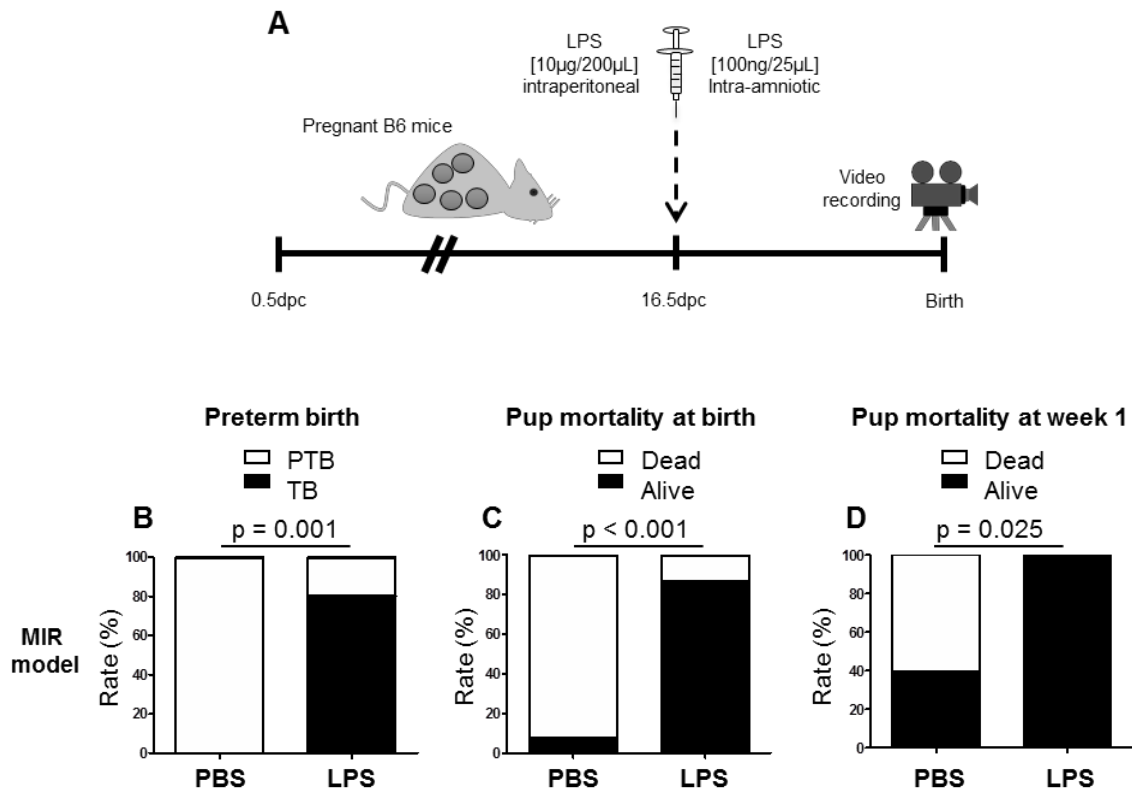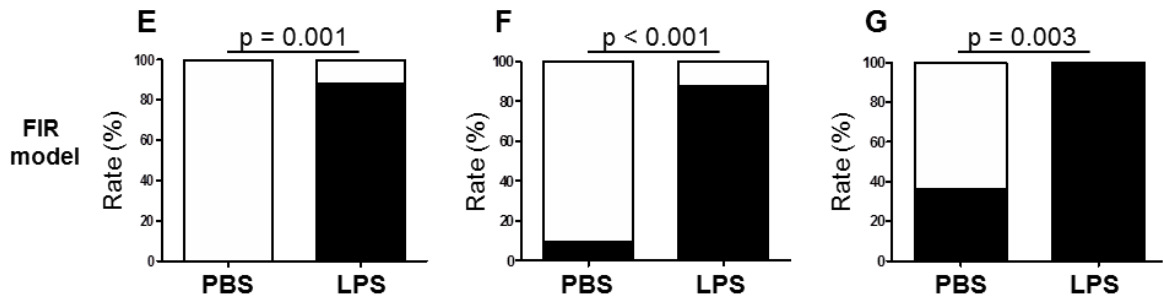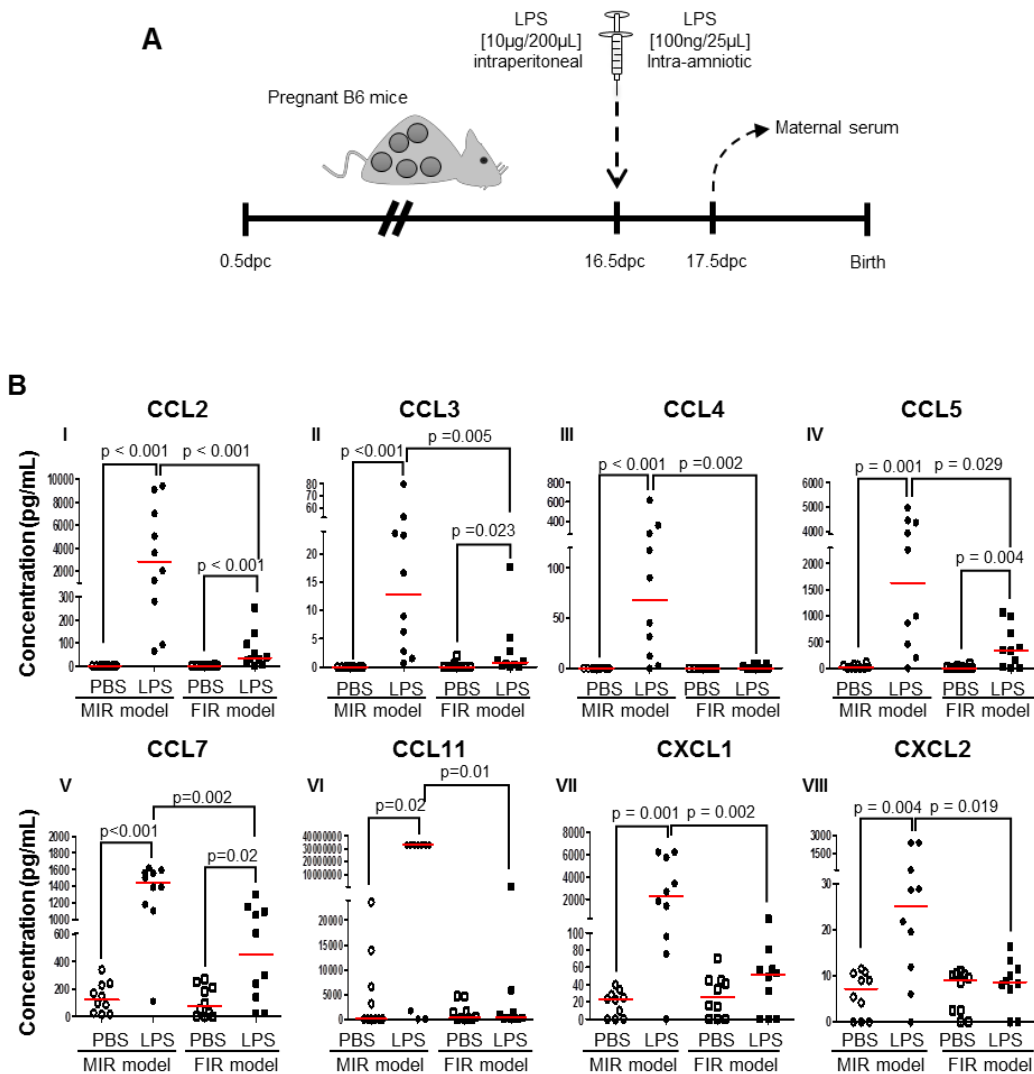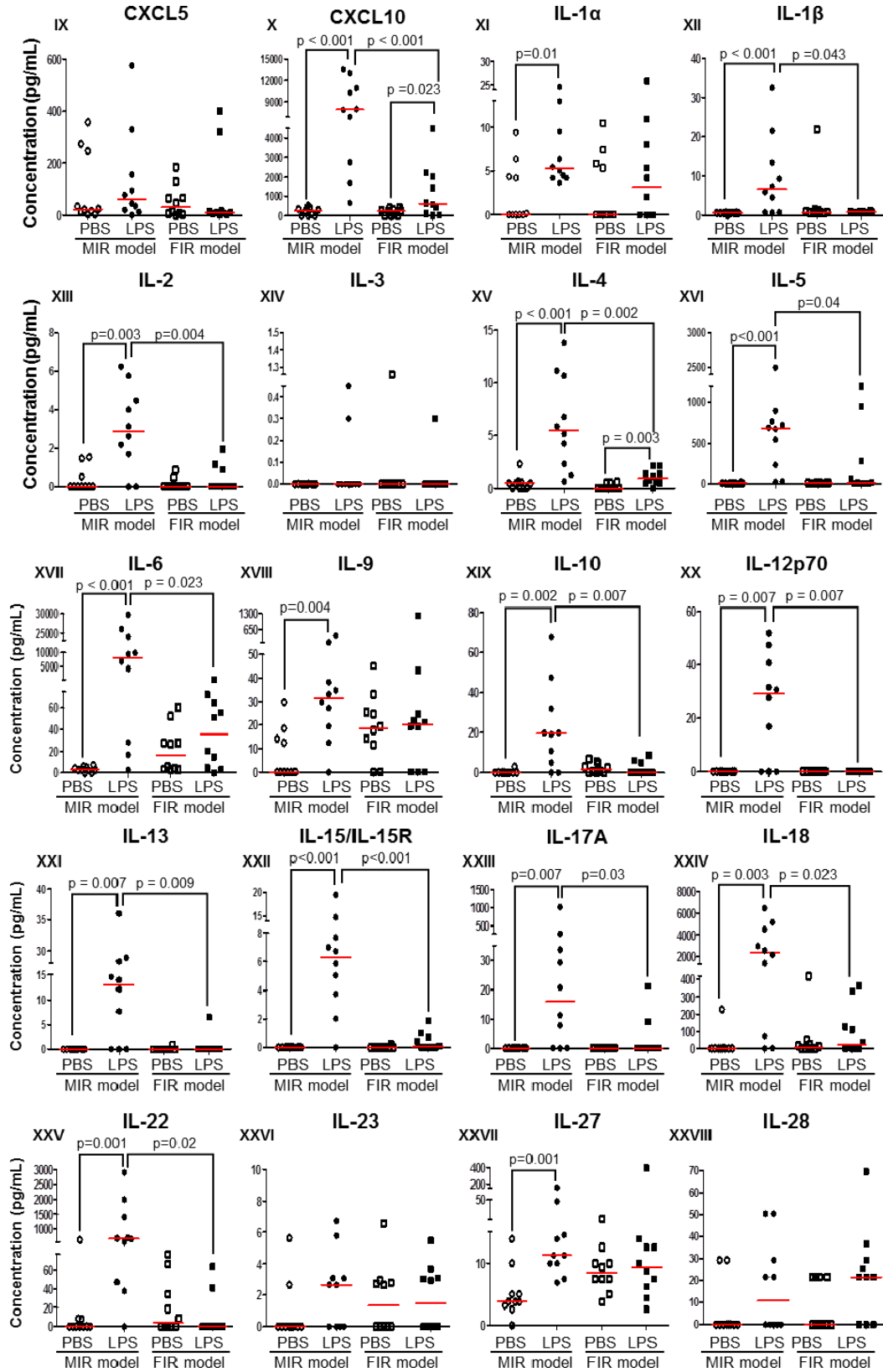
Figure 1: Models of inflammation-induced preterm birth and adverse neonatal outcomes. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (MIR model) (10µg/200µL) or intra-amniotically (FIR model) (100ng/25µL) injected with lipopolysaccharide (LPS) or 1X phosphate-buffered saline (PBS) (200 µL or 25 µL) and mice were monitored until delivery. B&E). Rate of preterm or term birth in the MIR and FIR models. C&F). Rate of neonatal mortality at birth in the MIR and FIR models. D&G). Rate of neonatal mortality at one week of age in the MIR and FIR models. n=7-10 dams with litters per group.

The rate of pup mortality at birth was greater than 85% in both the MIR and FIR models, which was significantly higher than that of controls (Figure 1C&1F). At one week of age, no pups from dams that received either systemic or local administration of LPS survived (Figure 1D&1G). These results demonstrate that a large LPS insult administered systemically or a lower dose given intra-amniotically induces adverse pregnancy and neonatal outcomes.

The maternal cytokine response in the MIR and FIR models

Next, cytokine concentrations were measured in the maternal circulation to evaluate the systemic inflammatory response in both the MIR and FIR models (Figure 2A). In the MIR model there were significantly higher serum concentrations of 30 cytokines compared to PBS controls (Figure 2B). In the FIR model, however, only 7 cytokine concentrations were higher compared to controls (Figure 2B). Interestingly, from the 34 cytokine concentrations reported herein, 25 of these were significantly higher in the MIR model than those in the FIR model (Figure 2B, I-VIII, X, XIII, XV-XVII, XIX-XXV, XXIX, XXX and XXXII-XXIV).
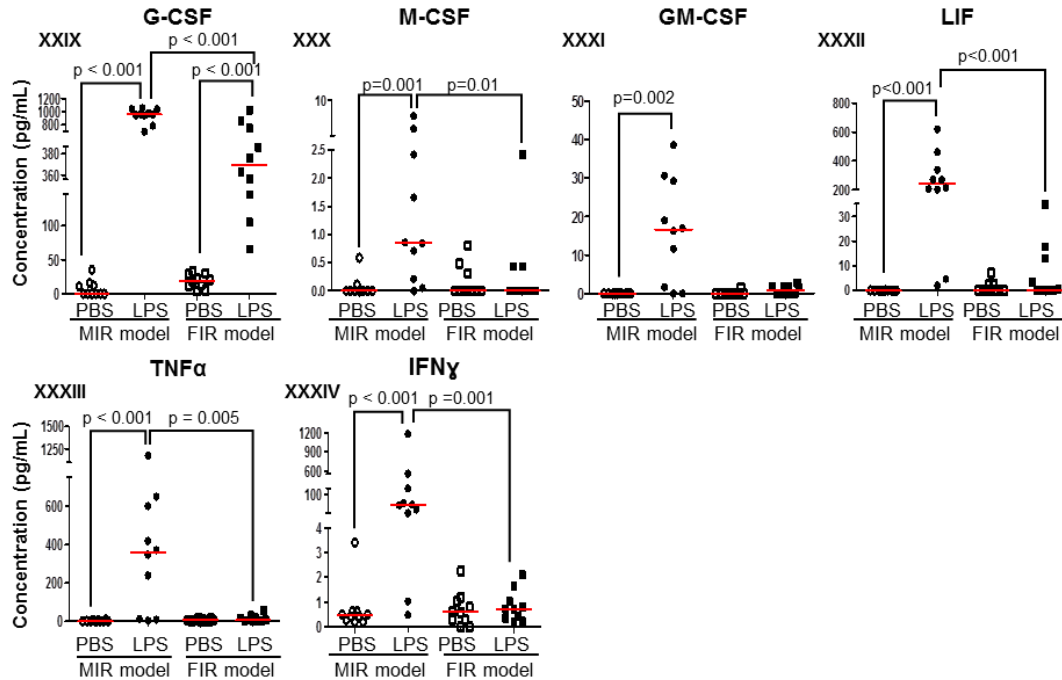
Figure 2: The maternal cytokine response in the MIR and FIR models. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (MIR model) (10μg/200μL) or intra-amniotically (FIR model) (100ng/25μL) injected with lipopolysaccharide (LPS) or 1X phosphate-buffered saline (PBS) (200 μL or 25 μL), and on 17.5 dpc maternal serum was collected for cytokine multiplex analysis. B) Concentrations 34 cytokines were measured in the maternal serum. n=10 dams per group.

Therefore, the MIR model is characterized by a stronger maternal cytokine response than the FIR model.

The fetal cytokine response in the MIR and FIR models

The fetal inflammatory response is associated with elevated IL-6 in the amniotic fluid [75, 76]. Therefore, amniotic fluid was collected from the MIR and FIR models and measured cytokine concentrations (Figure 3A). No apparent differences were found between fetuses of dams injected with LPS intraperitoneally (MIR model) and their control counterparts; yet, fetuses of dams injected with LPS intra-amniotically (FIR model) seemed smaller and friable compared to those from PBS injected controls (Figure 3B). Higher concentrations of 16 cytokines were found in the amniotic fluid in the MIR model when compared with its control (Figure 3C, II-IV, IX, X, XV, XVIII, XXIII-XXIX, XXXI and XXXII). In the FIR model, an elevation in the amniotic fluid concentrations of 12 cytokines was observed compared to its control (Figure 3C, II-IV, VIII-X, XII, XVI, XXIII, XXIX, XXXIII and XXXIV). However, the concentrations of 15 amniotic fluid cytokines were significantly higher in the FIR model than in the MIR model (Figure 3C, II-IV, VII-X, XII, XVI, XIX, XX, XXIII, XXXIII and XXXIV).

B

MIR model

PBS   LPS

FIR model

PBS   LPS

C

CCL2, CCL3, CCL4, CCL5 (row with I, II, III, IV)

I — CCL2: p = 0.003

II — CCL3: p = 0.006, p = 0.004, p<0.001

III — CCL4: p < 0.001, p = 0.002, p = 0.004

IV — CCL5: p < 0.001, p = 0.003, p = 0.004

V — CCL7

VI — CCL11

VII — CXCL1: p = 0.010

VIII — CXCL2: p = 0.001, p = 0.009

IX — CXCL5: p = 0.045, p <0.001, p = 0.017

X — CXCL10: p < 0.001, p = 0.002, p = 0.004

XI — IL-1α: p=0.019

XII — IL-1β: p = 0.001, p = 0.009

Concentration(pg/mL)

PBS LPS PBS LPS
MIR model FIR model

22

IL-3 XIII

IL-4 XIV

IL-5 XV

IL-6 XVI

IL-9 XVII

IL-10 XVIII

IL-12p70 XIX

IL-13 XX

IL-15/IL-15R XXI

IL-17A XXII

IL-18 XXIII

IL-22 XXIV

IL-23 XXV

IL-27 XXVI

IL-28 XXVII

IL-31 XXVIII

G-CSF XXIX

M-CSF XXX

GM-CSF XXXI

LIF XXXII

Figure 3: The fetal inflammatory response in the MIR and FIR models. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (MIR model) (10µg/200µL) or intra-amniotically (FIR model) (100ng/25µL) injected with lipopolysaccharide (LPS) or 1X phosphate-buffered saline (PBS) (200 µL or 25 µL), and on 17.5 dpc amniotic fluid was collected for cytokine multiplex analysis. Photographs of fetuses from dams with MIR or FIR are shown. B) Concentrations of 35 cytokines were measured in the amniotic fluid. n=5-14 dams per group.

These results indicate that the fetal inflammatory response is more dramatic when the insult is given intra-amniotically than when given systemically, which explains why women with intra-amniotic infection are mostly asymptomatic [21, 23, 122, 123].

Inflammatory gene expression in the fetal lung

Intra-amniotic inflammation is associated with fetal lung damage [124-126] and bronchopulmonary disorder [127-130]. Then, evaluated the expression of inflammation-associated genes was evaluated in fetal lungs in both the MIR and FIR models (Figure 4A). No apparent differences were found between the lungs from fetuses of dams intraperitoneally injected with LPS (MIR model) compared to their controls; yet, the lungs from fetuses of dams with FIR seemed pallid compared to controls (Figure 4B). The heatmap array shown in Figure 4C indicated that, in the MIR model, there is a downregulation of inflammation-related genes in the fetal lungs, whereas in the FIR model there is an upregulation of such genes (Figure 4C). When the expression of specific inflammatory genes in the fetal lung was plotted, *Il1b*, *Il6*, *Ccl2*, *Ccl3*, *Ccl5*, and *Cxcl1* were significantly upregulated in the FIR model compared to its control (Figure 4D, I-VI). In the MIR model, however, only *Ccl3* was upregulated in the fetal lungs compared to its control (Figure 4D-IV). Indeed, the expression of *Ccl2* and *Ccl5* was downregulated in the MIR model (Figure 4D-III and V).
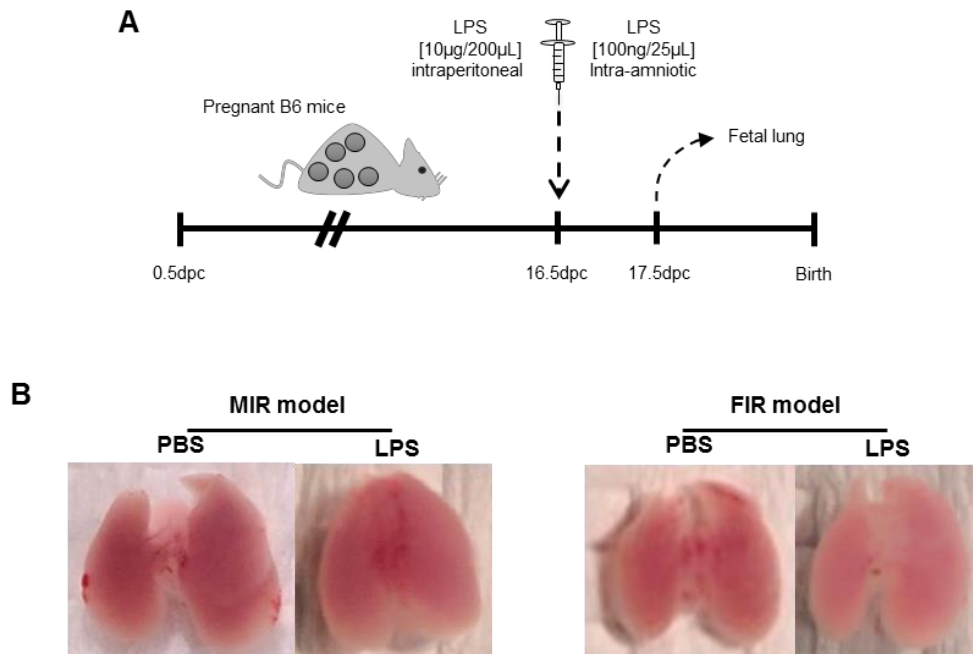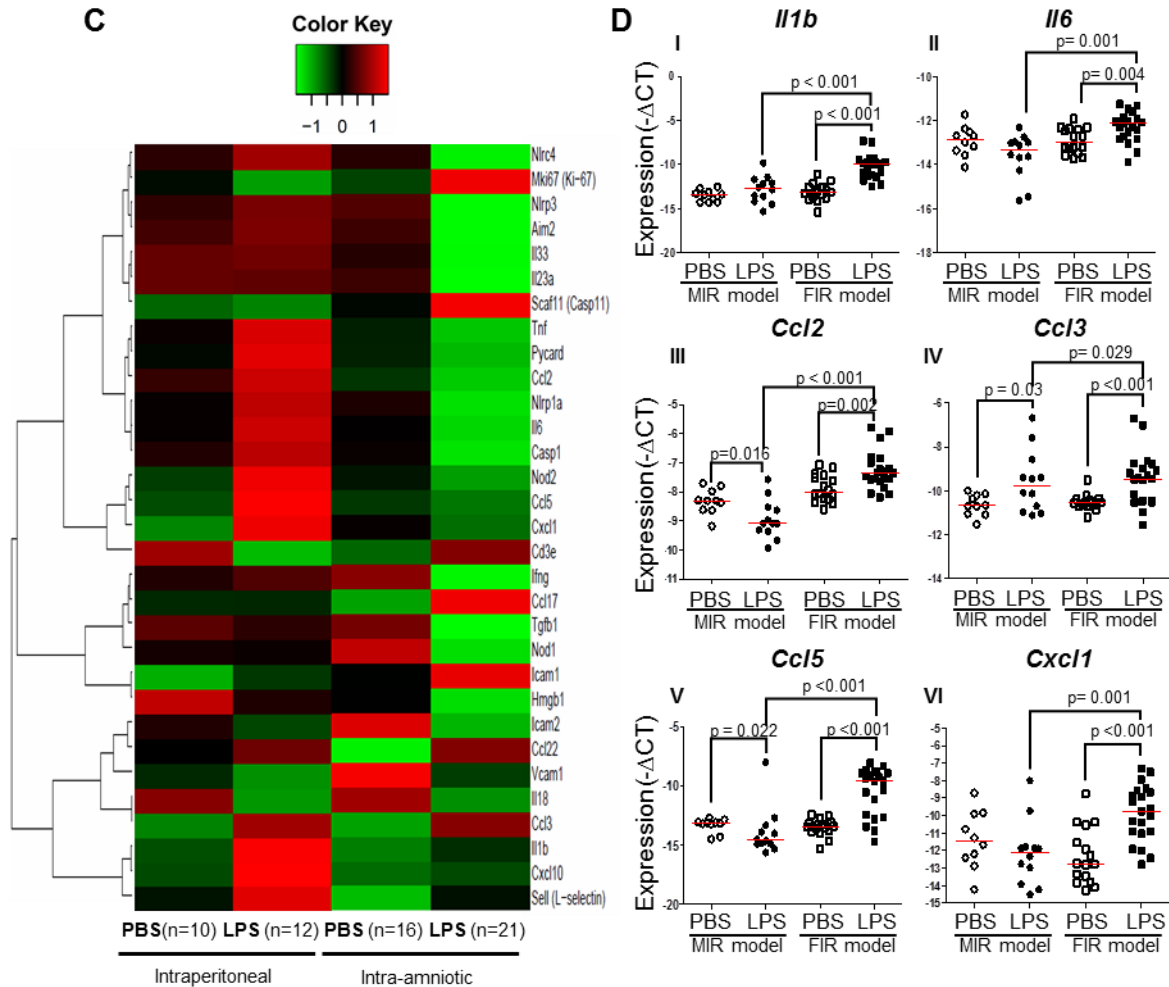
Figure 4. Inflammatory gene expression in the fetal lung. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (MIR model) (10µg/200µL) or intra-amniotically (FIR model) (100ng/25µL) injected with lipopolysaccharide (LPS) or 1X phosphate-buffered saline (PBS) (200 µL or 25 µL), and on 17.5 dpc fetal lung was collected for gene expression analysis. B) Pictures of fetal lungs from dams with MIR or FIR are shown. C) Heat map visualization of gene expression in fetal lung tissue. D) Expression of I) Il1b, II) IL6, III) Ccl2, IV) Ccl3, V) Ccl5 and VI) Cxcl1 in the fetal lung. n=10-21 dams with litters per group.

Together, these data demonstrate that intra-amniotic microbial products can cause an overexpression of inflammation-related genes in the fetal lungs, whereas maternal systemic inflammation seems to have the opposite effect.

Treatment with exendin-4 improves adverse pregnancy and neonatal outcomes

In order to dampen the inflammation caused by the MIR and FIR models, an anti-inflammatory peptide, exendin-4 [92, 94, 95, 131-133], was evaluated for its capacity to reduce or prev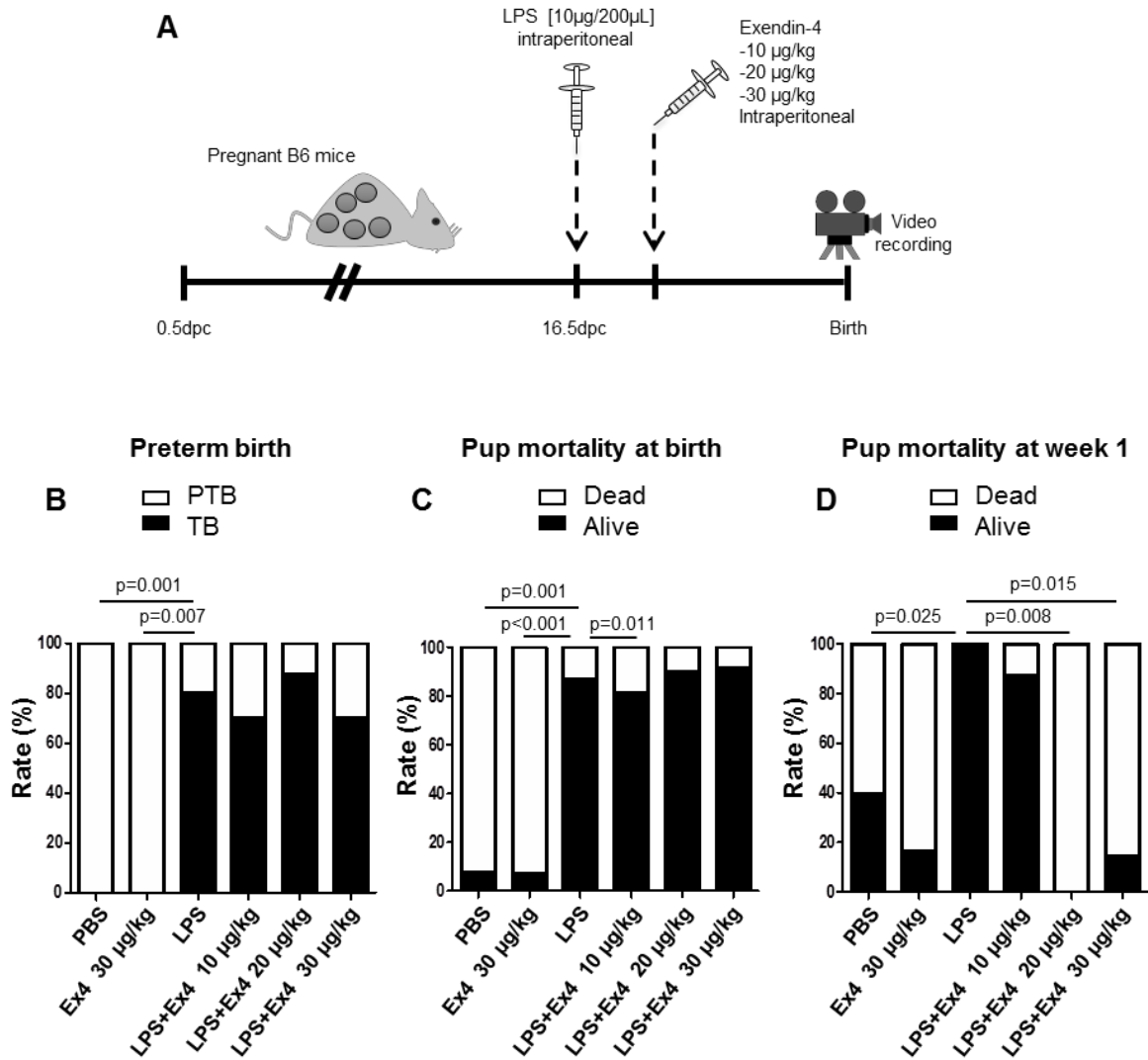ent adverse pregnancy and neonatal outcomes. First, treatment with exendin-4 was used to prevent preterm birth in the MIR model, therefore pregnant mice were injected with LPS and treated with exendin-4, 10µg/g, 20µg/g or 30µg/g, and their pregnancy outcomes were video monitored (Figure 5A).

Treatment with 10 or 30 µg/kg of exendin-4 reduced the rate of LPS-induced PTB by 10% (Figure 5B), whereas treatment with 20 µg/kg of exendin-4 did not reduce the rate of LPS-induced PTB (Figure 5B). Injection of exendin-4 (30 µg/kg) alone did not induce PTB.

The rate of stillbirth induced by LPS (86.67%) significantly reduced upon treatment with 10 µg/kg of exendin-4 (Figure 5C), however the of stillbirth induced by LPS did not reduce upon treatment with 20 µg/kg or 30 µg/kg of exendin-4 (Figure 5C). Injection of exendin-4 alone did not cause stillbirth (data not shown).

All dams injected with LPS delivered preterm and term pups that died within the first week after birth. Therefore, the rate of neonatal mortality in mice injected with LPS alone was 100% (Figure 5D). Importantly, live-born pups from the MIR model which had received exendin-4 treatment continued to thrive, whereas those born to dams without treatment died shortly after birth. The rate of neonatal mortality decreased in LPS-injected mice treated with 10 µg/kg, 20 µg/kg or 30 µg/kg of exendin-4 [Figure 5D: 87.5% (LPS+ 10 µg/kg of exendin-4; not-significant), 0% (LPS+ 20 µg/kg of exendin-4; p=0.008) and 14.29% (LPS+ 30 µg/kg of exendin-4; p=0.015) vs. 100% (10µg of LPS alone)]. Mice injected with exendin-4 alone (30 µg/kg) only delivered viable pups and the rate of neonatal mortality is comparable to controls injected with 1X PBS.

Figure 5. Treatment with exendin-4 improves adverse pregnancy and neonatal outcomes. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally injected with 10µg/200µL of lipopolysaccharide (LPS) or 1X phosphate-buffered saline (PBS) (200 µL), and injected intraperitoneally with 10 µg/kg , 20 µg/kg or 30 µg/kg of exendin-4 (Ex4). Mice were monitored until delivery. B) Rate of preterm birth, C) Rate of neonatal mortality at birth and D) Rate of neonatal mortality at one week of age in the MIR model. n=5-10 dams with litters per group.

Next, treatment with exendin-4 was also used to prevent preterm birth in the FIR model. Dams treated with exendin-4 had a 37.5% decrease in the rate of preterm birth (Figure 6E). And, in addition, pups from the FIR model treated with exendin-4 had a 26.3% decrease in mortality at birth compared to those born to

untreated dams (Figure 6F); however, none of the pups from the FIR model survived to one week of age regardless of exendin-4 treatment (Figure 6G).
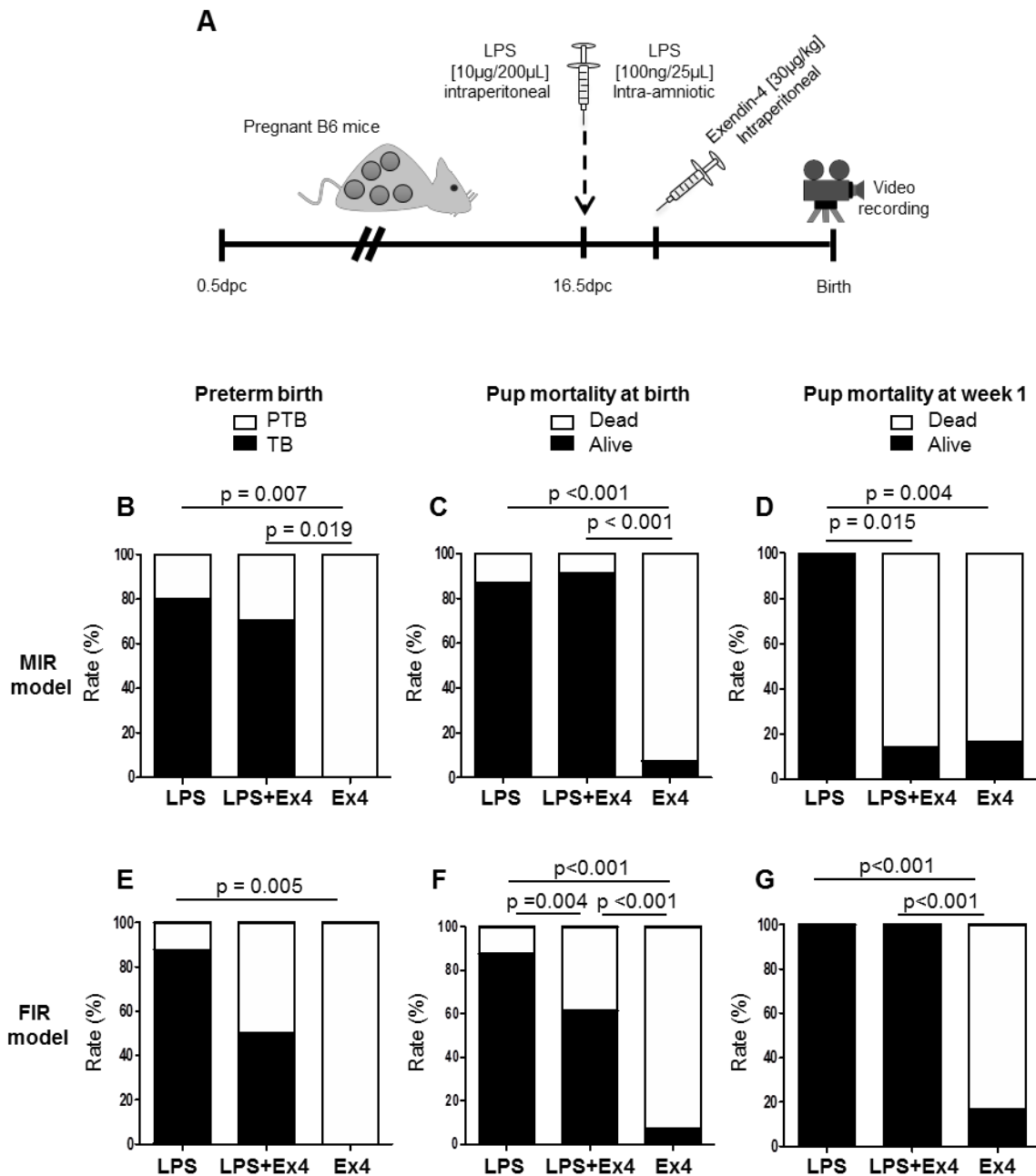
Figure 6. Treatment with exendin-4 improves adverse pregnancy and neonatal outcomes in the MIR and FIR models. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (MIR model) (10μg/200μL) or intra-amniotically (FIR model) (100ng/25μL) injected with lipopolysaccharide (LPS) or 1X phosphate-buffered saline (PBS) (200 μL or 25 μL), and injected intraperitoneally with 30 μg/kg exendin-4 (Ex4). Mice were monitored until delivery.

B&E) Rate of preterm birth in the MIR and FIR models. C&F) Rate of neonatal mortality at birth in the MIR and FIR models. D&G) Rate of neonatal mortality at one week of age in the MIR and FIR models. H) Photographs of thriving neonates born to dams injected with PBS, LPS+exendin-4, or exendin-4 alone are shown. n=5-10 dams with litters per group.

In both the MIR and FIR models, treatment with exendin-4 alone did not induce adverse pregnancy or neonatal outcomes (Figure 6B-G).

Collectively, these results show that exendin-4 treatment can modestly reduce the rate of preterm delivery in dams with MIR or FIR. Importantly, exendin-4 treatment can alleviate adverse neonatal outcomes in dams with systemic maternal inflammation but not in those with intra-amniotic inflammation.

Exendin-4 is localized in the uterus and maternal-fetal interface

Since treatment with exendin-4 had beneficial effects in the MIR model, the localization of this peptide in the maternal and fetal tissues was investigated using a Fluorescein-TRP[25]-exendin-4 (FLEX), which fluoresces after binding to the GLP-1 receptor [134] (Figure 7A). No signal was observed in the whole uterus from control mice injected with PBS or LPS alone (Figure 7B). FLEX was detected in the uterus of mice injected with LPS and treated with FLEX and, in mice injected with FLEX only (Figure 7B).
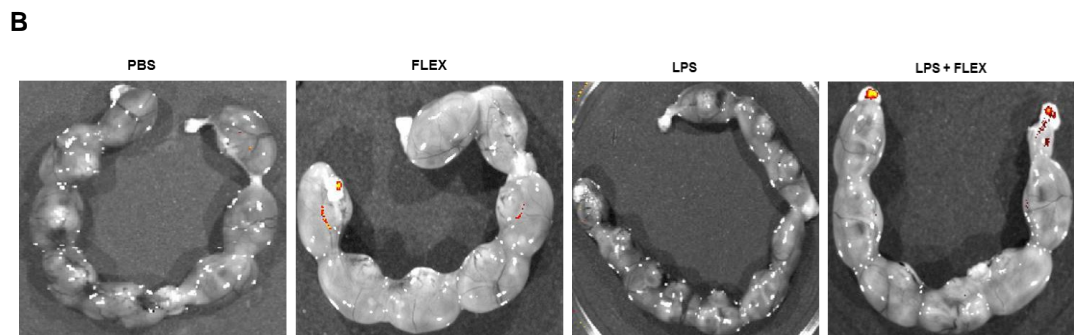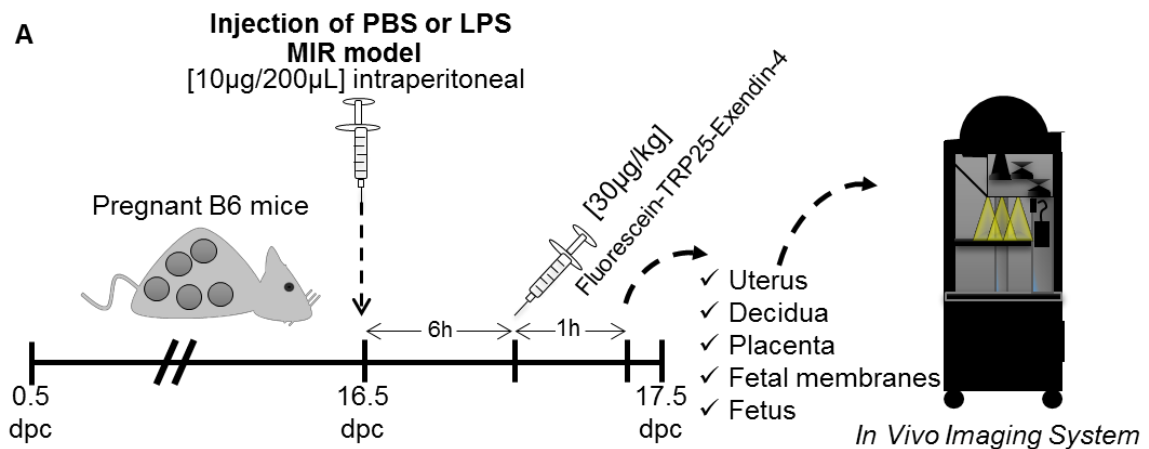
FLEX: FluoresceinTRP[25]-exendin-4

Figure 7. Treatment with exendin-4 improves adverse pregnancy and neonatal outcomes in the MIR and FIR models. Exendin-4 is localized in the uterus and maternal-fetal interface. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally injected with: 1) 1X phosphate-buffered saline (PBS) (200 µL); 2) lipopolysaccharide (LPS) (10µg/200µL); 3) Fluorescein-TRP[25]-exendin-4 (FLEX) (30µg/kg) alone; and 4) LPS followed by treatment with FLEX (30µg/kg). Imaging was performed 1 hour after the second injection. B) Representative images taken with the In Vivo Imaging System showing the fluorescence of FLEX in the uterus. n=3 per group.

In order to determine whether FLEX was present in fetal and maternal tissues, the dissection of the whole uterus was performed to evaluate fluorescence *in utero*, decidua, placenta, fetal membranes and fetus (Figure 8). No signal was observed in the control tissues from mice injected with PBS or LPS alone (Figure 8).
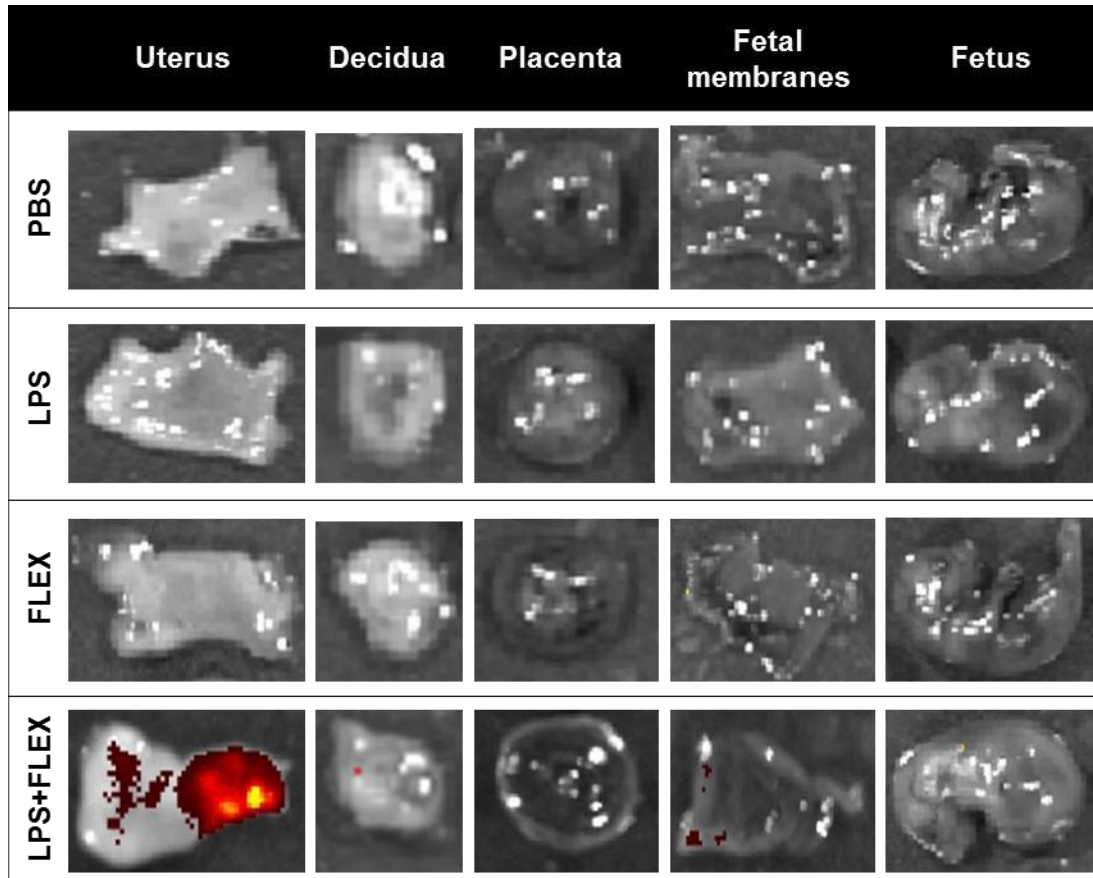
FLEX: FluoresceinTRP[25]-Exendin-4

Figure 8: Exendin-4 is localized in the uterus and maternal-fetal interface. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally injected with: 1) 1X phosphate-buffered saline (PBS) (200 µL); 2) lipopolysaccharide (LPS) (10µg/200µL); 3) Fluorescein-TRP[25]-exendin-4 (FLEX) (30µg/kg) alone; and 4) LPS followed by treatment with FLEX (30µg/kg). Imaging was performed 1 hour after the second injection. B) Representative images taken with the In Vivo Imaging System showing the fluorescence of FLEX in the uterus, decidua, placenta, fetal membranes, and fetus. n=3 per group.

Exendin-4 was strongly detected in the dissected uterus from mice injected with LPS and FLEX (Figure 8). A few traces of exendin-4 were also detected in the decidua and fetal membranes from mice injected with LPS and FLEX (Figure 8). However, exendin-4 was not detected in any of the maternal or fetal tissues in mice injected with FLEX alone (Figure 8).

Whether exendin-4 crossed placenta and present inside of the fetus and/or fetal-maternal tissues was investigated by microscopy in cryosections from whole fetuses (with placenta, and surrounded of fetal membranes and uterus) (Figure 9A). No differences in fluorescence were observed in mice injected with PBS, LPS, LPS+FLEX or FLEX (Figure 9B-E).

FLEX: FluoresceinTRP[25]-Exendin-4
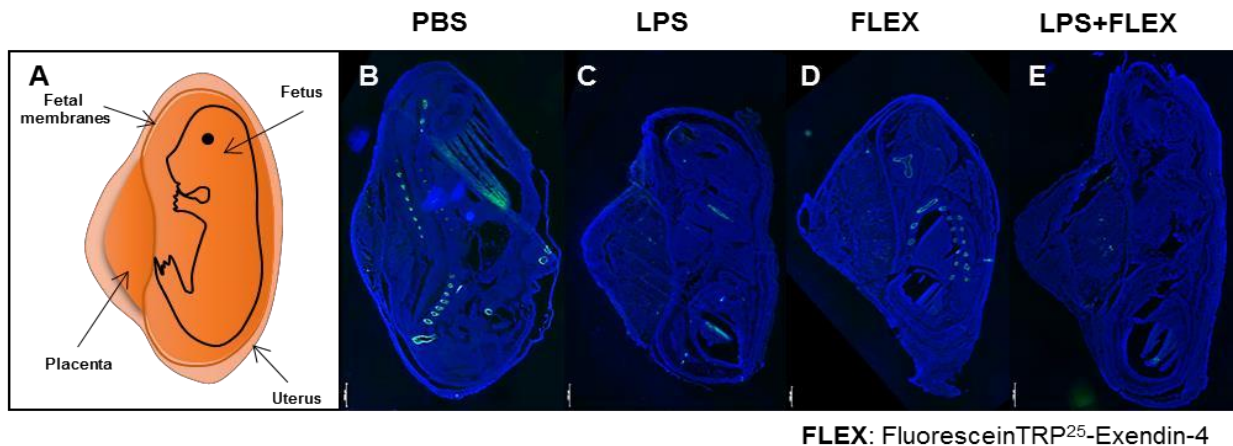
Figure 9. Exendin-4 is not localized inside of fetal tissues. A) Diagram of cryosections from the whole pup with placenta, and surrounded of fetal membranes and uterus. Cryosections of whole pups from pregnant mice were intraperitoneally injected on 16.5 days *post coitum* (dpc), with B) 1X phosphate-buffered saline (PBS) (200 µL);C) lipopolysaccharide (LPS) (10µg/200µL); D) Fluorescein-TRP[25]-exendin-4 (FLEX) (30µg/kg) alone; and E) LPS followed by treatment with FLEX (30µg/kg).

These findings suggest that systemic inflammation facilitates the diffusion of exendin-4 through the uterus and the maternal-fetal interface but not in the fetuses.

Neonates born to dams with systemic inflammation and treated with exendin-4 display a similar cytokine profile to healthy neonates

Since exendin-4 improved neonatal survival in pups born to dams with MIR, the anti-inflammatory effects of exendin-4 was investigated in the thriving pups. First, the plasma cytokine profile of 15 day old neonates was determined (Figure 10A). Neonates born to dams with MIR which received exendin-4 treatment had comparable plasma cytokine concentrations (31 out of 35 cytokines) to healthy neonates (pups born to dams injected with PBS alone) (Figure 10C, II-III, V-IX, XI-XVII, XX-XXXII, XXXIV and XXXV). Indeed, the plasma cytokine concentrations of CCL5, CXCL10, TNFα, and IL-12p70 were decreased in neonates born to dams with MIR which received exendin-4 treatment (Figure 10C, IV, X, XIX and XXXIII) compared to healthy pups. Healthy pups treated with exendin-4 alone had comparable, or even lower, plasma cytokine concentrations to healthy pups from dams injected with PBS alone.

C

CCL2 (I), CCL3 (II), CCL4 (III), CCL5 (IV) p = 0.047

CCL7 (V), CCL11 (VI), CXCL1 (VII), CXCL2 (VIII)

CXCL5 (IX), CXCL10 (X) p = 0.003, IL-1α (XI), IL-1β (XII)

IL-3 (XIII), IL-4 (XIV), IL-5 (XV), IL-6 (XVI)

IL-9 (XVII), IL-10 (XVIII), IL-12p70 (XIX) p = 0.003, IL-13 (XX)

All panels: Concentration(pg/mL) vs PBS (Healthy pup) and LPS+Ex4 (Treated pup)

35
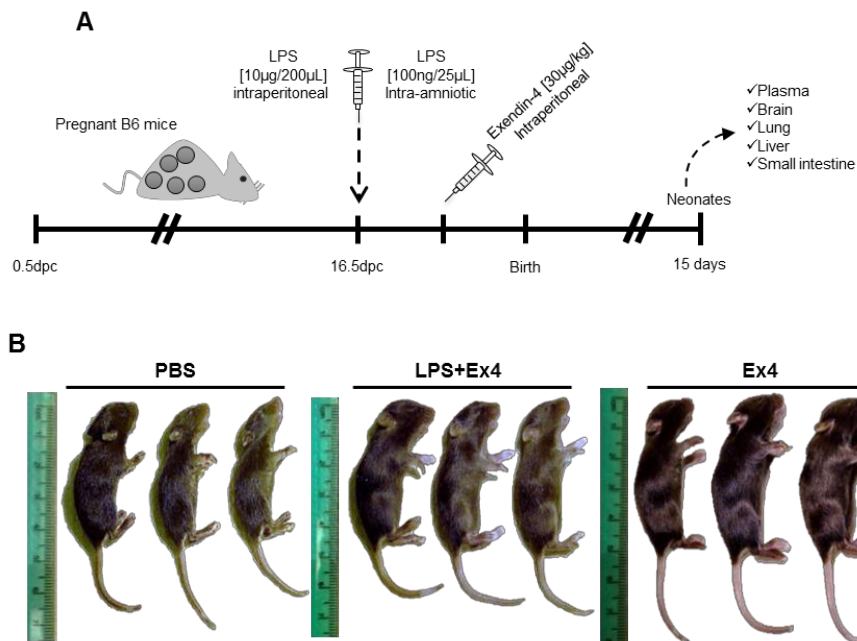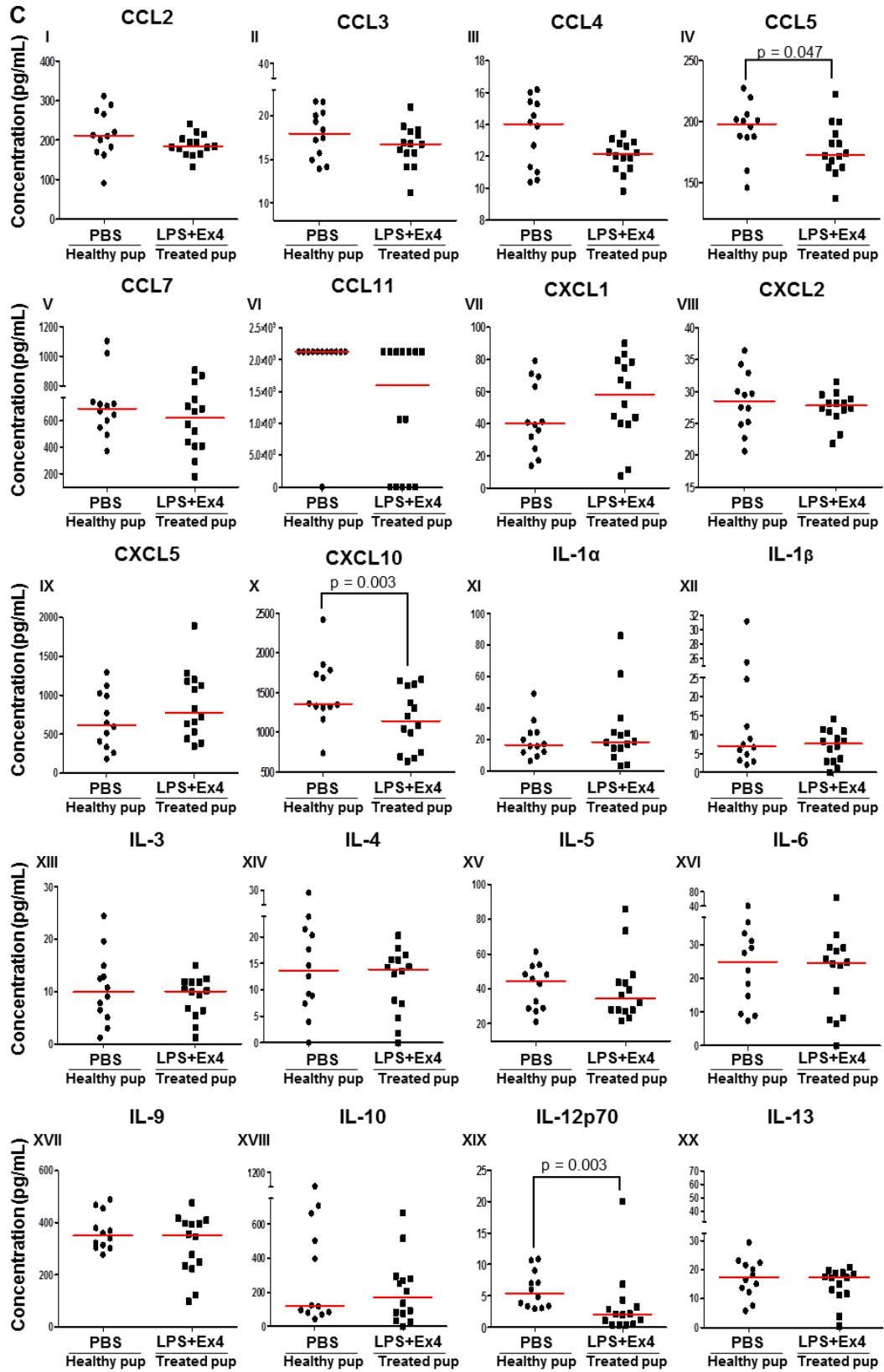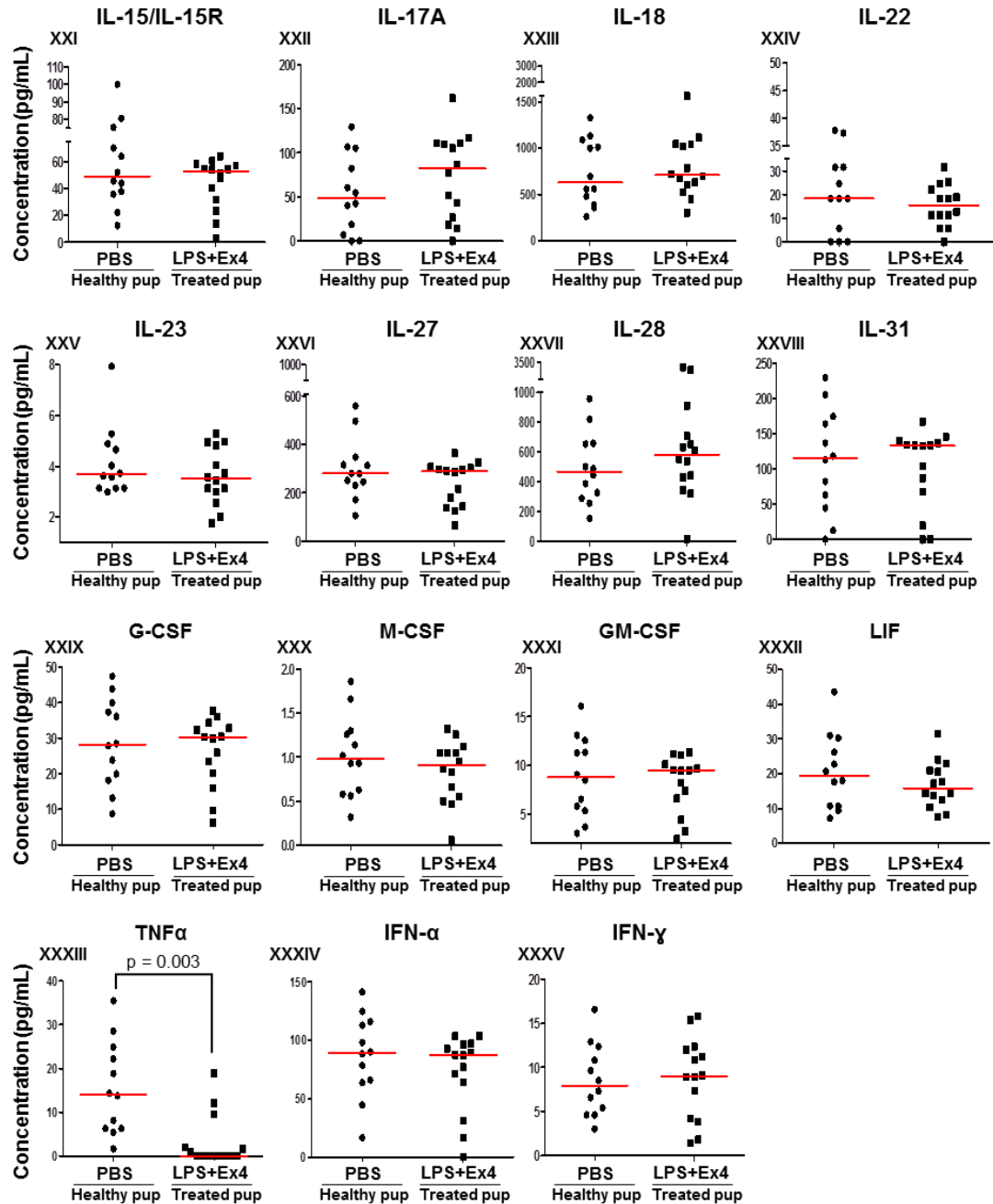
Figure 10: The cytokine profile of neonates born to dams with MIR and treated with exendin-4. A) On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (10µg/200µL) injected with lipopolysaccharide (LPS) followed by treatment with exendin-4 (30µg/kg). Controls were injected with 1X phosphate-buffered saline (PBS, 200µL) alone. At 15 days of age, neonatal plasma was collected for cytokine multiplex analysis. B) Concentrations of 35 cytokines were measured in neonatal plasma. n=12-14 neonates per group.

The expression of inflammation-related genes was determined in the neonatal brain, lung, liver, and small intestine (Figure 11).The expression of *Il1b*, *Il6*, *Ccl2*, *Ccl3*, *Ccl5*, and *Cxcl1* in the brain, lung, liver, and small intestine from pups born to MIR dams which received exendin-4 treatment was comparable to that of healthy pups (Figure 11, I-VI).
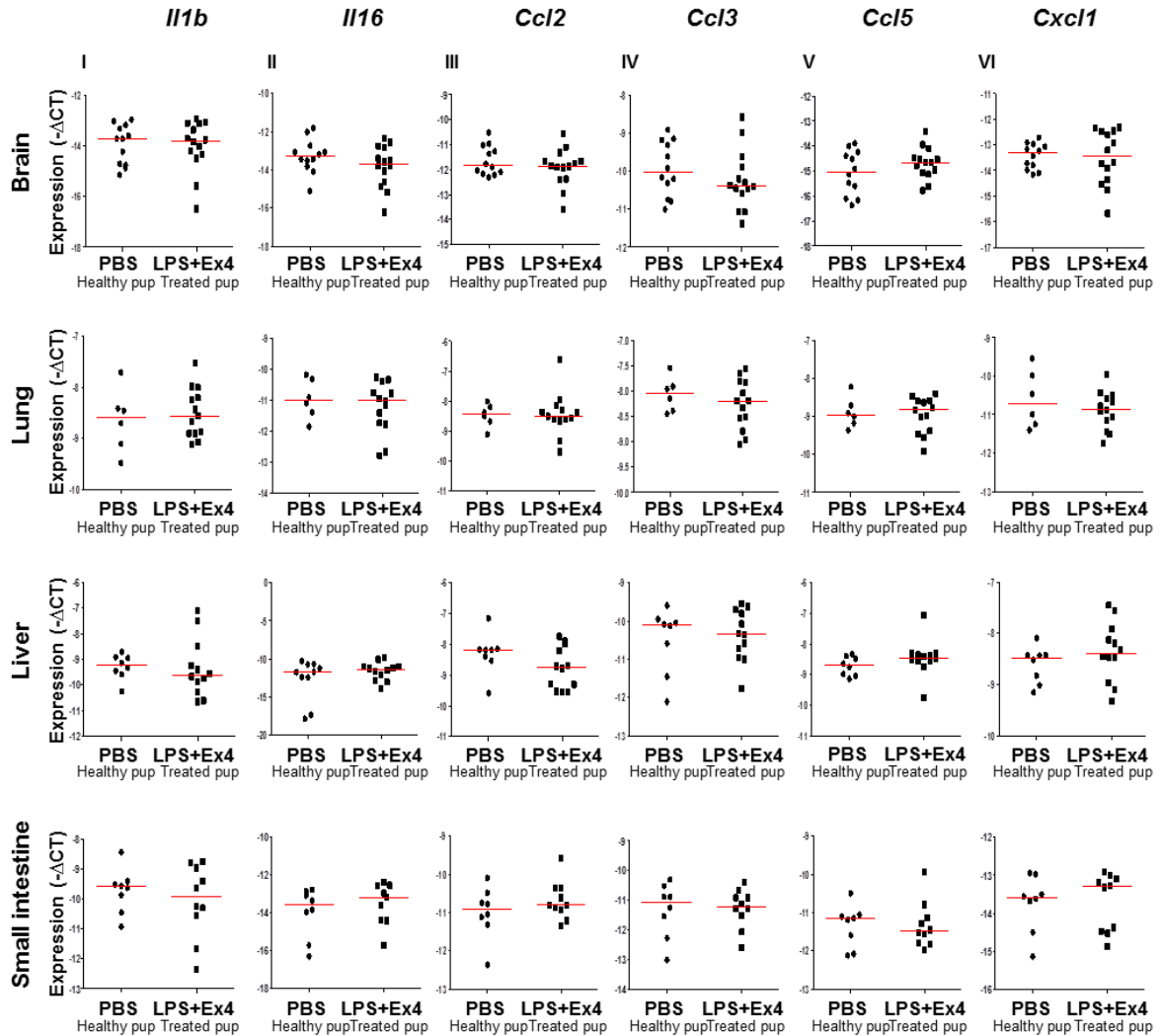
Figure 11: Inflammatory gene expression in neonates born to dams with MIR and treated with exendin-4. On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (10μg/200μL) injected either with lipopolysaccharide (LPS) followed by treatment with exendin-4 (30μg/kg). Controls were injected with 1X phosphate-buffered saline (PBS, 200μL) alone. At 15 days of age the neonatal brain, lung, liver, and small intestine were collected for gene expression analysis. Expression of I) Il1b, II) Il6, III) Ccl2, IV) Ccl3, V) Ccl5 and VI) Cxcl1 in the neonatal brain, lung, liver and small intestine. n=12-14 neonates per group.

No differences in the expression of such genes were observed between healthy pups born to dams injected with PBS (controls) or exendin-4 alone (Figure 12).
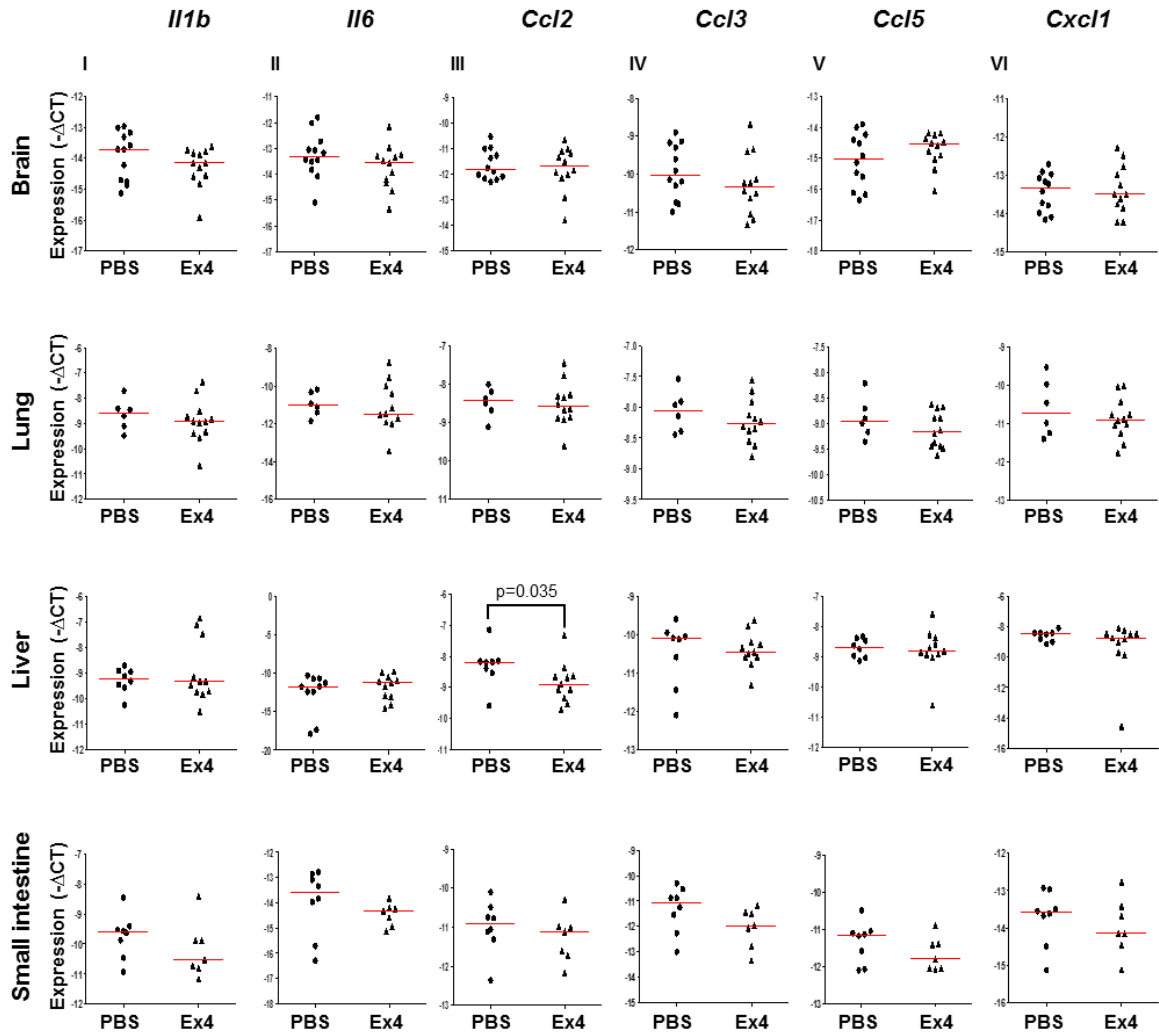
Figure 12: Inflammatory gene expression in neonates born to exendin-4-treated dams compared to those from 1X phosphate-buffered saline-treated controls. Expression levels of I) *Il1b, II) Il6, III) Ccl2, IV) Ccl3, V) Ccl5,* and *VI) Cxcl1* in the neonatal brain, lung, liver, and small intestine. n=12 neonates per group.

Collectively, these data show that exendin-4 has anti-inflammatory properties in dams with MIR, which results in thriving and healthy neonates.

Exendin-4 treatment induces an M1→M2 macrophage polarization in the neonate

The innate immune system has a central role in fetal and neonatal life [112, 135]; therefore, neonates born to dams with MIR and treated with exendin-4 were investigated for effects on lung, liver, and large intestine M1/M2 macrophage phenotypes. Macrophage immunophenotyping was performed in neonatal tissues (Figure 13A). The numbers of macrophages in the neonatal lung and liver were significantly reduced in neonates born to MIR dams which received exendin-4 treatment when compared to those from controls (Figure 13B, I and IV). The number of macrophages in the large intestine from neonates born to dams with MIR and treated with exendin-4 was comparable to that of healthy pups (Figure 13 B, VII).

Next, M1-like (CD11b+F4/80+iNOS+) and M2-like (CD11b+F4/80+IL-10+) macrophages were immunophenotyped in these tissues (Figure 13A), as previously reported [82, 83, 136, 137]. There was an increase in the number of M2-like macrophages in the lung and large intestine of neonates born to MIR dams with exendin-4 treatment compared to controls (Figure 13 B, II and VIII). The number of M2-like macrophages in the liver from neonates born to dams with MIR and treated with exendin-4 was comparable to that of healthy pups (Figure 13B, V). Conversely, there was a decrease in the number of M1-like macrophages in the liver of neonates born to MIR dams with exendin-4 treatment (Figure 13B, VI). The number of M1-like macrophages in the lung and large intestine from neonates born to dams with MIR and treated with exendin-4 was comparable to that of healthy pups (Figure 13B, III and IX).
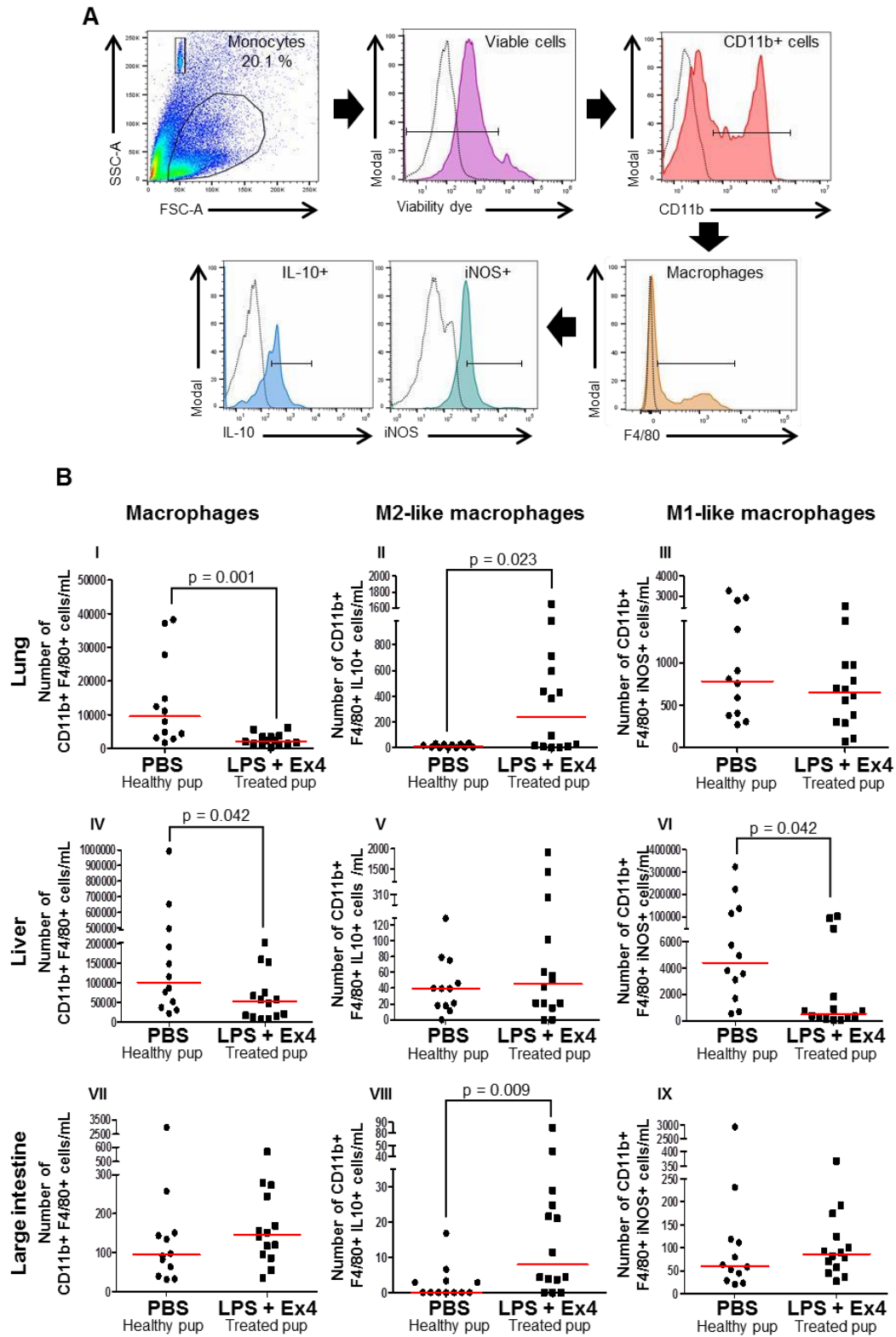
Figure 13: Exendin-4 treatment induces an M1→M2 macrophage polarization in neonates. On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally

(10µg/200µL) injected either with lipopolysaccharide (LPS) followed by treatment with exendin-4 (30µg/kg). Controls were injected with 1X phosphate-buffered saline (PBS. 200µL) alone. At 15 days of age, the neonatal lung, liver, and large intestine were collected for immunophenotyping. A) Gating strategy for M1- and M2-like macrophages. Dead cells were excluded using a viability dye. Empty histograms represent the autofluorescence control and colored histograms represent antibody fluorescent signals. B) Numbers of macrophages in the neonatal lung (I), liver (IV), and large intestine (VII). Numbers of M2-like macrophages in the neonatal lung (II), liver (V), and large intestine (VIII). Numbers of M1-like macrophages in the neonatal lung (III), liver (VI), and large intestine (IX). n=12-14 neonates per group.

Treatment of dams with exendin-4 alone caused an M2 macrophage polarization in the neonatal tissues (Figure 14).
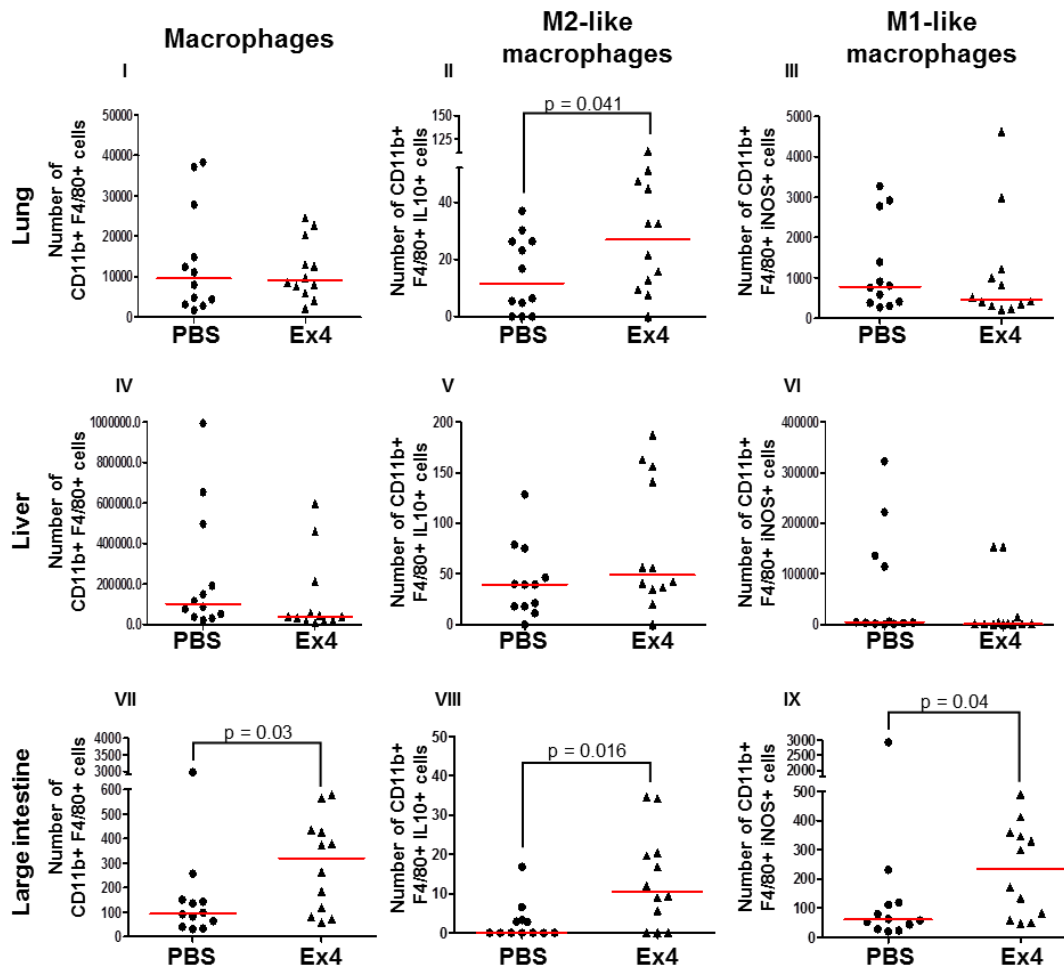
Figure 14: Tissue macrophages in neonates from exendin-4-treated dams compared to those from 1X phosphate-buffered saline-treated controls. Numbers

of total macrophages and M1- or M2-polarized macrophages in the neonatal lung (I-III), liver (IV-VI), and large intestine (VII-IX). n=12 neonates per group.

Taken together, these results indicate that exendin-4 treatment of dams with MIR decreases the overall number of macrophages in the neonatal tissues, while promoting an M1→M2 macrophage polarization. Exendin-4 treatment induces a neutrophil polarization in the neonate

The total numbers of neutrophils (CD11b+Ly6G+) as well as their expression of pro-inflammatory (iNOS) and anti-inflammatory (IL-10) cytokines were determined in the neonatal lung, liver, and large intestine (Figure 15A). The total numbers of neonatal neutrophils were increased in the lung and large intestine of neonates born to MIR dams with exendin-4 treatment when compared to PBS controls (Figure 15B, I and VII). The number of neutrophils in the liver from neonates born to dams with MIR and treated with exendin-4 was comparable to that of healthy pups (Figure 15B, IV).

Neutrophil immunophenotyping in neonatal tissues was also performed (Figure 15). There was an increase in the number of IL-10-expressing neutrophils in the lung, liver, and large intestine of neonates born to MIR dams with exendin-4 treatment compared to controls (Figure 15B, II, V and VIII). In contrast, there was a decrease in the number of iNOS-expressing neutrophils in the liver of neonates born to MIR dams with exendin-4 treatment compared to healthy pups (Figure 15B, VI).

A

B

| Neutrophils | IL-10-expressing neutrophils | iNOS-expressing neutrophils |
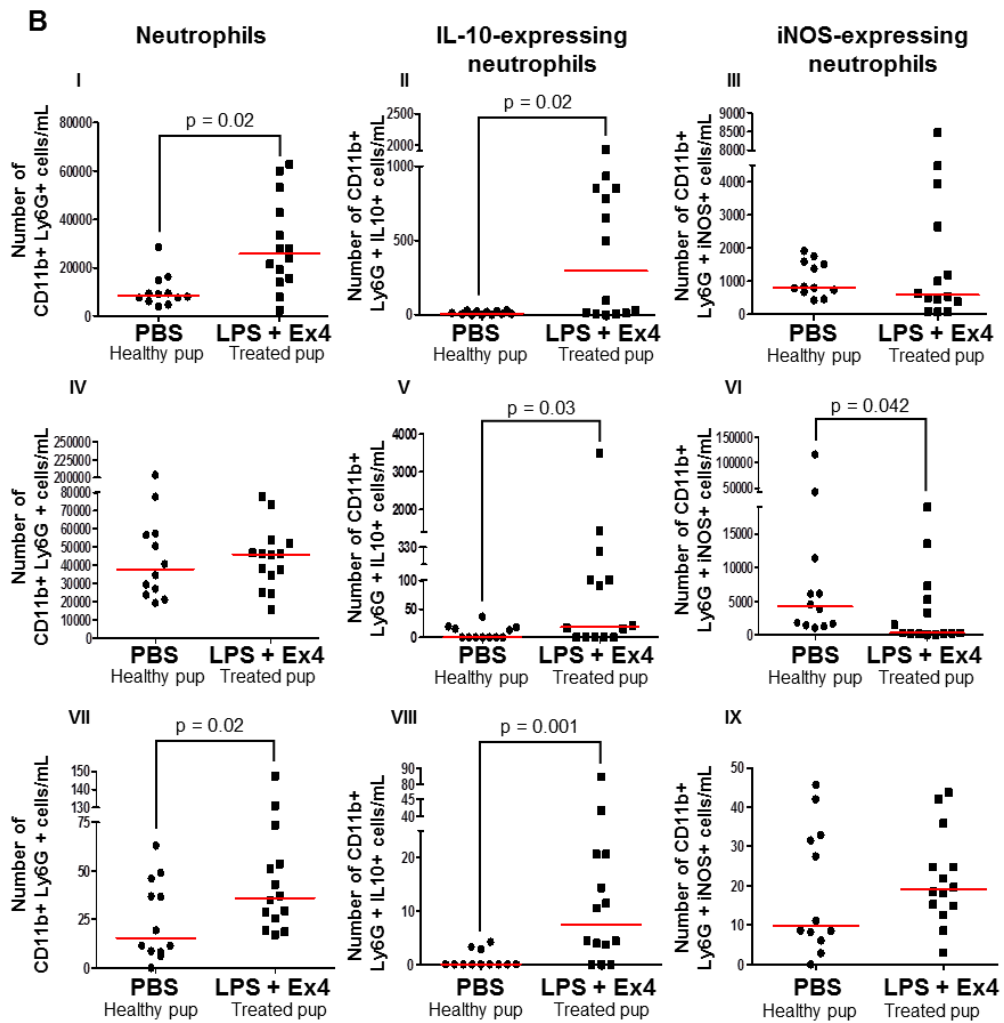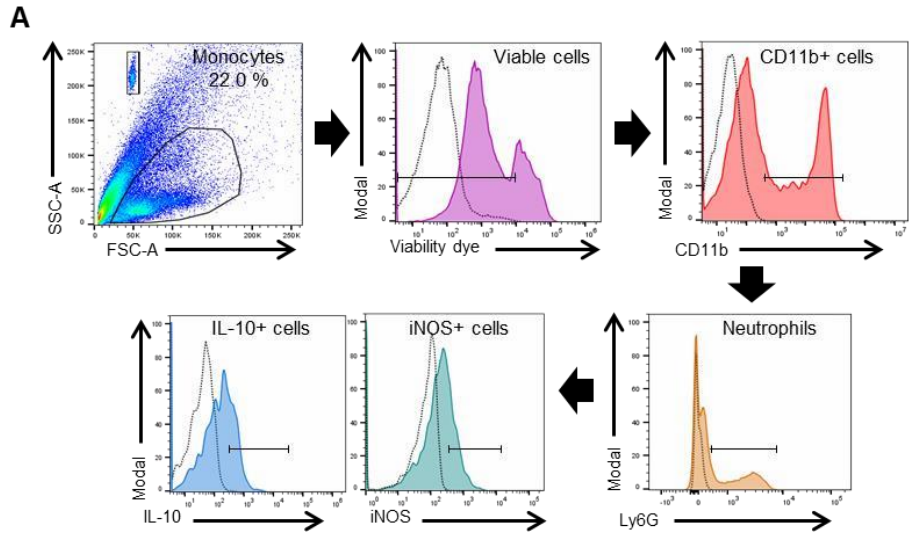
Figure 15: Exendin-4 treatment induces an increase in anti-inflammatory neutrophils in neonates. On 16.5 days post coitum (dpc), pregnant mice were intraperitoneally (10µg/200µL) injected with lipopolysaccharide (LPS) followed by treatment with exendin-4 (30µg/kg). Controls were injected with 1X phosphate-buffered saline (PBS, 200µL) alone. At 15 days of age, the neonatal lung, liver, and large intestine were collected for immunophenotyping. A) Gating strategy for neutrophil polarization. Dead cells were excluded using a viability dye. Empty histograms represent the autofluorescence control and colored histograms represent antibody fluorescent signals. B) Numbers of neutrophils in the neonatal lung (I), liver (IV), and large intestine (VII). Numbers of IL-10-expressing neutrophils in the neonatal lung (II), liver (V), and large intestine (VIII). Numbers of iNOS-expressing neutrophils in the neonatal lung (III), liver (VI), and large intestine (IX). n=12-14 neonates per group.

The number of iNOS-expressing neutrophils in the lung and large intestine from neonates born to dams with MIR and treated with exendin-4 was comparable to that of healthy pups (Figure 16).
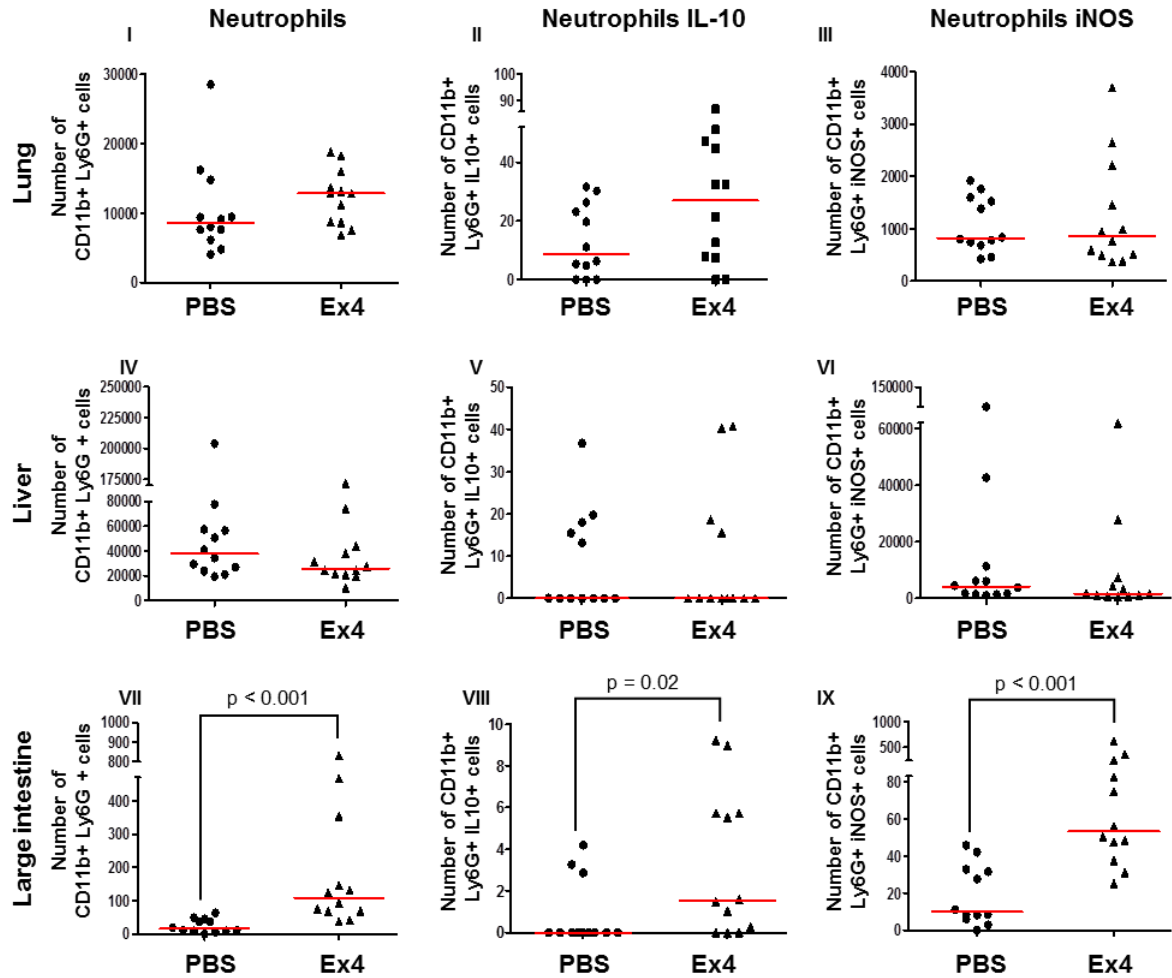
Figure 16: Tissue neutrophils in neonates from exendin-4-treated dams compared to those from 1X phosphate-buffered saline-treated controls. Numbers of total neutrophils and IL-10- or iNOS-expressing neutrophils in the neonatal lung (I-III), liver (IV-VI), and large intestine (VII-IX). n=12 neonates per group.

Treatment of dams with exendin-4 alone only caused an increase of pro- and anti-inflammatory neutrophils in the neonatal large intestine (Figure 16, VII to IX). These results show that exendin-4 treatment of dams with MIR increases the number of anti-inflammatory neutrophils in the neonatal tissues.

Exendin-4 treatment reduces neonatal CD8+ regulatory T cells

Regulatory T cells (Tregs) play a central role in both the developing fetus and in the neonate [112, 135, 138-141]. The next series of experiments were aimed to determine whether exendin-4 treatment of dams with MIR is altering neonatal Treg subsets (CD3+CD4+CD25+FoxP3+ and CD3+CD8+CD25+FoxP3+ cells) in the neonatal spleen and thymus (Figure 17A). No differences were observed in the number of splenic and thymic CD4+ Tregs between neonates born to exendin-4 treated dams with MIR and healthy neonates (Figure 17B, I and II). The number of CD8+ Tregs was reduced in the spleen of neonates born to MIR dams with exendin-4 treatment when compared to healthy neonates (Figure 17, III); however, no differences were observed in thymic CD8+ Tregs (Figure 17B, IV).

B
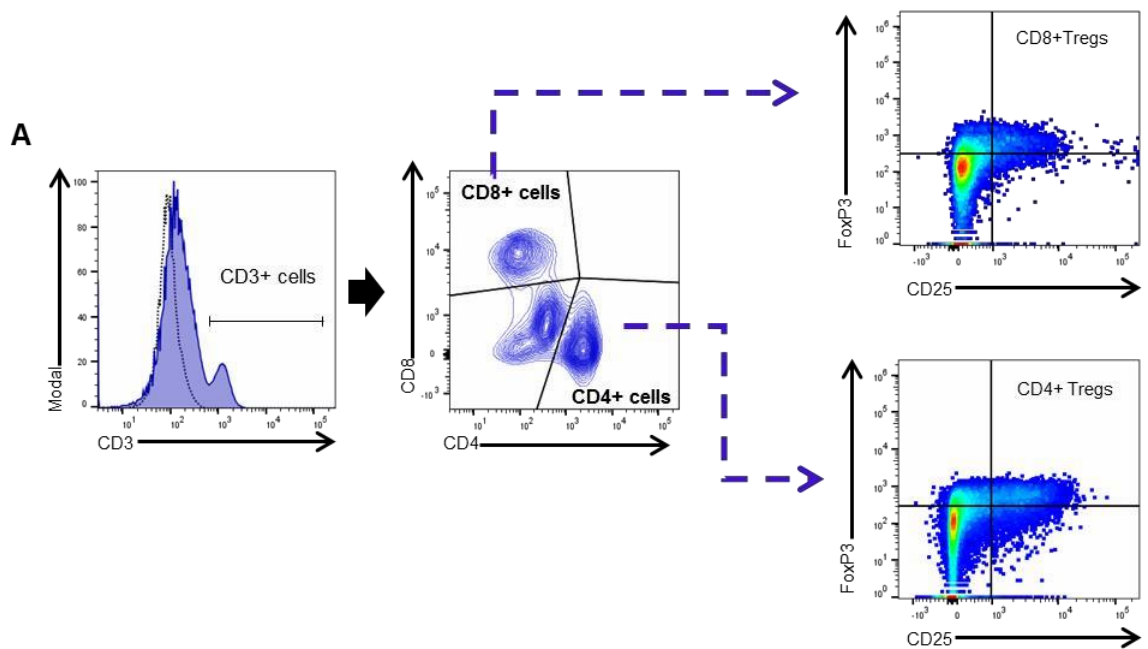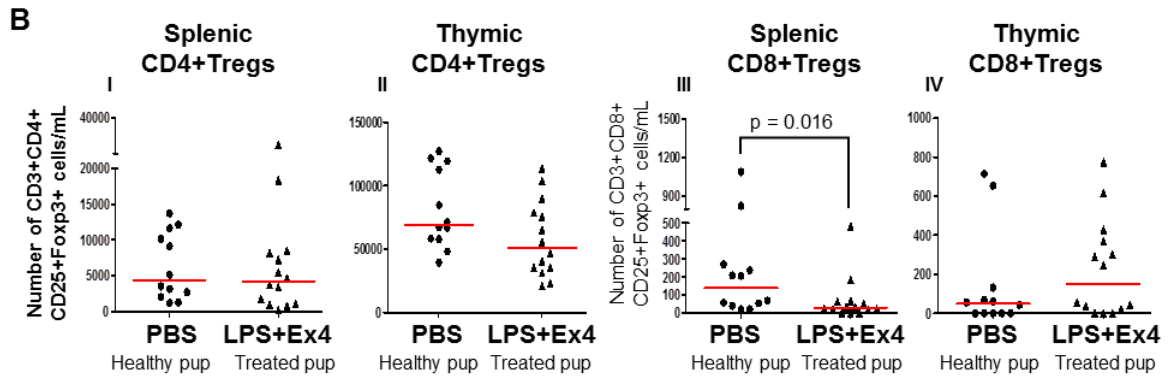
Figure 17: Exendin-4 treatment reduces neonatal CD8+ regulatory T cells. On 16.5 days *post coitum* (dpc), pregnant mice were intraperitoneally (10μg/200μL) injected either with lipopolysaccharide (LPS) followed by treatment with Exendin-4 (30μg/kg). Controls were injected with 1X phosphate-buffered saline (PBS, 200μL) alone. At 15 days of age, the neonatal spleen and thymus were collected for immunophenotyping. A) Gating strategy for CD4+ and CD8+ T regulatory cells. Dead cells were excluded using a viability dye. Dotted histograms represent the autofluorescence control and colored histograms represent antibody fluorescent signals. CD4+ and CD8+ regulatory T cells co-expressed CD25 and FoxP3. B) Number of splenic and thymic CD4+ regulatory T cells (I and II). Number of splenic and thymic CD8+ regulatory T cells (III and IV). n=12-14 neonates per group.

No differences in the number of CD4+ and CD8+ Tregs were observed between neonates born to dams treated with exendin-4 alone and those from PBS controls (Figure18).
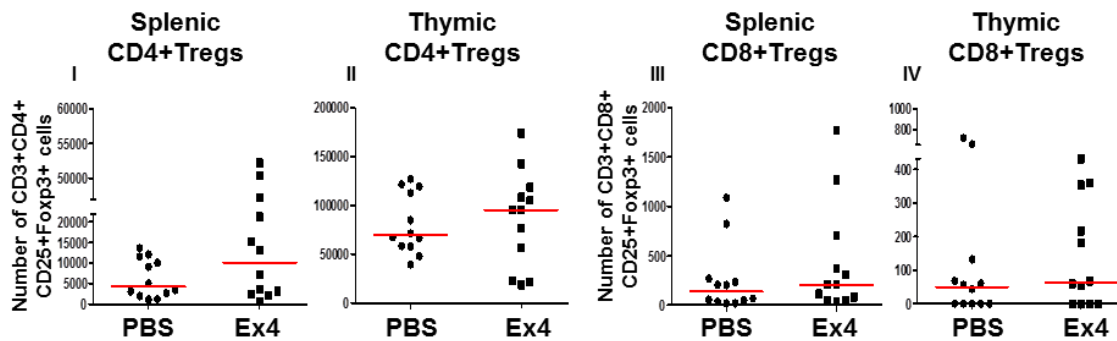

Figure 18: Regulatory T cells in neonates from exendin-4-treated dams compared to those from 1X phosphate-buffered saline-treated controls. Numbers of CD4+ I and II) and CD8+ (III and IV) regulatory T cells in the neonatal spleen and thymus. n=12 neonates per group.

These results indicate that exendin-4 may reduce neonatal inflammation by inhibiting the expansion of splenic CD8+ Tregs, which may have pro-inflammatory properties [84, 116, 136].

7. Discussion

Intra-amniotic infection is commonly associated with invasion of genital mycoplasmas, Gram-negative, and Gram-positive bacteria into the amniotic cavity [21, 42, 51, 142, 143]. This infection can result in a maternal and/or fetal inflammatory response [32, 46, 51-54, 75, 76, 81, 144-146]. This is consistent with the findings reported herein, in which the intra-amniotic administration of a microbial product (LPS) resulted in both a maternal and fetal inflammatory response. In addition, the intra-amniotic administration of LPS induced preterm birth and neonatal death, as previously reported [120]. This model is similar to the subclinical syndrome of preterm birth since (1) a low dose of LPS was injected, simulating the amniotic fluid concentrations of endotoxin found in women with spontaneous preterm labor [147]; and (2) the intra-amniotic injection of low doses of LPS did not cause hypothermia, which is consistent with the fact that most of the intra-amniotic infections in women with spontaneous preterm labor occur in the absence of a temperature change [148, 149].

The systemic administration of LPS induced a severe maternal cytokine response but a mild fetal cytokine response, which caused preterm birth and neonatal death. A systemic maternal inflammatory response is observed in women with clinical chorioamnionitis [45] and acute pyelonephritis [150, 151], both clinical conditions associated with preterm birth [123, 152-155] and adverse neonatal outcomes [153, 154, 156]. However, clinical chorioamnionitis results from intra-amniotic infection [45, 51-54], a condition which was not present in our model. On the other hand, acute pyelonephritis occurs independently of intra-amniotic infection and is not associated with a fetal inflammatory response [157], which resembles our MIR model.

The adverse pregnancy and neonatal outcomes observed in the MIR model were ameliorated by treatment with exendin-4 peptide. This is consistent with a previous report showing that a GLP-1 analogue, such as exendin-4, dampened inflammatory pathways in a rat model of sepsis [158]. GLP-1 receptors are present in the maternal [159] and fetal tissues [159-161]. Herein, exendin-4 was mainly

localized in the uterus and to a lesser extent in the decidua. These findings suggest that treatment with exendin-4 has anti-inflammatory effects in the MIR model by primarily targeting the maternal tissues. Such scenario explains why treatment with exendin-4 did not rescue the adverse neonatal outcomes in the FIR model. Treatment with exendin-4, however, did reduce the rate of preterm birth and neonatal mortality at birth in the FIR model, suggesting that a combined approach that targets both the maternal and fetal tissues may be the optimal strategy.

Neonates born to dams with systemic inflammation and treated with exendin-4 thrived and displayed plasma and tissue cytokine profiles comparable to healthy neonates. Previous studies have shown that the GLP-1 receptor is expressed in fetal tissues [159, 161], including the placenta [160]. In addition, administration of agonists exendin-4 and liraglutide increased expression of surfactant protein A and B in lung and amniotic fluid, which demonstrates the importance of the GLP-1 system in fetal development [160, 161]. In the current study, exendin-4 was modestly detected in the fetal membranes, suggesting that this peptide could have partial effects on the tissues surrounding the fetus, which translated into thriving neonates.

Treatment with exendin-4 induced an M2 macrophage polarization in neonates born to dams with systemic inflammation. This is consistent with previous studies demonstrating that glucagon-like peptides, such as exendin-4, induce an M2 macrophage polarization *in vitro* [162] and *in vivo* [163]. M2 macrophages are considered alternatively activated [164-170] and display anti-inflammatory properties through the production of IL-10 and upregulation of arginase-1 [166, 171-178]. In addition, decidual M2 macrophages participate in maternal-fetal tolerance throughout pregnancy [82, 179-185], suggesting that exendin-4 may also have effects at the maternal-fetal interface. Further studies are required to investigate the effects of GLP-1 analogues in the reproductive tissues and maternal-fetal interface.

Although adult neutrophils are a major component of the innate immune system, neonatal neutrophils tend to have quantitative and qualitative defects [135]. For example, neonatal neutrophils have impaired chemotaxis, rolling adhesion, transmigration, and lamellipodia formation [97]. Such innate immune cells also display impairments in anti-microbial mechanisms and are reduced in newborns presenting bacterial sepsis [97]. In the current study, treatment with exendin-4 caused an increase in anti-inflammatory neutrophils in neonates born to dams with systemic inflammation. These findings are in line with a previous report demonstrating that exendin-4 can modulate neutropenia and dampen pro-inflammatory cytokines [93]. Together, these results indicate that exendin-4 treatment of dams with systemic inflammation modulates the fetal inflammatory response, which resulted in thriving neonates with increased anti-inflammatory neutrophils.

CD4+ Tregs play a central role in the immune response by preventing autoimmunity (inhibiting anti-self-immune responses) and suppressing defensive immune responses to prevent host tissue damage [186-190]. In the fetus, CD4+ Tregs are generated during pregnancy in order to participate in self-tolerance and tolerance to non-inherited antigens on chimeric maternal cells [138, 139]. CD4+ Tregs are also implicated in the development of neonatal tolerance, where they suppress the development of donor-specific CD8+ T cell responses [112, 191]. The fact that neonates born to dams with systemic inflammation and treated with exendin-4 had normal numbers of CD4+ Tregs, which were comparable to those of healthy neonates, provides evidence that this peptide does not have deleterious effects on neonatal CD4+ Treg homeostasis.

CD8+CD25+ T cells expressing FoxP3 seem to share phenotypic, functional, and mechanistic actions with classical CD4+ regulatory T cells [113, 192] and therefore are termed CD8+ Tregs. In neonates, CD8+ Tregs modulate Th2-cell-mediated pathology and autoimmunity [193, 194], suggesting that such cells shape the development of the immune system [112]. In late pregnancy, however, maternal/decidual CD8+CD25+FoxP3+ T cells seem to have pro-

inflammatory functions [116, 117, 136]. Herein, treatment with exendin-4 suppressed the expansion of CD8+ Tregs in neonates born to dams with systemic inflammation. The fact that exendin-4 reduces CD8+ Tregs in the spleen of neonates born to dams with systemic inflammation suggests that such cells have pro-inflammatory rather than immunosuppressive functions. Yet, a functional assessment of neonatal CD8+ Tregs in the context of infection requires further investigation.

In summary, the findings presented herein provide evidence that exendin-4 improves adverse pregnancy and neonatal outcomes by modestly decreasing the rate of preterm birth and drastically improving neonatal survival in a model of maternal systemic inflammation. Moreover, exendin-4 treatment of dams with systemic inflammation confers protective effects on the neonates by reducing the expression and systemic concentrations of inflammatory cytokines and promoting an anti-inflammatory phenotype of neonatal immune cells.

8. Conclusions

1) Systemic administration of the microbial product LPS (MIR model) induced adverse pregnancy and neonatal outcomes by causing a severe maternal cytokine response and a mild fetal cytokine response, which was characterized by the downregulation of inflammatory genes in the lung.

2) Local administration of the microbial product LPS (FIR model) induced adverse pregnancy and neonatal outcomes by causing a mild maternal cytokine response and a severe fetal cytokine response in the amniotic fluid and fetal lung.

3) Exendin-4 treatment of dams with MIR improved adverse pregnancy and neonatal outcomes by reducing the rate of preterm birth by 10% and neonatal mortality by 87.7%; importantly, these neonates continued to thrive.

4) Exendin-4 treatment of dams with FIR improved adverse pregnancy and neonatal outcomes by reducing the rate of preterm birth by 37.5% and neonatal mortality at birth by 26.3%; however, these neonates failed to thrive.

5) Systemic inflammation facilitated the diffusion of exendin-4 through the uterus and the maternal-fetal interface.

6) Neonates born to exendin-4 treated dams with systemic inflammation displayed a similar cytokine profile to healthy control neonates; and 7) treatment with exendin-4 had immunomodulatory effects by inducing an M2 macrophage polarization and increasing anti-inflammatory neutrophils, as well as suppressing the expansion of CD8+ Tregs, in neonates born to dams with systemic inflammation.

These results provide evidence that dampening maternal systemic inflammation through novel interventions such as exendin-4 can improve the quality of life for neonates born to women with this clinical condition.

9. References

1. Romero, R., et al., *The preterm labor syndrome.* Ann N Y Acad Sci, 1994. **734**: p. 414-29.
2. Romero, R., *Prenatal medicine: the child is the father of the man. 1996.* J Matern Fetal Neonatal Med, 2009. **22**(8): p. 636-9.
3. Romero, R., et al., *The preterm parturition syndrome.* BJOG, 2006. **113 Suppl 3**: p. 17-42.
4. Gotsch, F., et al., *The preterm parturition syndrome and its implications for understanding the biology, risk assessment, diagnosis, treatment and prevention of preterm birth.* J Matern Fetal Neonatal Med, 2009. **22 Suppl 2**: p. 5-23.
5. Romero, R. and L. CJ., *Pathogenesis of spontaneous preterm labor*, in *Creasy and Resnik's Maternal–Fetal Medicine: Principles and Practice*, C. RK, R. R, and I. J, Editors. 2009, Elsevier: Philadelphia, PA. p. 521-543.
6. Muglia, L.J. and M. Katz, *The enigma of spontaneous preterm birth.* N Engl J Med, 2010. **362**(6): p. 529-35.
7. Blencowe, H., et al., *National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.* Lancet, 2012. **379**(9832): p. 2162-72.
8. Hamilton, B.E., et al., *Annual summary of vital statistics: 2010-2011.* Pediatrics, 2013. **131**(3): p. 548-58.
9. Liu, L., et al., *Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis.* Lancet, 2015. **385**(9966): p. 430-40.
10. Berkowitz, G.S., et al., *Risk factors for preterm birth subtypes.* Epidemiology, 1998. **9**(3): p. 279-85.
11. Goldenberg, R.L., et al., *Epidemiology and causes of preterm birth.* Lancet, 2008. **371**(9606): p. 75-84.
12. Romero, R., S.K. Dey, and S.J. Fisher, *Preterm labor: one syndrome, many causes.* Science, 2014. **345**(6198): p. 760-5.
13. Bobitt, J.R. and W.J. Ledger, *Unrecognized amnionitis and prematurity: a preliminary report.* J Reprod Med, 1977. **19**(1): p. 8-12.
14. Bobitt, J.R. and W.J. Ledger, *Amniotic fluid analysis. Its role in maternal neonatal infection.* Obstet Gynecol, 1978. **51**(1): p. 56-62.
15. Miller, J.M., Jr., M.J. Pupkin, and G.B. Hill, *Bacterial colonization of amniotic fluid from intact fetal membranes.* Am J Obstet Gynecol, 1980. **136**(6): p. 796-804.
16. Bobitt, J.R., C.C. Hayslip, and J.D. Damato, *Amniotic fluid infection as determined by transabdominal amniocentesis in patients with intact membranes in premature labor.* Am J Obstet Gynecol, 1981. **140**(8): p. 947-52.
17. Wallace, R.L. and C.N. Herrick, *Amniocentesis in the evaluation of premature labor.* Obstet Gynecol, 1981. **57**(4): p. 483-6.

18. Wahbeh, C.J., et al., *Intra-amniotic bacterial colonization in premature labor.* Am J Obstet Gynecol, 1984. **148**(6): p. 739-43.
19. Romero, R. and M. Mazor, *Infection and preterm labor.* Clin Obstet Gynecol, 1988. **31**(3): p. 553-84.
20. Romero, R., et al., *Infection in the pathogenesis of preterm labor.* Semin Perinatol, 1988. **12**(4): p. 262-79.
21. Romero, R., et al., *Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes.* Am J Obstet Gynecol, 1989. **161**(3): p. 817-24.
22. Romero, R., et al., *The role of systemic and intrauterine infection in preterm parturition.* Ann N Y Acad Sci, 1991. **622**: p. 355-75.
23. Gibbs, R.S., et al., *A review of premature birth and subclinical infection.* Am J Obstet Gynecol, 1992. **166**(5): p. 1515-28.
24. Watts, D.H., et al., *The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor.* Obstet Gynecol, 1992. **79**(3): p. 351-7.
25. Gomez, R., et al., *Pathogenesis of preterm labor and preterm premature rupture of membranes associated with intraamniotic infection.* Infect Dis Clin North Am, 1997. **11**(1): p. 135-76.
26. Romero, R., et al., *The role of infection in preterm labour and delivery.* Paediatr Perinat Epidemiol, 2001. **15 Suppl 2**: p. 41-56.
27. Yoon, B.H., et al., *Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes.* Am J Obstet Gynecol, 2001. **185**(5): p. 1130-6.
28. Yoon, B.H., et al., *The frequency and clinical significance of intra-amniotic inflammation in patients with a positive cervical fetal fibronectin.* Am J Obstet Gynecol, 2001. **185**(5): p. 1137-42.
29. Romero, R., et al., *Infection and prematurity and the role of preventive strategies.* Semin Neonatol, 2002. **7**(4): p. 259-74.
30. Shim, S.S., et al., *Clinical significance of intra-amniotic inflammation in patients with preterm premature rupture of membranes.* Am J Obstet Gynecol, 2004. **191**(4): p. 1339-45.
31. Romero, R., et al., *Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury.* Nutr Rev, 2007. **65**(12 Pt 2): p. S194-202.
32. Lee, S.E., et al., *The intensity of the fetal inflammatory response in intraamniotic inflammation with and without microbial invasion of the amniotic cavity.* Am J Obstet Gynecol, 2007. **197**(3): p. 294 e1-6.
33. Lee, S.E., et al., *The frequency and significance of intraamniotic inflammation in patients with cervical insufficiency.* Am J Obstet Gynecol, 2008. **198**(6): p. 633 e1-8.
34. Lee, S.E., et al., *Amniotic fluid volume in intra-amniotic inflammation with and without culture-proven amniotic fluid infection in preterm premature rupture of membranes.* J Perinat Med, 2010. **38**(1): p. 39-44.

35. Madan, I., et al., *The frequency and clinical significance of intra-amniotic infection and/or inflammation in women with placenta previa and vaginal bleeding: an unexpected observation.* J Perinat Med, 2010. **38**(3): p. 275-9.

36. Kim, S.M., et al., *The frequency and clinical significance of intra-amniotic inflammation in women with preterm uterine contractility but without cervical change: do the diagnostic criteria for preterm labor need to be changed?* J Matern Fetal Neonatal Med, 2012. **25**(8): p. 1212-21.

37. Romero, R., et al., *The role of inflammation and infection in preterm birth.* Semin Reprod Med, 2007. **25**(1): p. 21-39.

38. Vrachnis, N., et al., *Intrauterine inflammation and preterm delivery.* Ann N Y Acad Sci, 2010. **1205**: p. 118-22.

39. Romero, R., et al., *Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance.* J Matern Fetal Neonatal Med, 2014: p. 1-17.

40. Romero, R., et al., *Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes.* Am J Reprod Immunol, 2014. **72**(5): p. 458-74.

41. Romero, R., et al., *Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes.* J Matern Fetal Neonatal Med, 2015. **28**(12): p. 1394-409.

42. Romero, R., et al., *Clinical chorioamnionitis at term I: microbiology of the amniotic cavity using cultivation and molecular techniques.* J Perinat Med, 2015. **43**(1): p. 19-36.

43. Romero, R., et al., *Clinical chorioamnionitis at term II: the intra-amniotic inflammatory response.* J Perinat Med, 2016. **44**(1): p. 5-22.

44. Romero, R., et al., *Clinical chorioamnionitis at term III: how well do clinical criteria perform in the identification of proven intra-amniotic infection?* J Perinat Med, 2016. **44**(1): p. 23-32.

45. Romero, R., et al., *Clinical chorioamnionitis at term IV: the maternal plasma cytokine profile.* J Perinat Med, 2016. **44**(1): p. 77-98.

46. Romero, R., et al., *Clinical chorioamnionitis at term V: umbilical cord plasma cytokine profile in the context of a systemic maternal inflammatory response.* J Perinat Med, 2016. **44**(1): p. 53-76.

47. Romero, R., et al., *Clinical chorioamnionitis at term VI: acute chorioamnionitis and funisitis according to the presence or absence of microorganisms and inflammation in the amniotic cavity.* J Perinat Med, 2016. **44**(1): p. 33-51.

48. Gomez-Lopez, N., et al., *Intra-Amniotic Administration of HMGB1 Induces Spontaneous Preterm Labor and Birth.* Am J Reprod Immunol, 2016. **75**(1): p. 3-7.

49. Plazyo, O., et al., *HMGB1 Induces an Inflammatory Response in the Chorioamniotic Membranes That Is Partially Mediated by the Inflammasome.* Biol Reprod, 2016. **95**(6): p. 130.

50. Chaiyasit, N., et al., *Clinical chorioamnionitis at term VIII: a rapid MMP-8 test for the identification of intra-amniotic inflammation.* J Perinat Med, 2017. **45**(5): p. 539-550.

51. Gibbs, R.S., et al., *Quantitative bacteriology of amniotic fluid from women with clinical intraamniotic infection at term.* J Infect Dis, 1982. **145**(1): p. 1-8.

52. Gibbs, R.S., et al., *A randomized trial of intrapartum versus immediate postpartum treatment of women with intra-amniotic infection.* Obstet Gynecol, 1988. **72**(6): p. 823-8.

53. Gilstrap, L.C., 3rd and S.M. Cox, *Acute chorioamnionitis.* Obstet Gynecol Clin North Am, 1989. **16**(2): p. 373-9.

54. Gibbs, R.S. and P. Duff, *Progress in pathogenesis and management of clinical intraamniotic infection.* Am J Obstet Gynecol, 1991. **164**(5 Pt 1): p. 1317-26.

55. Romero, R., et al., *Amniotic fluid white blood cell count: a rapid and simple test to diagnose microbial invasion of the amniotic cavity and predict preterm delivery.* Am J Obstet Gynecol, 1991. **165**(4 Pt 1): p. 821-30.

56. Romero, R., et al., *The diagnostic and prognostic value of amniotic fluid white blood cell count, glucose, interleukin-6, and gram stain in patients with preterm labor and intact membranes.* Am J Obstet Gynecol, 1993. **169**(4): p. 805-16.

57. Romero, R., et al., *A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count, interleukin-6, and gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes.* Am J Obstet Gynecol, 1993. **169**(4): p. 839-51.

58. Gomez, R., et al., *The value of amniotic fluid interleukin-6, white blood cell count, and gram stain in the diagnosis of microbial invasion of the amniotic cavity in patients at term.* Am J Reprod Immunol, 1994. **32**(3): p. 200-10.

59. Yoon, B.H., et al., *Maternal blood C-reactive protein, white blood cell count, and temperature in preterm labor: a comparison with amniotic fluid white blood cell count.* Obstet Gynecol, 1996. **87**(2): p. 231-7.

60. Martinez-Varea, A., et al., *Clinical chorioamnionitis at term VII: the amniotic fluid cellular immune response.* J Perinat Med, 2017. **45**(5): p. 523-538.

61. Romero, R., et al., *Evidence of perturbations of the cytokine network in preterm labor.* Am J Obstet Gynecol, 2015. **213**(6): p. 836 e1-836 e18.

62. Romero, R., et al., *Amniotic fluid prostaglandin levels and intra-amniotic infections.* Lancet, 1986. **1**(8494): p. 1380.

63. Kircher, S. and G. Lubec, *Urinary excretion of acid glycosaminoglycans and its relationship to proteinuria.* Nephron, 1986. **42**(3): p. 275-6.

64. Romero, R., et al., *Amniotic fluid prostaglandin E2 in preterm labor.* Prostaglandins Leukot Essent Fatty Acids, 1988. **34**(3): p. 141-5.

65. Romero, R., et al., *Amniotic fluid concentrations of prostaglandin F2 alpha, 13,14-dihydro-15-keto-prostaglandin F2 alpha (PGFM) and 11-deoxy-13,14-dihydro-15-keto-11, 16-cyclo-prostaglandin E2 (PGEM-LL) in preterm labor.* Prostaglandins, 1989. **37**(1): p. 149-61.

66. Romero, R., et al., *Amniotic fluid arachidonate lipoxygenase metabolites in preterm labor.* Prostaglandins Leukot Essent Fatty Acids, 1989. **36**(2): p. 69-75.

67. Bry, K. and M. Hallman, *Prostaglandins, inflammation, and preterm labor.* J Perinatol, 1989. **9**(1): p. 60-5.

68. Mazor, M., et al., *Changes in amniotic fluid concentrations of prostaglandins E2 and F2 alpha in women with preterm labor.* Isr J Med Sci, 1990. **26**(8): p. 425-8.

69. Hsu, C.D., et al., *Dual roles of amniotic fluid nitric oxide and prostaglandin E2 in preterm labor with intra-amniotic infection.* Am J Perinatol, 1998. **15**(12): p. 683-7.

70. Lee, S.E., et al., *Amniotic fluid prostaglandin F2 increases even in sterile amniotic fluid and is an independent predictor of impending delivery in preterm premature rupture of membranes.* J Matern Fetal Neonatal Med, 2009. **22**(10): p. 880-6.

71. Maddipati, K.R., et al., *Eicosanomic profiling reveals dominance of the epoxygenase pathway in human amniotic fluid at term in spontaneous labor.* FASEB J, 2014. **28**(11): p. 4835-46.

72. Park, J.Y., et al., *An elevated amniotic fluid prostaglandin F2alpha concentration is associated with intra-amniotic inflammation/infection, and clinical and histologic chorioamnionitis, as well as impending preterm delivery in patients with preterm labor and intact membranes.* J Matern Fetal Neonatal Med, 2016. **29**(16): p. 2563-72.

73. Maddipati, K.R., et al., *Lipidomic analysis of patients with microbial invasion of the amniotic cavity reveals up-regulation of leukotriene B4.* FASEB J, 2016. **30**(10): p. 3296-3307.

74. Maddipati, K.R., et al., *Clinical chorioamnionitis at term: the amniotic fluid fatty acyl lipidome.* J Lipid Res, 2016. **57**(10): p. 1906-1916.

75. Gomez, R., et al., *The fetal inflammatory response syndrome.* Am J Obstet Gynecol, 1998. **179**(1): p. 194-202.

76. Gotsch, F., et al., *The fetal inflammatory response syndrome.* Clin Obstet Gynecol, 2007. **50**(3): p. 652-83.

77. Romero, R., et al., *A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition.* Am J Obstet Gynecol, 1998. **179**(1): p. 186-93.

78. Pacora, P., et al., *Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome.* J Matern Fetal Neonatal Med, 2002. **11**(1): p. 18-25.

79. Kim, C.J., et al., *Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance.* Am J Obstet Gynecol, 2015. **213**(4 Suppl): p. S29-52.

80. Redline, R.W., *Classification of placental lesions.* Am J Obstet Gynecol, 2015. **213**(4 Suppl): p. S21-8.

81. Panaitescu, B., et al., *Intrauterine Infection, Preterm Parturition, and the Fetal Inflammatory Response Syndrome*, in *High-Risk Pregnancy:*

Management Options, D. James, et al., Editors. 2017, Cambridge University Press: Cambridge, UK. p. 579-603.

82. Xu, Y., et al., *An M1-like Macrophage Polarization in Decidual Tissue during Spontaneous Preterm Labor That Is Attenuated by Rosiglitazone Treatment.* J Immunol, 2016. **196**(6): p. 2476-2491.

83. St Louis, D., et al., *Invariant NKT Cell Activation Induces Late Preterm Birth That Is Attenuated by Rosiglitazone.* J Immunol, 2016. **196**(3): p. 1044-59.

84. Gomez-Lopez, N., et al., *In vivo activation of invariant natural killer T cells induces systemic and local alterations in T-cell subsets prior to preterm birth.* Clin Exp Immunol, 2017. **189**(2): p. 211-225.

85. Kadam, L., et al., *Rosiglitazone Regulates TLR4 and Rescues HO-1 and NRF2 Expression in Myometrial and Decidual Macrophages in Inflammation-Induced Preterm Birth.* Reprod Sci, 2017: p. 1933719117697128.

86. Mitka, M., *FDA eases restrictions on the glucose-lowering drug rosiglitazone.* JAMA, 2013. **310**(24): p. 2604.

87. Davidson, M.B., G. Bate, and P. Kirkpatrick, *Exenatide.* Nat Rev Drug Discov, 2005. **4**(9): p. 713-4.

88. Drucker, D.J., et al., *Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line.* Proc Natl Acad Sci U S A, 1987. **84**(10): p. 3434-8.

89. Korner, M., et al., *GLP-1 receptor expression in human tumors and human normal tissues: potential for in vivo targeting.* J Nucl Med, 2007. **48**(5): p. 736-43.

90. Lee, Y.S. and H.S. Jun, *Anti-Inflammatory Effects of GLP-1-Based Therapies beyond Glucose Control.* Mediators of inflammation, 2016. **2016**: p. 3094642.

91. Guo, C., et al., *Glucagon-like peptide 1 improves insulin resistance in vitro through anti-inflammation of macrophages.* Braz J Med Biol Res, 2016. **49**(12): p. e5826.

92. Wang, Y., et al., *Exendin-4 decreases liver inflammation and atherosclerosis development simultaneously by reducing macrophage infiltration.* Br J Pharmacol, 2014. **171**(3): p. 723-34.

93. Yanay, O., et al., *Effects of exendin-4, a glucagon like peptide-1 receptor agonist, on neutrophil count and inflammatory cytokines in a rat model of endotoxemia.* J Inflamm Res, 2015. **8**: p. 129-35.

94. Buldak, L., et al., *Exenatide (a GLP-1 agonist) expresses anti-inflammatory properties in cultured human monocytes/macrophages in a protein kinase A and B/Akt manner.* Pharmacol Rep, 2016. **68**(2): p. 329-37.

95. Buldak, L., et al., *Exenatide and metformin express their anti-inflammatory effects on human monocytes/macrophages by the attenuation of MAPKs and NFkappaB signaling.* Naunyn Schmiedebergs Arch Pharmacol, 2016. **389**(10): p. 1103-15.

96. He, L., et al., *Anti-inflammatory effects of exendin-4, a glucagon-like peptide-1 analog, on human peripheral lymphocytes in patients with type 2 diabetes.* J Diabetes Investig, 2013. **4**(4): p. 382-92.

97. Levy, O., *Innate immunity of the newborn: basic mechanisms and clinical correlates.* Nat Rev Immunol, 2007. **7**(5): p. 379-90.

98. Siegrist, C.A., *Vaccination in the neonatal period and early infancy.* Int Rev Immunol, 2000. **19**(2-3): p. 195-219.

99. Adkins, B., *Development of neonatal Th1/Th2 function.* Int Rev Immunol, 2000. **19**(2-3): p. 157-71.

100. Garcia, A.M., et al., *T cell immunity in neonates.* Immunol Res, 2000. **22**(2-3): p. 177-90.

101. Krishnan, S., et al., *Differences in participation of innate and adaptive immunity to respiratory syncytial virus in adults and neonates.* J Infect Dis, 2003. **188**(3): p. 433-9.

102. Firth, M.A., P.E. Shewen, and D.C. Hodgins, *Passive and active components of neonatal innate immune defenses.* Anim Health Res Rev, 2005. **6**(2): p. 143-58.

103. Qing, G., K. Rajaraman, and R. Bortolussi, *Diminished priming of neonatal polymorphonuclear leukocytes by lipopolysaccharide is associated with reduced CD14 expression.* Infect Immun, 1995. **63**(1): p. 248-52.

104. Levy, O., et al., *Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein.* Pediatrics, 1999. **104**(6): p. 1327-33.

105. Yost, C.C., et al., *Neonatal NET-inhibitory factor and related peptides inhibit neutrophil extracellular trap formation.* J Clin Invest, 2016. **126**(10): p. 3783-3798.

106. Gomez-Lopez, N., et al., *Neutrophil Extracellular Traps in the Amniotic Cavity of Women with Intra-Amniotic Infection: A New Mechanism of Host Defense.* Reprod Sci, 2017. **24**(8): p. 1139-1153.

107. Levy, O., et al., *Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848.* J Immunol, 2004. **173**(7): p. 4627-34.

108. de Jong, E., et al., *The phenotype and function of preterm infant monocytes: implications for susceptibility to infection.* J Leukoc Biol, 2017. **102**(3): p. 645-656.

109. Wong, O.H., F.P. Huang, and A.K. Chiang, *Differential responses of cord and adult blood-derived dendritic cells to dying cells.* Immunology, 2005. **116**(1): p. 13-20.

110. Siegrist, C.A. and R. Aspinall, *B-cell responses to vaccination at the extremes of age.* Nat Rev Immunol, 2009. **9**(3): p. 185-94.

111. Sharma, A.A., et al., *The developing human preterm neonatal immune system: a case for more research in this area.* Clin Immunol, 2012. **145**(1): p. 61-8.

112. Adkins, B., C. Leclerc, and S. Marshall-Clarke, *Neonatal adaptive immunity comes of age.* Nat Rev Immunol, 2004. **4**(7): p. 553-64.
113. Cosmi, L., et al., *Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes.* Blood, 2003. **102**(12): p. 4107-14.
114. Nakagawa, T., et al., *IL-6 positively regulates Foxp3+CD8+ T cells in vivo.* Int Immunol, 2010. **22**(2): p. 129-39.
115. Shao, L., et al., *Activation of CD8+ regulatory T cells by human placental trophoblasts.* J Immunol, 2005. **174**(12): p. 7539-47.
116. Gomez-Lopez, N., D.M. Olson, and S.A. Robertson, *Interleukin-6 controls uterine Th9 cells and CD8(+) T regulatory cells to accelerate parturition in mice.* Immunol Cell Biol, 2016. **94**(1): p. 79-89.
117. Arenas-Hernandez, M., et al., *An imbalance between innate and adaptive immune cells at the maternal-fetal interface occurs prior to endotoxin-induced preterm birth.* Cell Mol Immunol, 2016. **13**(4): p. 462-73.
118. Gomez-Lopez, N., et al., *In vivo activation of invariant natural killer T cells induces systemic and local alterations in T-cell subsets prior to preterm birth.* Clin Exp Immunol, 2017.
119. Reibke, R., et al., *CD8+ regulatory T cells generated by neonatal recognition of peripheral self-antigen.* Proc Natl Acad Sci U S A, 2006. **103**(41): p. 15142-7.
120. Gomez-Lopez, N., et al., *Intra-amniotic administration of lipopolysaccharide induces spontaneous preterm labor and birth in the absence of a body temperature change.* J Matern Fetal Neonatal Med, 2017: p. 1-8.
121. Arenas-Hernandez, M., et al., *Isolation of Leukocytes from the Murine Tissues at the Maternal-Fetal Interface.* J Vis Exp, 2015(99): p. e52866.
122. Gravett, M.G., et al., *Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis.* Obstet Gynecol, 1986. **67**(2): p. 229-37.
123. Romero, R., et al., *Intraamniotic infection and the onset of labor in preterm premature rupture of the membranes.* Am J Obstet Gynecol, 1988. **159**(3): p. 661-6.
124. Kramer, B.W., et al., *Dose and time response after intraamniotic endotoxin in preterm lambs.* Am J Respir Crit Care Med, 2001. **164**(6): p. 982-8.
125. Kallapur, S.G., et al., *Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(3): p. L527-36.
126. Ozalkaya, E., et al., *Morbidity in preterm infants with fetal inflammatory response syndrome.* Pediatr Int, 2016. **58**(9): p. 850-4.
127. Yoon, B.H., et al., *Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia.* Am J Obstet Gynecol, 1997. **177**(4): p. 825-30.

128. Ghezzi, F., et al., *Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia.* Eur J Obstet Gynecol Reprod Biol, 1998. **78**(1): p. 5-10.

129. Yoon, B.H., et al., *A systemic fetal inflammatory response and the development of bronchopulmonary dysplasia.* Am J Obstet Gynecol, 1999. **181**(4): p. 773-9.

130. Yoon, B.H., et al., *"Atypical" chronic lung disease of the newborn is linked to fetal systemic inflammation.* Am J Obstet Gynecol, 2002. **187**(6): p. S129.

131. Kodera, R., et al., *Glucagon-like peptide-1 receptor agonist ameliorates renal injury through its anti-inflammatory action without lowering blood glucose level in a rat model of type 1 diabetes.* Diabetologia, 2011. **54**(4): p. 965-78.

132. Carlessi, R., et al., *Exendin-4 attenuates brain death-induced liver damage in the rat.* Liver Transpl, 2015. **21**(11): p. 1410-8.

133. Robinson, E., et al., *Exendin-4 protects against post-myocardial infarction remodelling via specific actions on inflammation and the extracellular matrix.* Basic Res Cardiol, 2015. **110**(2): p. 20.

134. Chicchi, G.G., et al., *Fluorescein-Trp25-exendin-4, a biologically active fluorescent probe for the human GLP-1 receptor.* Peptides, 1997. **18**(2): p. 319-21.

135. Basha, S., N. Surendran, and M. Pichichero, *Immune responses in neonates.* Expert Rev Clin Immunol, 2014. **10**(9): p. 1171-84.

136. Furcron, A.E., et al., *Vaginal progesterone, but not 17alpha-hydroxyprogesterone caproate, has antiinflammatory effects at the murine maternal-fetal interface.* Am J Obstet Gynecol, 2015. **213**(6): p. 846 e1-846 e19.

137. Furcron, A.E., et al., *Human Chorionic Gonadotropin Has Anti-Inflammatory Effects at the Maternal-Fetal Interface and Prevents Endotoxin-Induced Preterm Birth, but Causes Dystocia and Fetal Compromise in Mice.* Biol Reprod, 2016. **94**(6): p. 136.

138. Mold, J.E., et al., *Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero.* Science, 2008. **322**(5907): p. 1562-5.

139. Burt, T.D., *Fetal regulatory T cells and peripheral immune tolerance in utero: implications for development and disease.* Am J Reprod Immunol, 2013. **69**(4): p. 346-58.

140. Kallapur, S.G., et al., *Intra-amniotic IL-1beta induces fetal inflammation in rhesus monkeys and alters the regulatory T cell/IL-17 balance.* J Immunol, 2013. **191**(3): p. 1102-9.

141. Rueda, C.M., et al., *Effect of chorioamnionitis on regulatory T cells in moderate/late preterm neonates.* Hum Immunol, 2015. **76**(1): p. 65-73.

142. *Romero, R., et al., A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic*

infection in preterm labor with intact membranes. Am J Reprod Immunol, 2014. **71**(4): p. 330-58.

143. Yoneda, N., et al., *Polymicrobial Amniotic Fluid Infection with Mycoplasma/Ureaplasma and Other Bacteria Induces Severe Intra-Amniotic Inflammation Associated with Poor Perinatal Prognosis in Preterm Labor.* Am J Reprod Immunol, 2016. **75**(2): p. 112-25.

144. Romero, R., T. Chaiworapongsa, and J. Espinoza, *Micronutrients and intrauterine infection, preterm birth and the fetal inflammatory response syndrome.* J Nutr, 2003. **133**(5 Suppl 2): p. 1668S-1673S.

145. Lee, S.E., et al., *Funisitis in term pregnancy is associated with microbial invasion of the amniotic cavity and intra-amniotic inflammation.* J Matern Fetal Neonatal Med, 2006. **19**(11): p. 693-7.

146. Son, G.H., et al., *Comparative Analysis of Midtrimester Amniotic Fluid Cytokine Levels to Predict Spontaneous Very Pre-term Birth in Patients with Cervical Insufficiency.* Am J Reprod Immunol, 2016. **75**(2): p. 155-61.

147. Romero, R., et al., *Labor and infection. II. Bacterial endotoxin in amniotic fluid and its relationship to the onset of preterm labor.* Am J Obstet Gynecol, 1988. **158**(5): p. 1044-9.

148. Sheinberg, M., et al., *Application of telethermography in the evaluation of preterm premature rupture of the fetal membranes.* Biomed Instrum Technol, 1996. **30**(6): p. 526-30.

149. Goodlin, R.C. and P.G. Brooks, *Abdominal wall hot spots in pregnant women.* J Reprod Med, 1987. **32**(3): p. 177-80.

150. Gotsch, F., et al., *Maternal serum concentrations of the chemokine CXCL10/IP-10 are elevated in acute pyelonephritis during pregnancy.* J Matern Fetal Neonatal Med, 2007. **20**(10): p. 735-44.

151. Madan, I., et al., *The peripheral whole-blood transcriptome of acute pyelonephritis in human pregnancya.* J Perinat Med, 2014. **42**(1): p. 31-53.

152. Tita, A.T. and W.W. Andrews, *Diagnosis and management of clinical chorioamnionitis.* Clin Perinatol, 2010. **37**(2): p. 339-54.

153. Martinelli, P., et al., *Chorioamnionitis and prematurity: a critical review.* J Matern Fetal Neonatal Med, 2012. **25 Suppl 4**: p. 29-31.

154. Farkash, E., et al., *Acute antepartum pyelonephritis in pregnancy: a critical analysis of risk factors and outcomes.* Eur J Obstet Gynecol Reprod Biol, 2012. **162**(1): p. 24-7.

155. Oh, K.J., et al., *Twenty-four percent of patients with clinical chorioamnionitis in preterm gestations have no evidence of either culture-proven intraamniotic infection or intraamniotic inflammation.* Am J Obstet Gynecol, 2017. **216**(6): p. 604 e1-604 e11.

156. Westover, T. and R.A. Knuppel, *Modern management of clinical chorioamnionitis.* Infect Dis Obstet Gynecol, 1995. **3**(3): p. 123-32.

157. Dulay, A.T., et al., *Compartmentalization of acute phase reactants Interleukin-6, C-Reactive Protein and Procalcitonin as biomarkers of intra-amniotic infection and chorioamnionitis.* Cytokine, 2015. **76**(2): p. 236-243.

158. Steven, S., et al., *Gliptin and GLP-1 analog treatment improves survival and vascular inflammation/dysfunction in animals with lipopolysaccharide-induced endotoxemia.* Basic Res Cardiol, 2015. **110**(2): p. 6.

159. Campos, R.V., Y.C. Lee, and D.J. Drucker, *Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse.* Endocrinology, 1994. **134**(5): p. 2156-64.

160. Ouhilal, S., et al., *Hypoglycemia, hyperglucagonemia, and fetoplacental defects in glucagon receptor knockout mice: a role for glucagon action in pregnancy maintenance.* Am J Physiol Endocrinol Metab, 2012. **302**(5): p. E522-31.

161. Romani-Perez, M., et al., *Pulmonary GLP-1 receptor increases at birth and exogenous GLP-1 receptor agonists augmented surfactant-protein levels in litters from normal and nitrofen-treated pregnant rats.* Endocrinology, 2013. **154**(3): p. 1144-55.

162. Shiraishi, D., et al., *Glucagon-like peptide-1 (GLP-1) induces M2 polarization of human macrophages via STAT3 activation.* Biochem Biophys Res Commun, 2012. **425**(2): p. 304-8.

163. Darsalia, V., et al., *Exendin-4 reduces ischemic brain injury in normal and aged type 2 diabetic mice and promotes microglial M2 polarization.* PLoS One, 2014. **9**(8): p. e103114.

164. Stein, M., et al., *Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation.* J Exp Med, 1992. **176**(1): p. 287-92.

165. Doyle, A.G., et al., *Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma.* Eur J Immunol, 1994. **24**(6): p. 1441-5.

166. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm.* J Immunol, 2000. **164**(12): p. 6166-73.

167. Anderson, C.F. and D.M. Mosser, *A novel phenotype for an activated macrophage: the type 2 activated macrophage.* J Leukoc Biol, 2002. **72**(1): p. 101-6.

168. Zhao, P., et al., *Response gene to complement 32 (RGC-32) expression on M2-polarized and tumor-associated macrophages is M-CSF-dependent and enhanced by tumor-derived IL-4.* Cell Mol Immunol, 2015. **12**(6): p. 692-9.

169. Nair, M.G. and D.R. Herbert, *Immune polarization by hookworms: taking cues from T helper type 2, type 2 innate lymphoid cells and alternatively activated macrophages.* Immunology, 2016. **148**(2): p. 115-24.

170. Xie, Y., et al., *Toxoplasma gondii GRA15II effector-induced M1 cells ameliorate liver fibrosis in mice infected with Schistosomiasis japonica.* Cell Mol Immunol, 2016.

171. Mosser, D.M., *The many faces of macrophage activation.* J Leukoc Biol, 2003. **73**(2): p. 209-12.

172. Verreck, F.A., et al., *Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4560-5.

173. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends Immunol, 2004. **25**(12): p. 677-86.

174. Pesce, J.T., et al., *Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis.* PLoS Pathog, 2009. **5**(4): p. e1000371.

175. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.* Nat Immunol, 2010. **11**(10): p. 889-96.

176. Biswas, S.K. and A. Mantovani, *Orchestration of metabolism by macrophages.* Cell Metab, 2012. **15**(4): p. 432-7.

177. Moore, S.M., et al., *Fatty acid-binding protein 5 limits the anti-inflammatory response in murine macrophages.* Mol Immunol, 2015. **67**(2 Pt B): p. 265-75.

178. Shiratori, H., et al., *THP-1 and human peripheral blood mononuclear cell-derived macrophages differ in their capacity to polarize in vitro.* Mol Immunol, 2017. **88**: p. 58-68.

179. Gustafsson, C., et al., *Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype.* PLoS One, 2008. **3**(4): p. e2078.

180. Svensson, J., et al., *Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10.* J Immunol, 2011. **187**(7): p. 3671-82.

181. Kim, S.Y., et al., *Methylome of fetal and maternal monocytes and macrophages at the feto-maternal interface.* Am J Reprod Immunol, 2012. **68**(1): p. 8-27.

182. Gomez-Lopez, N., et al., *Immune cells in term and preterm labor.* Cell Mol Immunol, 2014. **11**(6): p. 571-81.

183. Kwan, M., et al., *Dynamic changes in maternal decidual leukocyte populations from first to second trimester gestation.* Placenta, 2014. **35**(12): p. 1027-34.

184. Svensson-Arvelund, J. and J. Ernerudh, *The Role of Macrophages in Promoting and Maintaining Homeostasis at the Fetal-Maternal Interface.* Am J Reprod Immunol, 2015. **74**(2): p. 100-9.

185. Svensson-Arvelund, J., et al., *The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages.* J Immunol, 2015. **194**(4): p. 1534-44.

186. Itoh, M., et al., *Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance.* J Immunol, 1999. **162**(9): p. 5317-26.

187. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.

188. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
189. Khattri, R., et al., *An essential role for Scurfin in CD4+CD25+ T regulatory cells.* Nat Immunol, 2003. **4**(4): p. 337-42.
190. Chang, J.H., H. Hu, and S.C. Sun, *Survival and maintenance of regulatory T cells require the kinase TAK1.* Cell Mol Immunol, 2015. **12**(5): p. 572-9.
191. Gao, Q., et al., *CD4+CD25+ cells regulate CD8 cell anergy in neonatal tolerant mice.* Transplantation, 1999. **68**(12): p. 1891-7.
192. Li, S., et al., *A naturally occurring CD8(+)CD122(+) T-cell subset as a memory-like Treg family.* Cell Mol Immunol, 2014. **11**(4): p. 326-31.
193. Adams, B., et al., *CD8+ T lymphocytes regulating Th2 pathology escape neonatal tolerization.* J Immunol, 2003. **171**(10): p. 5071-6.
194. Field, A.C., et al., *Regulatory CD8+ T cells control neonatal tolerance to a Th2-mediated autoimmunity.* J Immunol, 2003. **170**(5): p. 2508-15.

Supplemental material

Supplementary Table 1. Antibodies used for immunophenotyping.

Antigen	Symbol	Fluorophore	Clone	Company	Catalog number
CD11b	CD11b	PE-CF594	M1/70	BD Biosciences	562287
Lymphocyte antigen 6G	Ly6G	APC	1A8	BD Biosciences	560599
F4/80	F4/80	APC-eFluor 780	BM8	eBioscience	47-4801-82
CD3	CD3	FITC	145-2C11	BD Biosciences	533062
CD4	CD4	APC	RM4-5	BD Biosciences	553051
CD8	CD8	PE-CF594	53-6.7	BD Biosciences	562283
CD25	CD25	PECy7	PC61	BD Biosciences	552880
Inducible nitric oxide synthase 2	iNOS	PE	CXNFT	eBioscience	12-5920-82
Interleukin 10	IL-10	AF700	JES5-16E3	eBioscience	56-7101-82
Forkhead box p3	Foxp3	V450	MF23	BD Biosciences	561293

Supplementary Table 2. TaqMan® gene expression assays used in this study

Gene name	Gene Symbol	Assay ID
Actin, beta	*Actb*	Mm04394036_g1
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	Mm99999915_g1
Glucuronidase, beta	*Gusb*	Mm01197698_m1
Heat shock protein 90 alpha (cytosolic), class B member 1	*Hsp90ab1*	Mm00833431_g1
Interleukin 1 beta	*Il1b*	Mm00434228_m1
Interferon gamma	*Ifng*	Mm01168134_m1
Interleukin 6	*Il6*	Mm00446190_m1
Interleukin 18	*Il18*	Mm00434226_m1
Interleukin 23, alpha subunit p19	*Il23a*	Mm00518984_m1
Interleukin 33	*Il33*	Mm00505403_m1
Transforming growth factor, beta 1	*Tgfb1*	Mm01178820_m1
NLR family, pyrin domain containing 3	*Nlrp3*	Mm00840904_m1
Caspase 1	*Casp1*	Mm00438023_m1
SR-related CTD-associated factor 11	*Scaf11 (Casp11)*	Mm01297328_m1
Antigen identified by monoclonal antibody Ki 67	*Mki67 (Ki-67)*	Mm01278617_m1
Chemokine (C-C motif) ligand 2	*Ccl2*	Mm00441242_m1
Chemokine (C-C motif) ligand 3	*Ccl3*	Mm00441259_g1
Chemokine (C-C motif) ligand 5	*Ccl5*	Mm01302427_m1
Chemokine (C-C motif) ligand 17	*Ccl17*	Mm01244826_g1
Chemokine (C-C motif) ligand 22	*Ccl22*	Mm00436439_m1
Chemokine (C-X-C motif) ligand 1	*Cxcl1*	Mm04207460_m1
Chemokine (C-X-C motif) ligand 10	*Cxcl10*	Mm00445235_m1

Tumor necrosis factor	*Tnf*	Mm00443258_m1
Gene name	**Gene Symbol**	**Assay ID**
Selectin, lymphocyte	*Sell (L-selectin)*	Mm00441291_m1
Intercellular adhesion molecule 1	*Icam1*	Mm00516023_m1
Intercellular adhesion molecule 2	*Icam2*	Mm00494862_m1
Vascular cell adhesion molecule 1	*Vcam1*	Mm01320970_m1
CD3 antigen, epsilon polypeptide	*Cd3e*	Mm01179194_m1
Nucleotide-binding oligomerization domain containing 1	*Nod1*	Mm00805062_m1
Nucleotide-binding oligomerization domain containing 2	*Nod2*	Mm00467543_m1
High mobility group box 1	*Hmgb1*	Mm00849805_gH
NLR family, CARD domain containing 4	*Nlrc4*	Mm01233151_m1
NLR family, pyrin domain containing 1A	*Nlrp1a*	Mm03047263_m1
Absent in melanoma 2	*Aim2*	Mm01295719_m1
PYD and CARD domain containing	*Pycard*	Mm00445747_g1