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Iroh

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RESUMEN

El trabajo de parto pre-término precede comúnmente al nacimiento prematuro, y a nivel mundial es la causa principal de morbilidad y mortalidad perinatal. La mayoría de los estudios realizados se han enfocado a establecer una conexión entre la activación del sistema inmune innato y la inflamación patológica que induce el parto y nacimiento pre-término. Sin embargo, el papel de los linfocitos T efectores de origen materno en la patogenicidad del parto pre-término no ha sido estudiado en detalle. En el presente trabajo demostramos que las células T efectoras y de memoria efectora que expresaron granzima B y perforina estaban enriquecidas en la interface materno-fetal de mujeres que presentaron parto pre-término. Desarrollamos un modelo murino de activación sistémica de linfocitos T inyectando un anticuerpo monoclonal anti-CD3ε a hembras preñadas en el día 16.5 *post coitum*. En este modelo se observó activación de células T en la interfase materno-fetal y en el miometrio, nacimientos prematuros del 80-100% de los animales, y efectos adversos a nivel fetal y neonatal. También reportamos que antes de la inducción del nacimiento pre-término, la activación de células T causó respuestas inflamatorias en la interfase materno-fetal, el miometrio y el cérvix, se indujo una respuesta inflamatoria fetal (líquido amniótico), así como un incremento de macrófagos pro-inflamatorios con fenotipo M1 y ausencia de infiltración de neutrófilos en la interfase materno-fetal. Posteriormente se demostró que el tratamiento con progesterona puede servir como estrategia para prevenir el parto y nacimiento prematuro, así como los efectos adversos en los neonatos a través de la atenuación de las respuestas pro-inflamatorias causadas por la activación de las células T en la interfase materno-fetal y en el cérvix. Estos resultados indican que las células T efectoras causan inflamación patológica en la interfase materno-fetal, en la madre y en el feto, e inducen el parto y el nacimiento pre-término, al igual que efectos adversos en los neonatos. Estos efectos adversos pueden ser prevenidos por el tratamiento con progesterona, una estrategia clínicamente aprobada.

ABSTRACT

Preterm labor commonly precedes preterm birth, the leading cause of perinatal morbidity and mortality worldwide. Most research has focused on establishing a causal link between innate immune activation and pathological inflammation leading to preterm labor and birth. However, the role of maternal effector/activated T cells in the pathogenesis of preterm labor/birth is poorly understood. In this study, we first demonstrated that effector memory and activated maternal T cells expressing granzyme B and perforin are enriched at the maternal-fetal interface (decidua) of women with spontaneous preterm labor. Next, using a murine model, the monoclonal anti-CD3ε antibody was injected in pregnant dams at 16.5 days *post coitum* and induced T-cell activation at the maternal-fetal interface and myometrium, resulting in 80-100% of preterm labor/birth and adverse fetal and neonatal outcomes. We also reported that prior to inducing preterm birth, *in vivo* T cell activation caused inflammatory responses at the maternal-fetal interface, myometrium, and cervix, fetal inflammatory response (i.e. amniotic fluid), as well as an increased in proinflammatory macrophages with phenotype M1, and the absence of an increased influx of neutrophils at the maternal-fetal interface. Then, we showed that treatment with progesterone can serve as a strategy to prevent preterm labor/birth and adverse neonatal outcomes by attenuating the proinflammatory responses at the maternal-fetal interface and cervix induced by T cell activation. Collectively, these findings provide mechanistic evidence showing that effector and activated T cells cause pathological inflammation at the maternal-fetal interface, in the mother, and in the fetus, inducing preterm labor and birth and adverse neonatal outcomes. Such adverse effects can be prevented by treatment with progesterone, a clinically approved strategy.

ABBREVIATIONS

µL – Microliters

AI = Acute inflammatory lesions of the placenta

Anti-CD3ε – Anti-mouse CD3 epsilon

Arg1 – Arginase 1

CCL – C-C Motif Chemokine Ligand

cDNA – Complementary deoxyribonucleic acid

CI = Chronic inflammatory lesions of the placenta

CXCL – Chemokine (C-X-C motif) ligand

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

dpc – days post coitum

DT – Diphtheria toxin

FcR – Fc Receptor

Foxp3 – Forkhead box P3

g – Grams

G-CSF – Granulocyte colony-stimulating factor

GM-CSF – Granulocyte-Macrophage colony-stimulating factor

h – Hours

i.p. – Intraperitoneal

IFN – Interferon

IL – Interleukin

iNOS – Inducible nitric oxide synthase 2

IQR – Interquartile range

L – Litters

LIF – Leukemia inhibitor factor

LPS – Lipopolysaccharide

M-CSF – Macrophage colony-stimulating factor

MHz – Megahertz

min – Minutes

mL – Milliliters

P4 – Progesterone

PBS – Phosphate buffered saline

PreAmp – Pre-amplification

PTL – Women who delivered preterm with labor

PTNL – Women who delivered preterm without labor

qRT-PCR –Quantitative RT-PCR

RNA – Ribonucleic acid

RT-PCR – Reverse Transcription Polymerase Chain Reaction

RU486 – Mifepristone (progesterone receptor antagonist)

s.c. – Subcutaneous

SO – Sesame oil

T_{CM} – Central Memory T cells

Teff – Effector T cells

T_{EM} – Effector Memory T cells

T_{EMRA} – Terminally Differentiated Effector Memory T cells

TIL – Women who delivered at term with labor

T_N – Naïve T cells

TNF – Tumor necrosis factor

TNL – Women who delivered at term without labor

1. INTRODUCTION

1.1. Pregnancy

Pregnancy is the term used to describe the period from fecundation and the development of the fetus inside the uterus. During pregnancy, women undergo significant anatomical and physiological changes in order to nourish the developing fetus [1]. These changes begin upon conception and every organ system in their bodies are affected [2]. For most women experiencing an uncomplicated pregnancy, these changes resolve after pregnancy with minimal residual effects [1].

Term pregnancy has been defined as one in which 260–294 days (in average 40 weeks) have elapsed since the first day of the last menstrual period [3]. Neonates born before this interval (less than 37 weeks of pregnancy) are classified as preterm, whereas neonates delivered beyond this interval (42 weeks and beyond) are designated postterm [3].

1.2. Preterm birth

Preterm birth, defined as birth before 37 weeks of gestation, is the leading cause of perinatal morbidity and mortality worldwide [4, 5]. Nearly two-thirds of all cases of preterm birth are preceded by spontaneous preterm labor [6-8], a syndrome of multiple pathological processes [9, 10]. Of all the putative causes associated with spontaneous preterm labor, only pathological inflammation has been causally linked to preterm birth [11-15].

Inflammation is implicated in the physiological and pathological processes of labor in term and preterm gestations [9, 16-18]. Pathological inflammation can be triggered by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (i.e. alarmins) [19-22]. PAMPs and DAMPs are sensed by pattern recognition receptors (PRRs), which are mainly present in innate immune cells [23]. Therefore, most of the perinatal immunology research has focused on the role of innate immunity in the mechanisms that lead to

preterm labor [24-37]. Indeed, the stimulation of neutrophils/macrophages by administration of an endotoxin [38, 39] or activation of invariant natural killer T cells via alpha-galactosylceramide [40, 41] induces preterm labor and birth. However, pathological inflammation can also be mediated by T cells, the cellular component of the adaptive immune system [42].

1.3. Maternal-fetal interface

The maternal-fetal interface represents the site of immune interactions between the mother and the conceptus [43-47]. In humans, the maternal-fetal interface includes: 1) the decidua parietalis, the area of contact between the myometrium and the chorioamniotic membranes, and 2) the decidua basalis, the interface between the myometrium and the placenta [48, 49]. T cells have been implicated in implantation [50-53] and pregnancy maintenance through the mediation of maternal-fetal tolerance [54-69], and their infiltration at the maternal-fetal interface (i.e. decidua) has been associated with the physiological process of labor at term [49, 70-73] and the syndrome of preterm labor and birth [74-76]. However, a mechanistic link between maternal T cells and the pathophysiology of preterm labor and birth is lacking.

1.4. T cells in term and preterm labor

We previously proposed that maternal circulating T cells infiltrate into the maternal-fetal interface prior to delivery and during labor at term [70, 71]. Decidual T cells are activated, and have both a regulatory and an effector phenotype [48, 77, 78]. Decidual CD4+ T cells are involved in term parturition [49]. Specifically, it was demonstrated that decidual CD4+ T cells are higher in term than in preterm gestations without labor. These T cells express CD45RO, but not CD45RA, which suggests that they are memory cells generated in early pregnancy, when fetal–antigen presentation occurs [49, 63, 79]. Moreover, it was demonstrated that decidual CD4+ T cells express IL-1β, TNF-α and MMP-9 during spontaneous labor at term [49]. The fact that decidual T cells express activation markers such as

CD25 [80], and labor mediators implicated in both term and preterm labor [81-90], suggests that the adaptive limb of the immune system participates during labor.

It was also demonstrated that, during term labor, T cells are preferentially recruited into the rupture zone of the fetal membranes by chemotactic processes facilitated by CXCL10 and CCL5 [49, 71, 91]. However, T-cell attraction to the rupture zone was significantly diminished in premature rupture of membrane (PROM) cases [91]. These data suggest that T-cell recruitment into the maternal-fetal interface is required for term pregnancy, and the dysregulation of this recruitment may lead to pathological rupture of membranes.

1.5. Chronic chorioamnionitis

A clinical observation is chronic chorioamnionitis, a placental lesion in which maternal T cells infiltrate the fetal tissues (e.g. chorioamniotic membranes) through the decidua [76], is strongly associated with preterm labor and birth [74], and women with this condition display a systemic T-cell mediated cytotoxicity [92]. In line with this evidence, it was also shown that transcriptional silencing limits the infiltration of T cells and other immune cells into the maternal-fetal interface [93-95], suggesting that an uncontrolled invasion of effector T cells may lead to pregnancy complications. These findings further support the idea that activation of T cells (i.e. effector T cells) at the maternal-fetal interface can lead to preterm labor/birth.

Therefore, we hypothesized that T-cell activation via CD3ε molecule in late pregnancy would initiate innate and adaptive immune responses at the maternal-fetal interface, developing pathological inflammation and leading to preterm labor/birth.

2. JUSTIFICATION

Preterm birth is the leading cause of neonatal morbidity and mortality worldwide. Preterm birth is preceded by preterm labor, which is a syndrome of multiple etiologies. Pathological inflammation is implicated in several of the putative causes of preterm labor. Therefore, it is highly important to understand the mechanisms that lead to pathological inflammation in late pregnancy which, in turn, could lead to preterm labor and birth.

3. HYPOTHESIS

We hypothesized that the activation of maternal T cells at the maternal-fetal interface will induce preterm labor and birth.

4. GENERAL AIM

To determine the role of effector/activated T cells in the pathogenesis of preterm labor/birth

5. PARTICULAR AIMS

1. To determine the activation of T cells at the human maternal-fetal interface in preterm birth
2. To induce the *in vivo* activation of T cells using a monoclonal anti-CD3ε during late pregnancy
3. To develop a new preterm birth animal model through T-cell activation during late pregnancy and compare it with two stablished preterm birth models (LPS- or RU486-induced preterm labor/birth)
4. To characterize the maternal and fetal inflammatory responses prior to *in vivo* T-cell activation-induced preterm birth
5. To elucidate whether *in vivo* T-cell activation affects the innate immunity at the maternal-fetal interface and myometrium prior to preterm birth
6. To determine whether *in vivo* T-cell activation-induced preterm birth be prevented

6. MATERIALS AND METHODS

Human subjects, clinical specimens, and definitions

Human placental basal plate (decidua basalis; Figure 1) and chorioamniotic membrane (decidua parietalis; Figure 1) samples were obtained at the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services, Wayne State University (Detroit, MI, USA), and the Detroit Medical Center (Detroit, MI, USA). The collection and use of human materials for research purposes were approved by the Institutional Review Boards of the National Institute of Child Health and Human Development and Wayne State University. All participating women provided written informed consent prior to sample collection. The study groups included women who delivered at term with labor (TIL), at term without labor (TNL), preterm with labor (PTL), or preterm without labor (PTNL). Three separate cohorts of women were used in this study: one for the immunophenotyping of effector T cells, and a second cohort for the immunophenotyping of activated T cells. The demographic and clinical characteristics of the study groups are shown in Tables I and II. Labor was defined by the presence of regular uterine contractions at a frequency of at least two contractions every 10 min with cervical changes resulting in delivery. Preterm delivery was defined as delivery before 37 weeks of gestation. Patients with multiple births or neonates that had congenital or chromosomal abnormalities were excluded from this study.

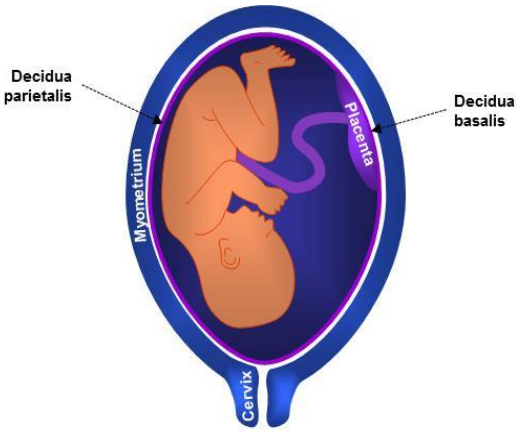


Figure 1. Schematic representation showing the human decidua basalis and decidua parietalis.

Table I. Demographic and clinical characteristics of the study population for immunophenotyping of effector and naïve T cells in decidual tissues

	TNL (n=20)	**TIL (n=55)**	**PTNL (n=15)**	**PTL (n=50)**	**p value**
Age (y; median [IQR])[a]	27 (24-29.3)	25 (22-29)	29 (24.5-35.5)	23 (21.3-26.3)	0.01
Body mass index (kg/m^2; median [IQR])[a]	27.6 (24.5-29.7)	29.3 (24.6-34)	27.5 (24.9-35.8)	24.9 (21.6-29.3)	0.03
Gestational age at delivery (week; median [IQR])[a]	39.1 (39-39.3)	39.4 (38.6-40.7)	33.9 (28.9-36.3)	34.2 (31.2-35.9)	<0.001
Birth weight (g; median [IQR])[a]	3232.5 (2911.3-3658.8)	3205 (3007.5-3506.3)	1930 (1082.5-2385)	1977.5 (1466.3-2343.8)	<0.001
Race (n[%])[b] African-American Caucasian Hispanic Asian Other	 13 (65%) 3 (15%) 2 (10%) 2 (10%) 0 (0%)	 51 (92.7%) 3 (5.5%) 0 (0%) 0 (0%) 1 (1.8%)	 11 (73.3%) 3 (20%) 0 (0%) 0 (0%) 1 (6.7%)	 44 (88%) 1 (2%) 0 (0%) 2 (4%) 3 (6%)	0.004
Primiparity (n[%])[b]	2 (10%)	7 (12.7%)	3 (20%)	11 (22%)	NS
Cesarean section (n[%])[b]	20 (100%)	6 (10.9%)	15 (100%)	9 (18%)	<0.001

Data are given as median (interquartile range, IQR) and percentage (n/N).
TNL = term without labor; TIL = term with labor; PTNL = preterm without labor; PTL = preterm labor
[a]Kruskal-Wallis test
[b]Fisher's exact test

Table II. Demographic and clinical characteristics of the study population for immunophenotyping of activated T cells in decudual tissues

	TNL (n=20)	TIL (n=37)	PTNL (n=18)	PTL (n=53)	p value
Age (y; median [IQR])[a]	24 (22-30.3)	26 (22-31)	30 (26-33)	24 (20-29)	0.02
Body mass index (kg/m^2; median [IQR])[a]	33.7 (27.2-37.1)	28.5 (24.2-35.6)	27.8 (24-35.4)	26.4 (21.5-31.3)	NS
Gestational age at delivery (week; median [IQR])[a]	39 (38.8-39.3)	38.9 (38.3-39.4)	33.4 (30.5-34.4)	34.9 (33.9-35.7)	<0.001
Birth weight (g; median [IQR])[a]	3355 (2872.5-3511.3)	3185 (2735-3495)	1420 (1205.3-2095)	2255 (1830-2540)	<0.001
Race (n[%])[b] African-American Caucasian Hispanic Asian Other	 15 (75%) 3 (15%) 0 (0%) 1 (5%) 1 (5%)	 35 (94.6%) 1 (2.7%) 0 (0%) 0 (0%) 1 (2.7%)	 12 (66.6%) 4 (22.2%) 0 (0%) 1 (5.6%) 1 (5.6%)	 46 (86.8%) 5 (9.4%) 0 (%) 0 (%) 2 (3.8%)	NS
Primiparity (n[%])[b]	0 (0%)	10 (27%)	3 (16.7%)	14 (26.4%)	0.03
Cesarean section (n[%])[b]	20 (100%)	4 (10.8%)	18 (100%)	12 (22.6%)	<0.001

Data are given as median (interquartile range, IQR) and percentage (n/N).
TNL = term without labor; TIL = term with labor; PTNL = preterm without labor; PTL = preterm labor
[a]Kruskal-Wallis test
[b]Fisher's exact test

Decidual leukocyte isolation from human samples

Decidual leukocytes from human decidual tissue were isolated as previously described [96]. Briefly, the decidua basalis was collected from the basal plate of the placenta, and the decidua parietalis was separated from the chorioamniotic membranes. Decidual tissue was homogenized using a gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA) in StemPro Cell Dissociation Reagent (Life Technologies, Grand Island, NY, USA). Homogenized tissues were incubated for 45 min at 37°C with gentle agitation. After incubation, tissues were washed with

ice-cold 1X PBS and filtered through a 100-μm cell strainer. Cell suspensions were collected and centrifuged at 300 x g for 10 min at 4°C, and the cell pellet was suspended in stain buffer (Cat. no. 554656; BD Biosciences, San Jose, CA, USA). Mononuclear cells were purified using a density gradient (Ficoll-Paque Plus; GE Healthcare Bio-Sciences, Uppsala, Sweden), following the manufacturer's instructions. Then, mononuclear cell suspensions were washed using stain buffer before immunophenotyping.

Immunophenotyping of human decidual leukocytes

Mononuclear cell suspensions from decidual tissues were stained with BD Horizon Fixable Viability Stain 510 dye (BD Biosciences) prior to incubation with extracellular and intracellular monoclonal antibodies. Mononuclear cell suspensions were washed with stain buffer and centrifuged. Cell pellets were incubated for 10 min with 20 μL of human FcR Blocking Reagent (Miltenyi Biotec) in 80 μL of stain buffer. Next, mononuclear cell suspensions were incubated with extracellular fluorochrome-conjugated anti-human monoclonal antibodies (Table III) for 30 min at 4°C in the dark. After extracellular staining, the cells were fixed. For intracellular staining, the cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) prior to incubation with intracellular monoclonal antibodies (Table III). Finally, mononuclear cell suspensions were washed and resuspended in 0.5 mL of stain buffer and acquired using the BD LSRFortessa flow cytometer and FACSDiva 6.0 software. Leukocyte subsets were gated within the viability gate. Immunophenotyping included the identification of effector memory T cells (T_{EM}; CD3+CD4+ or CD3+CD4− CD45RA−CCR7−), naive T cells (T_N; CD3+CD4+ or CD3+CD4− CD45RA+CCR7+), central memory T cells (T_{CM}; CD3+CD4+ or CD3+CD4− CD45RA−CCR7+), and terminally differentiated effector memory T cells (T_{EMRA}; CD3+CD4+ or CD3+CD4− CD45RA+CCR7−). Activated CD4+ and CD8+ T cells were evaluated by the expression of granzyme B and/or perforin. The data analysis was performed using FlowJo software v10 (TreeStar, Ashland, OR, USA).

Table III. List of anti-human antibodies used for immunophenotyping

Anti-human antibodies			
Cell marker	**Fluorochrome**	**Clone**	**Company**
CD3ε	BUV737	UCHT1	BD Biosciences
CD4	APC-H7	RPA-T4	BD Biosciences
CD8α	BUV395	RPA-T8	BD Biosciences
Granzyme B	PE-CF594	GB11	BD Biosciences
Perforin	Alexa Fluor 488	δG9	BD Biosciences
IgG1κ Isotype control	PE-CF594	X40	BD Biosciences
IgG2b κ Isotype control	Alexa Fluor 488	27-35	BD Biosciences
CD45	APC-Cy7	2D1	BD Biosciences
CD3	BV421	UCHT1	BD Biosciences
CD4	Alexa Fluor 488	RPA-T4	BD Biosciences
CD45RA	Alexa Fluor 700	HI100	BD Biosciences
CCR7	PE-Cy7	3D12	BD Biosciences

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred in the animal care facility at the C.S. Mott Center for Human Growth and Development, Wayne State University, Detroit, MI, and housed under a circadian cycle (light:dark = 12:12 h). Eight- to twelve-week-old C57BL/6 females were mated with C57BL/6 males of proven fertility. Female mice were examined daily between 8:00 AM and 9:00 AM for the presence of a vaginal plug, which indicated 0.5 days *post coitum* (dpc). Upon observation of vaginal plugs, female mice were removed from the mating cages and housed separately. A weight gain more than 2 g confirmed pregnancy at 12.5 dpc. All animal experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol No. A-09-08-12, A-07-03-15, and 18-03-0584), following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal models of preterm labor and birth

Anti-CD3ε–induced preterm labor/birth [97]. Dams were injected i.p. with 10 μg/200 μL monoclonal anti-CD3ε antibody (Clone 145-2C11; BD Biosciences) dissolved in sterile 1X PBS (Fisher Scientific Chemicals, Fair Lawn, NJ, USA) using a 26-gauge needle on 16.5 dpc. Controls were i.p. injected with 10 μg/200 μL of IgG1 κ isotype control (Clone A19-3; BD Biosciences) dissolved in sterile 1X PBS (n = 4–7 each).

LPS-induced preterm labor/birth [38, 98]. Dams were injected i.p. with 15 μg/150μL LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, St Louis, MO, USA) dissolved in sterile 1X PBS using a 26-gauge needle on 16.5 dpc. Controls were injected with only 150 μL of sterile 1X PBS (n = 7–8 each).

RU486-induced preterm labor/birth [99]. Dams were injected s.c. with 150 μg/100 μL of RU486 (mifepristone) (Sigma Aldrich) dissolved in DMSO (Sigma-Aldrich) and diluted 1:13 in sterile 1X PBS or 100 μL of DMSO diluted 1:13 in sterile 1X PBS as a control on 15.5 dpc (n = 3–8 each).

Following injection, pregnant mice were monitored using a video camera with infrared light (Sony, Tokyo, Japan) until delivery. Gestational length was calculated from the presence of the vaginal plug (0.5 dpc) until the observation of the first pup in the cage bedding. Preterm birth was defined as delivery < 18.5 dpc. Neonatal mortality was determined by the number of dead pups among the total number of the litter size.

In vivo imaging by ultrasound

Pregnant dams were anesthetized on 17.0 dpc by inhalation of 2-3% isoflurane (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. Dams were positioned on a heated platform and stabilized using adhesive tape. Fur was removed from the abdomen and thorax following the application of Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to these areas. Body temperature was maintained at 37±1˚C and monitored using a rectal probe. Respiratory and heart rates were monitored by electrodes embedded on the heated platform. An ultrasound probe from the Vevo 2100 (VisualSonics, Toronto, Ontario, Canada) was fixed and mobilized with a mechanical holder (VisualSonics), and the transducer was slowly moved over the abdomen to determine fetal heart rate and umbilical artery pulsatility index.

Leukocyte isolation from murine decidual, myometrial, and lymphatic tissues

Isolation of leukocytes from decidual and myometrial tissues was performed, as previously described [100]. Briefly, tissues were cut into small pieces using fine scissors and enzymatically digested with StemPro Cell Dissociation Reagent for 35 min at 37°C. The uterine-draining lymph nodes (ULN), and spleen were also collected and leukocyte suspensions were prepared, as previously reported [38]. Leukocytes were filtered using a 100 µm cell strainer and washed with FACS buffer [0.1% BSA (Sigma-Aldrich) and 0.05% sodium azide (Fisher Scientific Chemicals) in 1X PBS)] before immunophenotyping.

Immunophenotyping of decidual, myometrial, and lymphatic leukocytes

Leukocyte suspensions from decidual, myometrial, and lymphatic tissues were centrifuged at 1250 x g for 10 min at 4°C. Cell pellets were then incubated with the CD16/CD32 monoclonal antibodies (FcγIII/II receptor; BD Biosciences) for 10 min and subsequently incubated with specific fluorochrome-conjugated anti-mouse monoclonal antibodies (Table IV) for 30 min at 4°C in the dark. Leukocyte suspensions were fixed/permeabilized with the BD Cytofix/Cytoperm Fixation/Permeabilization kit prior to staining with intracellular Abs. Cells were acquired using the BD LSRFortessa flow cytometer and FACSDiva 8.0 software. Immunophenotyping included the identification of T cells (CD3+CD4+ and CD3+CD8+ cells) and their activation status by the expression of CD25, CD69, IL-2, and IFN-γ in the decidual, myometrial, and lymphatic tissues. M1- and M2-like macrophage phenotypes (CD11b+F4/80+iNOS+ or Arg1+), and neutrophils (CD45+F4/80−Ly6G+) were also determined in the decidual and myometrial tissues. Data were analyzed using FlowJo software v10.

Table IV. List of anti-mouse antibodies used for immunophenotyping

Anti-mouse antibodies			
Cell marker	**Fluorochrome**	**Clone**	**Company**
CD45	Alexa Fluor 700	30-F11	BD biosciences
CD3ε	APC-Cy7	145-2C11	BD biosciences
CD4	APC	RM4-5	BD biosciences
CD8α	PE-CF594	53-6.7	BD biosciences
CD45	AF700	30-F11	BD biosciences
CD3	BUV395	145-2C11	BD biosciences
CD4	PECy5	RM4-5	BD biosciences
CD8	BV421	53-6.7	BD biosciences
CD69	PE-CF594	H1.2F3	BD biosciences
CD25	BV510	PC61	BD biosciences
CD11b	PE-CF594	M1/70	BD biosciences
F4/80	APC-eFluor780	BM8	eBioscience
F4/80	PE	BM8	eBioscience
Ly6G	APC-Cy7	1A8	BD biosciences

IL-2	AF700	JES6-5H4	BD biosciences
IFN-γ	V450	XMG1.2	BD biosciences
iNOS	PE	CXNFT	eBioscience
Arginase-1 (Arg1)	FITC	Polyclonal	R&D Systems

Gene expression of M1 and M2 markers in decidual and myometrial macrophages

Dams were injected with anti-CD3ε, LPS, or RU486 (or their respective controls) (n = 6–8 per group). Mice were euthanized 12–16 h post-injection, and decidual and myometrial tissues from the implantation sites were collected. Leukocytes were isolated from the decidual and myometrial tissues as described above. Leukocyte suspensions were sequentially filtered using a 100-µm cell strainer followed by a 30-µm cell strainer and then washed with 1X PBS. After centrifugation at 300 × g for 10 min at 4°C, the leukocytes were resuspended in 1 mL of 1X PBS and counted using an automatic cell counter (Cellometer Auto 2000; Nexcelom Bioscience). Macrophages were then isolated from decidual and myometrial cells by magnetic separation using mouse Anti-F4/80 UltraPure MicroBeads (Miltenyi Biotec) and MS magnetic columns (Miltenyi Biotec), following the manufacturer's instructions. After elution from the column, a small aliquot of the isolated F4/80+ cells was taken to assess the purity by flow cytometry using the fluorochrome-conjugated anti-mouse monoclonal antibodies CD45, CD11b, and F4/80 (Table IV). Isolated F4/80+ cells were then centrifuged, and the cell pellet was resuspended in 350 µL RLT Lysis Buffer (Qiagen). Total RNA was isolated from F4/80+ cells using the RNeasy micro kit (Qiagen), following the manufacturer's instructions. RNA integrity was evaluated with the 2100 Bioanalyzer system (Agilent Technologies, Wilmington, DE) using the Agilent RNA 6000 Pico Kit (Agilent Technologies). cDNA was synthesized by using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies, Carlsbad, CA, USA) on the Applied Biosystems GeneAmp PCR System 9700 (Applied Biosystems, Life Technologies), following the manufacturer's instructions.

cDNA was amplified using the TaqMan PreAmp Master Mix (2x) (Applied Biosystems) on the Applied Biosystems 7500 Fast Real-time PCR System. mRNA expression was determined by quantitative RT-PCR (qRT-PCR) using a BioMark high-throughput qRT-PCR System (Fluidigm, San Francisco, CA, USA) and TaqMan gene expression assays (Thermo Fisher Scientific) (Table V).

Table V. List of the TaqMan gene expression assays from Applied Biosystems

Name	**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 alpha	*IL1a*	Mm00439620_m1
Interleukin 1 beta	*Il1b*	Mm00434228_m1
Interferon gamma	*Ifng*	Mm01168134_m1
Interleukin 2	*Il2*	Mm00434256_m1
Interleukin 3	*Il3*	Mm00439631_m1
Interleukin 4	*Il4*	Mm00445259_m1
Interleukin 5	*Il5*	Mm00439646_m1
Interleukin 6	*Il6*	Mm00446190_m1
Interleukin 9	*Il9*	Mm00434305_m1
Interleukin 10	*Il10*	Mm01288386_m1
Interleukin 12b (IL12p40)	*IL12b*	Mm01288989_m1
Interleukin 17A	*Il17a*	Mm00439618_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 (Casp11)	*Scaf11*	Mm01297328_m1
Antigen identified by monoclonal antibody Ki 67 (Ki-67)	*Mki67*	Mm01278617_m1
Programmed cell death 1 (PD-1)	*Pdcd1*	Mm01285676_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 9	*Cxcl9*	Mm00434946_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
Selectin, lymphocyte	*Sell*	Mm00441291_m1
Selectin, endothelial cell	*Sele*	Mm00441278_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
Cytotoxic T-lymphocyte-associated protein 4	*Ctla4*	Mm00486849_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
Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	*Nfkbiz*	Mm00600522_m1
Interferon regulatory factor 4	*Irf4*	Mm00516431_m1
Interferon regulatory factor 5	*Irf5*	Mm00496477_m1
Arginase liver,	*Arg1*	Mm00475988_m1
Nitric oxide synthase 2, inducible	*Nos2*	Mm00440502_m1
Interleukin 27	*Il27*	Mm00461162_m1
Interleukin 12a	*Il12a*	Mm00434169_m1
Resistin like alpha	*Retnla*	Mm00445109_m1
Chemokine (C-C motif) ligand 24	*Ccl24*	Mm00444701_m1
Chitinase-like 3	*Chil3*	Mm00657889_mH
Arachidonate 15-lipoxygenase	*Alox15*	Mm00507789_m1
Suppresor of cytokine signaling	*Socs1*	Mm00782550_s1
Suppresor of cytokine signaling 2	*Socs2*	Mm01236704_m1
Signal transducer and activator of transcription 6	*Stat6*	Mm01160477_m1
Signal transducer and activator of transcription 1	*Stat1*	Mm01257286_m1

Determination of proinflammatory genes in decidual and myometrial tissues

Dams were injected with anti-CD3ε, LPS, or RU486 (or their respective controls) (n = 7–9 each for anti-CD3ε, 9–13 each for LPS, and 11–16 each for RU486). Mice were euthanized 12–16 h post-injection, and decidual and myometrial tissues from the implantation sites were collected and placed in RNAlater Stabilization Solution (Life Technologies). Total RNA was isolated from decidual and myometrial tissues using the RNeasy mini kit (Qiagen), following the manufacturer's instructions. RNA concentrations and purity were assessed with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and RNA integrity was evaluated with the 2100 Bioanalyzer system using the Agilent RNA 6000 Nano Kit (Agilent Technologies). cDNA was synthesized by using the SuperScript III First-Strand Synthesis System for RT-PCR on the Applied Biosystems GeneAmp PCR System 9700, following the manufacturer's instructions. cDNA was amplified using the TaqMan PreAmp Master Mix (2X) on the Applied Biosystems 7500 Fast Real-time PCR System. mRNA expression was determined by qRT-PCR using a BioMark high-throughput qRT-PCR System and TaqMan gene expression assays (Table V).

Determination of cytokine concentrations in amniotic fluid and the maternal circulation

Dams were injected with anti-CD3ε, LPS, or RU486 (or their respective controls). Mice were euthanized 12–16 h post-injection, and peripheral blood was collected by cardiac puncture for serum separation (n = 11–12 each for anti-CD3ε, 10 each for LPS, and 10 each for RU486). Amniotic fluid was also obtained from each amniotic sac with a 26-gauge needle (n = 5 each for anti-CD3ε, 5 each for LPS, and 5 each for RU486). Maternal serum and amniotic fluid samples were centrifuged at 1300 × g for 10 min at 4°C, and the supernatants were separated and stored at −20°C until analysis.

The ProcartaPlex Mouse Cytokine and Chemokine Panel 1A 36-plex (Invitrogen by Thermo Fisher Scientific, Vienna, Austria) was used to measure the concentrations of IFN-γ, IFN-α, IL-12p70, IL-1β, TNF-α, GM-CSF, IL-18, IL-17A, IL-22, IL-23, IL-27, IL-9, IL-15/IL-15R, IL-13, IL-2, IL-4, IL-5, IL-6, IL-10, CCL11, IL-28, IL-3, LIF, IL-1α, IL-31, CXCL1, CCL3, CXCL10, CCL2, CCL7, CCL4, CXCL2, CCL5, G-CSF, M-CSF, and CXCL5 in serum and amniotic fluid samples, according to the manufacturer's instructions. Plates were read using the Luminex 100 SystemFill (Luminex, Austin, TX), and analyte concentrations were calculated with ProcartaPlex Analyst 1.0 Software from Affymetrix (San Diego, CA). The sensitivities of the assays are shown in Table VI and the inter-assay and the intra-assay coefficients of variation were <10%.

Table VI. The ProcartaPlex Mouse Cytokine and Chemokine assay sensitivities

Cytokine or Chemokine	**Sensitivity**
IFN-γ	0.09 pg/mL
IFN-α	3.03 pg/mL
IL-12p70	0.21 pg/mL
IL-1β	0.14 pg/mL
TNF-α	0.39 pg/mL
GM-CSF	0.19 pg/mL
IL-18	9.95 pg/mL
IL-17A	0.08 pg/mL
IL-22	0.24 pg/mL
IL-23	2.21 pg/mL
IL-27	0.34 pg/mL
IL-9	0.28 pg/mL
IL-15/IL-15R	0.42 pg/mL
IL-13	0.16 pg/mL
IL-2	0.10pg/mL
IL-4	0.03 pg/mL
IL-5	0.32 pg/mL
IL-6	0.21 pg/mL
IL-10	0.69 pg/mL
CCL11	0.01 pg/mL
IL-28	20.31 pg/mL
IL-3	0.11 pg/mL
LIF	0.28 pg/mL
IL-1α	0.32 pg/mL

IL-31	0.45 pg/mL
CXCL1	0.05 pg/mL
CCL3	0.13 pg/mL
CXCL10	0.26 pg/mL
CCL2	3.43 pg/mL
CCL7	0.15 pg/mL
CCL4	1.16 pg/mL
CXCL2	0.37 pg/mL
CCL5	0.35 pg/mL
G-CSF	0.19 pg/mL
M-CSF	0.02 pg/mL
CXCL5	5.67 pg/mL

Chemokine and cytokine concentrations in amniotic fluid were adjusted by protein concentrations, which were determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), following the manufacturer's instructions.

Determination of progesterone concentration in the maternal circulation

Dams were injected with anti-CD3ε or isotype control (n = 10–11 each). Mice were euthanized 16 h post-injection, and peripheral blood was collected by cardiac puncture for serum separation. Maternal serum was centrifuged at 1300 x g for 10 min at 4°C, and the supernatants were separated and stored at −20°C until analysis. Serum progesterone (P4) was measured using the PROG-EASIA ELISA kit (GenWay Biotech, San Diego, CA, USA), according to the manufacturer's instructions. The sensitivity of the assay was 0.08 ± 0.03 ng/mL. The intra-assay coefficient of variation was 10.5%.

Preterm birth rescue by treatment with progesterone

Dams were injected s.c. with either 1 mg/100 µL P4 (Sigma-Aldrich) diluted in sesame oil (SO; Sigma-Aldrich) or 100 µL of SO (control groups) on 15.5, 16.5, and 17.5 dpc. On 16.5 dpc, dams were also injected i.p. with either 10 µg/200 µL anti-CD3ε or isotype control (n = 5–10 each). Following the last injection, dams were monitored via video camera with infrared light until delivery. The rate of preterm birth and gestational length was recorded as described above. The rate of neonatal mortality was calculated as the number of pups found dead among the total litter size. Representative images of pups just after birth and at 1 d old were also obtained.

Determination of proinflammatory genes in decidual, myometrial, and cervical tissues after treatment with progesterone

Dams were injected s.c. with either 1 mg/100 µL P4 (Sigma-Aldrich) diluted in SO (Sigma-Aldrich) or 100 µL of SO (control groups) on 15.5 and 16.5 dpc. On 16.5 dpc, dams were also injected with either 10 µg/200 µL anti-CD3ε or isotype control (n = 5 each). Approximately 16 h after anti-CD3ε or isotype injection, mice were euthanized, and decidual and myometrial tissues from the implantation sites as well as cervical tissues were collected and placed in RNAlater Stabilization Solution (Life Technologies). Total RNA was isolated from tissues using the RNeasy mini kit (Qiagen), following the manufacturer's instructions. RNA concentrations and purity were assessed with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and RNA integrity was evaluated with the 2100 Bioanalyzer system using the Agilent RNA 6000 Nano Kit. cDNA was synthesized by using the SuperScript III First-Strand Synthesis System for RT-PCR on the Eppendorf AG (Eppendorf, Hamburg, Germany), following the manufacturer's instructions. cDNA was amplified using the TaqMan PreAmp Master Mix (2×) on the Applied Biosystems 7500 Fast Real-time PCR System.

mRNA expression was determined by qRT-PCR using a BioMark high-throughput qRT-PCR System and TaqMan gene expression assays (Table V).

Statistical Analyses

Statistical analyses were performed using SPSS v19.0 (IBM, Armonk, NY, USA) or the R package (https://www.r-project.org/). For human demographic data, the group comparisons were performed using the Fisher exact test for proportions and Kruskal–Wallis tests for nonnormally distributed continuous variables. When proportions are displayed, percentages and 95% confidence intervals are shown. Medians are shown with the interquartile range (IQR). Kaplan–Meier survival curves were used to plot and compare the gestational length data (Mantel–Cox test). For maternal heart rates, fetal heart rate, umbilical artery pulsatility index and fetal weights, the statistical significance of group comparisons was assessed using the t test, and the means are shown with the SEM. For the rates of preterm birth and neonatal mortality, the Fisher exact test was used. For multiplex, ELISA and flow cytometry data, the statistical significance between groups was determined using a Mann–Whitney U test. 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. Heat maps were created for the group mean expression matrix (gene x group mean), with each gene expression level being standardized first. The heat maps shown in Figures 10, 11, 12 and 16 represent the Z-scores of the mean (−ΔCt). The heat maps found in Figure 19 display the −ΔCt values of each group, centered on the −ΔCt value of the control group treated with SO + isotype. All heat maps show hierarchical clustering using correlation distance. The p values were determined by an unpaired t test or a Mann–Whitney U test. A p value of ≤0.05 was considered statistically significant.

7. RESULTS

7.1. Effector and activated maternal T cells are enriched at the human maternal-fetal interface during spontaneous preterm labor

Naïve T cells (T_N) travel throughout the circulation in search of antigens presented by dendritic cells [101]. Upon antigen presentation, T cells proliferate and differentiate into effector cells that can migrate to B-cell areas or inflamed tissues [102]. A proportion of these cells persist as circulating memory T cells conferring protection against the known antigen [103]. Memory T cells can be subdivided based on their phenotype and function into central memory T cells (T_{CM}), which display high proliferative potential but lack an immediate effector function, and effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) T cells that have low proliferative capacity but display rapid effector function [104, 105]. Such cells can be distinguished by the expression of CD45RA, which is expressed by naïve or terminally differentiated T cells, and CCR7, a lymph node homing receptor [104, 105]. We have previously shown that memory-like T cells with effector functions are present at the human maternal-fetal interface during the physiological process of term labor [49, 71]. Therefore, using immunophenotyping, we first investigated whether the different effector T-cell subsets were differentially distributed in the decidual tissues of women with spontaneous preterm labor (Figure 2A). Strikingly, both CD4+ and CD8+ T_{EM} were the most abundant T-cell subsets at the human maternal-fetal interface (i.e. decidua basalis and decidua parietalis) (Fig. 2B and Figure 3A). Indeed, CD4+ and CD8+ T_{EM} were enriched at the maternal-fetal interface of women who underwent spontaneous preterm labor compared to those who delivered at term (Figure 2B and Figure 3A). The increase in T_{EM} was accompanied by a reduction in both CD4+ and CD8+ T_N (Figure 2C) and T_{CM} (Figure 2D) at the maternal-fetal interface of women with spontaneous preterm labor (Figure 2C&D and Figure 3B&C). CD8+ T_{EMRA} were also greater at the maternal-fetal interface of women who underwent spontaneous preterm labor compared to those who delivered preterm without labor (Figure 2E and Figure 3D).

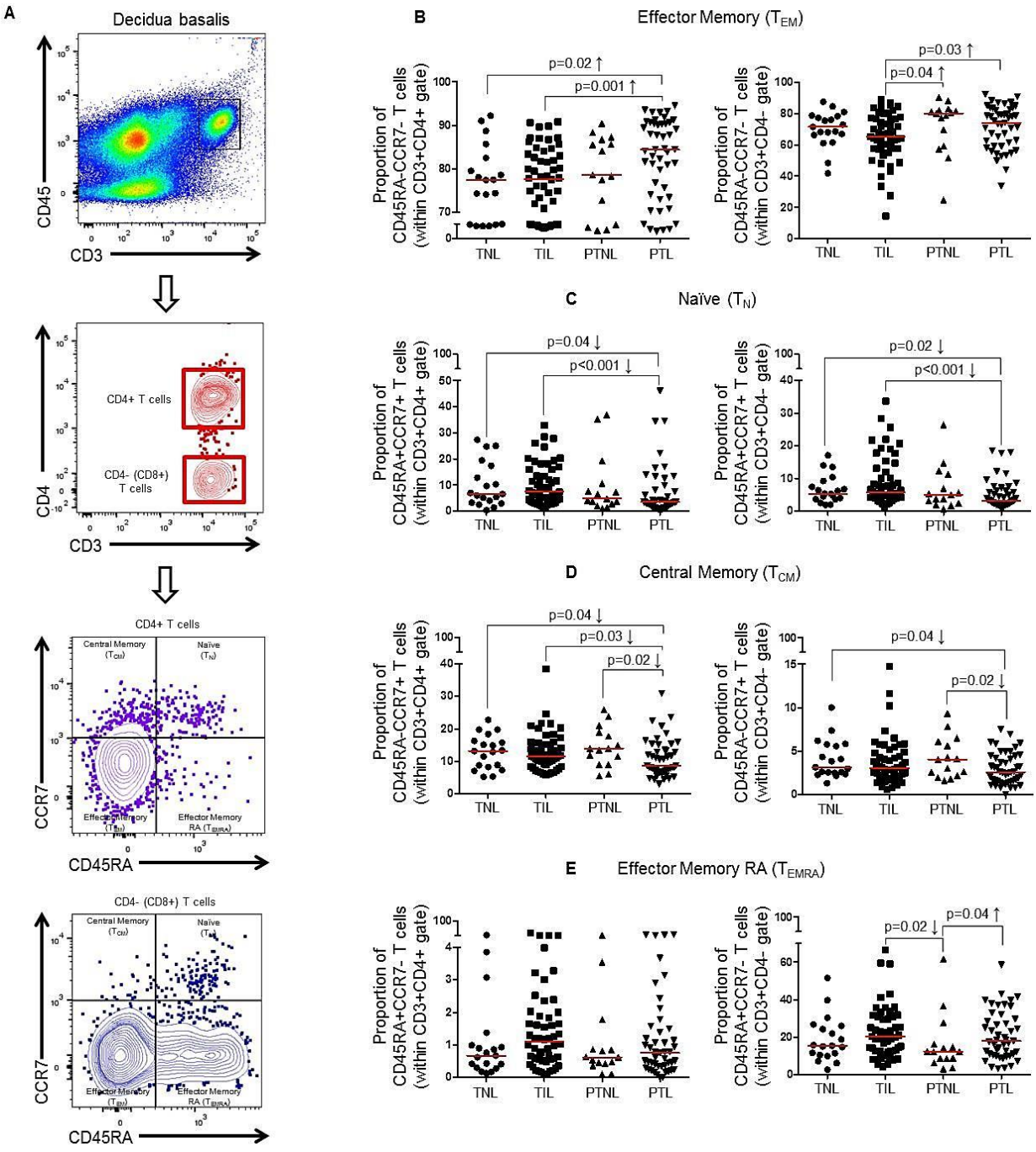


Figure 2. Immunophenotyping of effector and naive T cells in decidual tissues. (**A**) Gating strategy used to identify CD4+ and CD4- (CD8+) effector memory (T_{EM}; CD45RA-CCR7-), naïve (T_N; CD45RA+CCR7+), central memory (T_{CM}; CD45RA-CCR7+) and effector memory RA (T_{EMRA}; CD45RA+ CCR7-) T cells in the decidua basalis from women who delivered at term without labor (TNL), term with labor (TIL), preterm without labor (PTNL), or preterm with labor (PTL). (**B**) Proportions of T_{EM} cells. (**C**) Proportions of T_N cells. (**D**) Proportions of T_{CM} cells. (**E**) Proportions of T_{EMRA} cells.

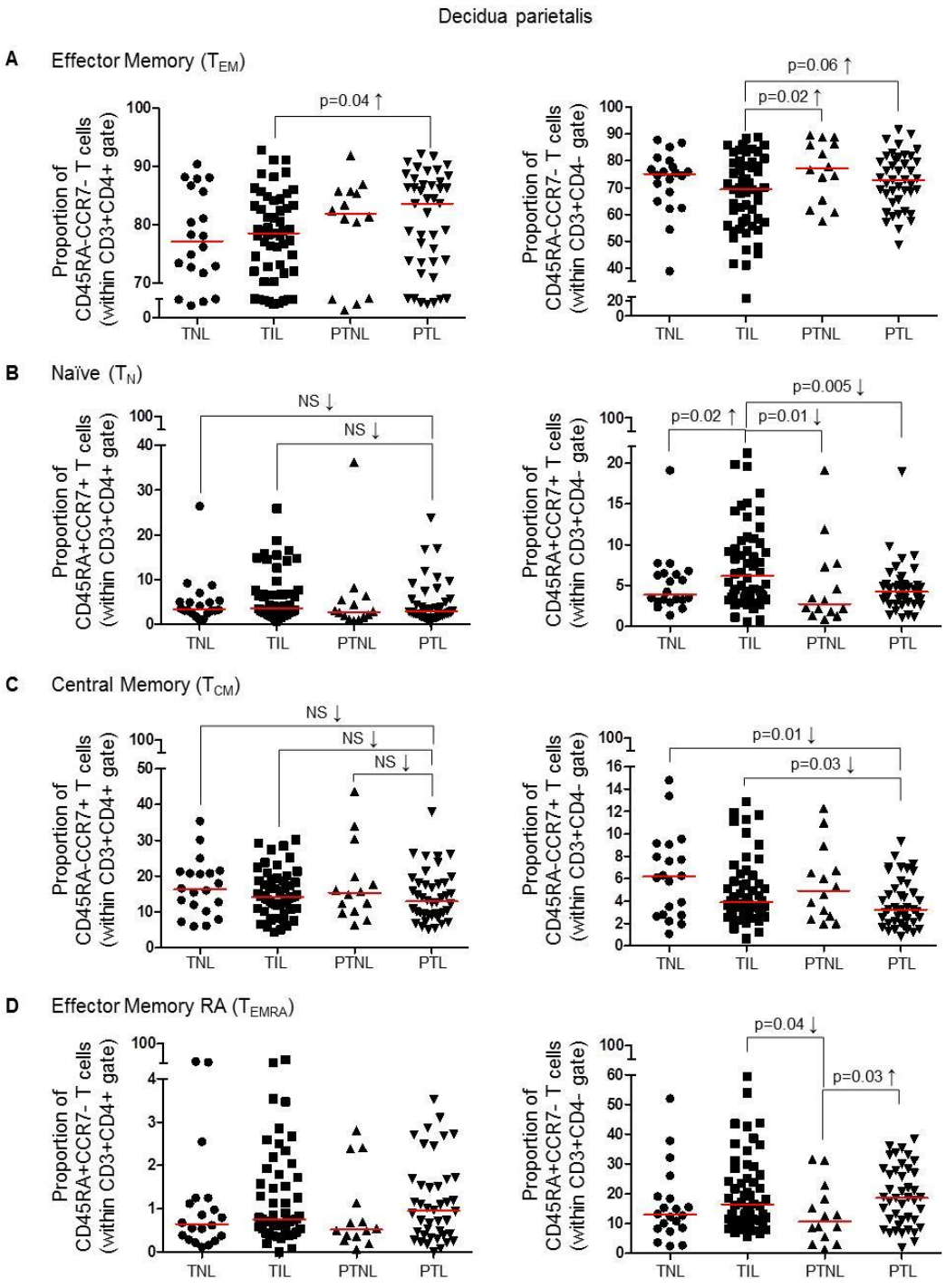


Figure 3. Immunophenotyping of effector memory T cells in the decidua parietalis. Proportions of CD4+ and CD4- (CD8+) effector memory (T_{EM}; CD45RA-CCR7-), naïve (T_N; CD45RA+CCR7+), central memory (T_{CM}; CD45RA-CCR7+) and effector memory RA (T_{EMRA}; CD45RA+ CCR7-) T cells in the decidua parietalis from women who delivered at term without labor (TNL), term with labor (TIL), preterm without labor (PTNL), or preterm with labor (PTL). (**A**) Proportions of T_{EM} cells. (**B**) Proportions of T_N cells. (**C**) Proportions of T_{CM} cells. (**D**) Proportions of T_{EMRA} cells.

After encountering their antigen, T_{EM} become activated and perform their effector functions through the release of inflammatory mediators such as granzyme B and perforin [106-110]. Therefore, we next investigated whether effector T cells expressed granzyme B and perforin at the human maternal-fetal interface (Figure 4A). Consistent with our previous findings, CD4+ and CD8+ T cells expressing granzyme B and perforin were enriched at the maternal-fetal interface of women with spontaneous preterm labor (Figure 4B&C and Figure 5A&B).

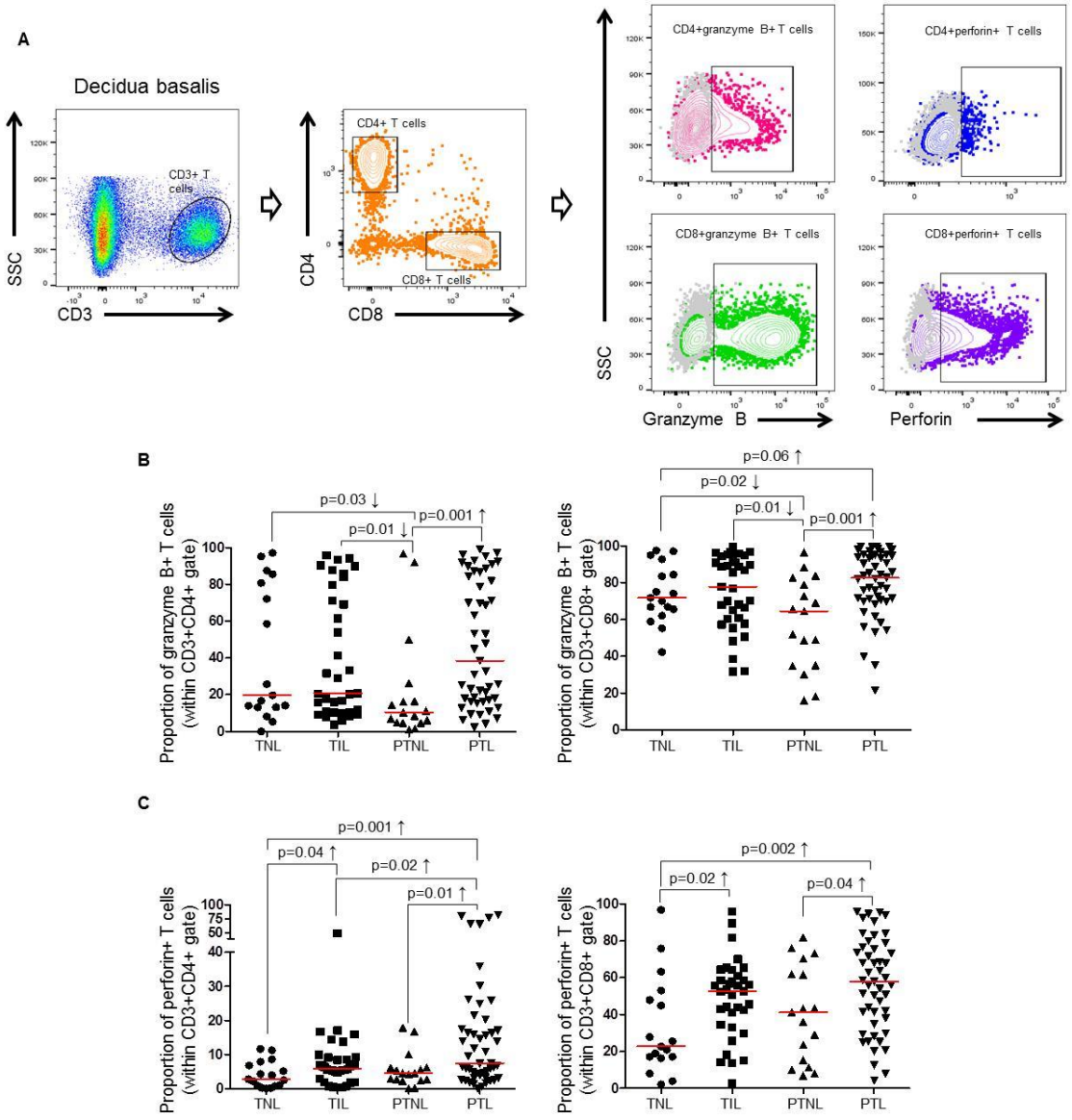


Figure 4. Immunophenotyping of activated T cells in decidual tissues. (**A**) Gating strategy used to identify CD4+ and CD8+ T cells expressing granzyme B or perforin in the decidua basalis from women who delivered at term without labor (TNL), term with labor (TIL), preterm without labor (PTNL), or preterm with labor (PTL). (**B**) Proportions of CD4+granzyme B+ or CD8+granzyme B+ T cells. (**C**) Proportions of CD4+perforin+ or CD8+perforin+ T cells.

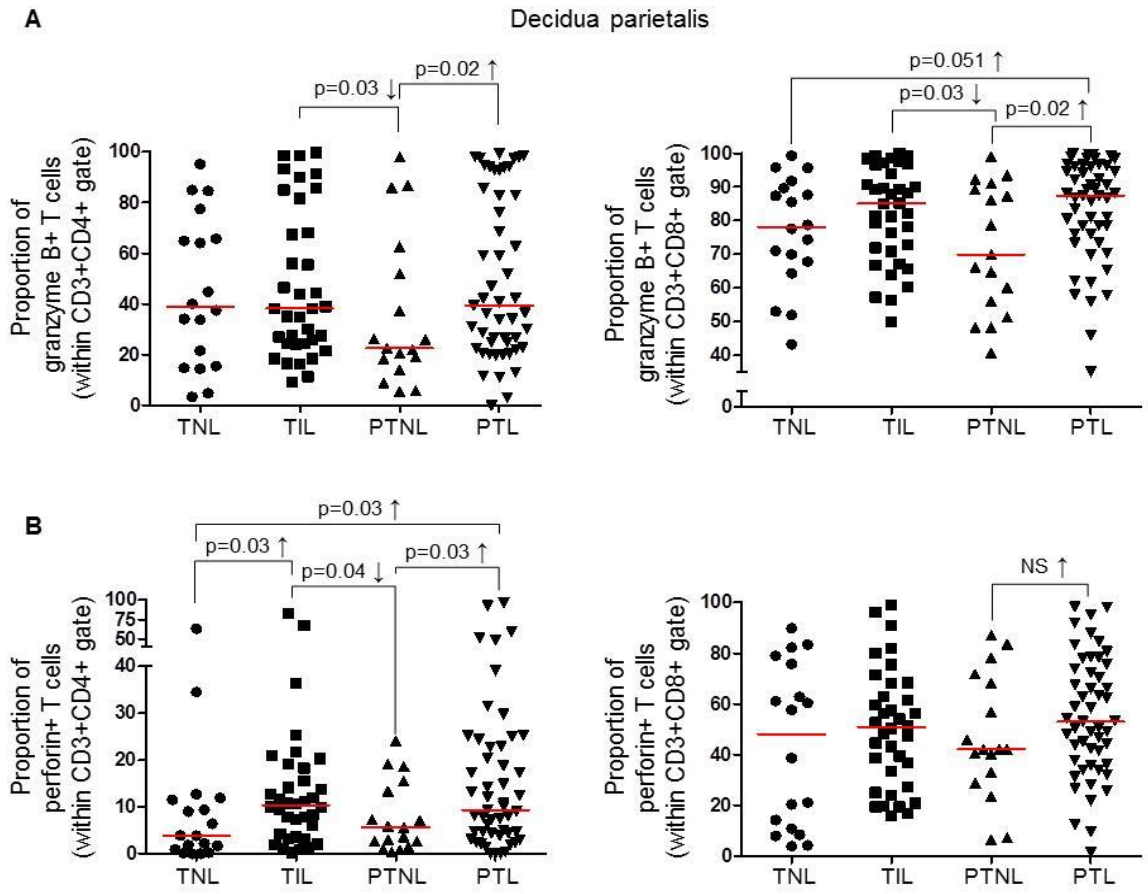


Figure 5. Immunophenotyping of activated T cells in the decidua parietalis. Proportions of CD4+ and CD8+ T cells expressing granzyme B or perforin in the decidua parietalis from women who delivered at term without labor (TNL), term with labor (TIL), preterm without labor (PTNL), or preterm with labor (PTL). (**A**) Proportions of CD4+granzyme B+ or CD8+granzyme B+ T cells. (**B**) Proportions of CD4+perforin+ or CD8+perforin+ T cells.

Together, these findings indicate that both CD4+ and CD8+ maternal T cells exhibit an effector/memory phenotype and release pro-inflammatory mediators at the human maternal-fetal interface during spontaneous preterm labor. In other words, the human syndrome of preterm labor is characterized by an increase in maternal effector/activated T cells at the maternal-fetal interface.

7.2. In vivo T-cell activation induces preterm birth and mortality at birth

Next, we investigated the mechanisms whereby effector/activated T cells could induce preterm labor and birth by using a murine model. *In vivo* T-cell activation is achieved by the administration of an anti-CD3 antibody which induces a cytokine-related syndrome [111-115]; therefore, we used this strategy in pregnant mice.

In order to prove that anti-CD3ε induced T-cell activation at the maternal-fetal interface and myometrium, we determined the expression of the activation markers CD25 [116] and CD69 [117] by CD4+ and CD8+ T cells, and the expression of IL-2 by CD4+ T cells [118] and IFNγ by CD8+ T cells [119], in the decidua and myometrium (Figure 6). In line with our hypothesis [97], anti-CD3ε induced an increased the expression of CD25 (Figure 7A&B) and CD69 (Figure 7C&D) by CD4+ and CD8+ T cells in both the decidua and myometrium. Upregulation of these activation markers was also observed in response to LPS (Figure 7A-D), although the increased expression of CD25 in decidual T cells did not reach significance (Figure 7A&B). In contrast, RU486 did not cause major changes in the expression of these activation markers by decidual and myometrial T cells (Figure 7A-D).

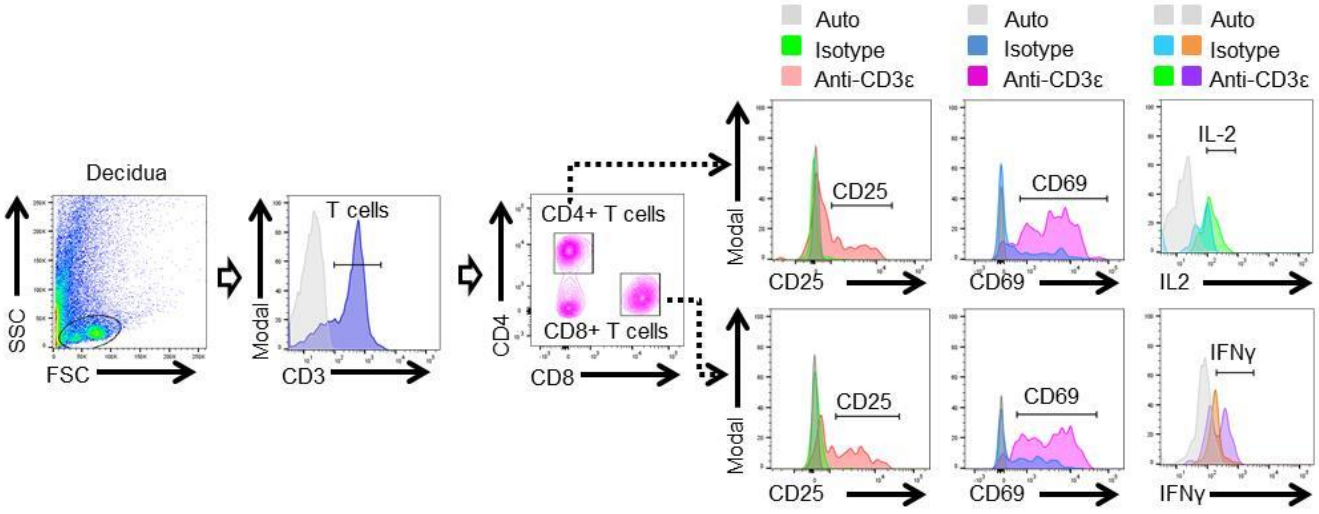


Figure 6. Activation of T at the maternal-fetal interface and in the myometrium prior to preterm birth. Gating strategy used to identify activated CD4+ (CD3+CD4+ cells expressing CD25, CD69, or IL-2) and CD8+ (CD3+CD8+ cells expressing CD25, CD69, or IFN-γ) T cells at the maternal-fetal interface. Gray histograms represent autofluorescence controls, and colored histograms represent the expression of CD25, CD69, IL-2, or IFN-γ.

Treatment with anti-CD3ε upregulated the expression of IL-2 by CD4+ T cells in the decidua and myometrium (Figure 7E). However, injection with anti-CD3ε increased the proportion of CD8+IFNγ+ T cells in the myometrium, but not in the decidua (Figure 7F). These data suggest that, upon T-cell activation, T-cell responses are differentially regulated at the maternal-fetal interface and in the reproductive tissues. We also observed that LPS increased the infiltration of decidual CD8+ T cells expressing IFN-γ, which is considered to be a response to microbial products [120]. CD8+ T cells expressing IFN-γ in the RU486 model were rare and did not vary (Figure 7F).

Dams injected with anti-CD3ε displayed greater proportions of CD4+ and CD8+ T cells expressing CD25 and CD69, but these dams did not have more IL-2-expressing CD4+ T cells or IFNγ-expressing CD8+ T cells, in the spleen and ULN compared to controls (Supplementary Figure 1). Dams injected with LPS also had higher proportions of CD4+ or CD8+ T cells expressing CD25 and CD69 in the spleen and ULN compared to controls, but no changes in IL-2-expressing CD4+ T cells or IFN-γ-expressing CD8+ T cells (Supplementary Figure 1). Treatment with RU486 did not induce any changes in the expression of activation markers by splenic and ULN T cells (Supplementary Figure 1).

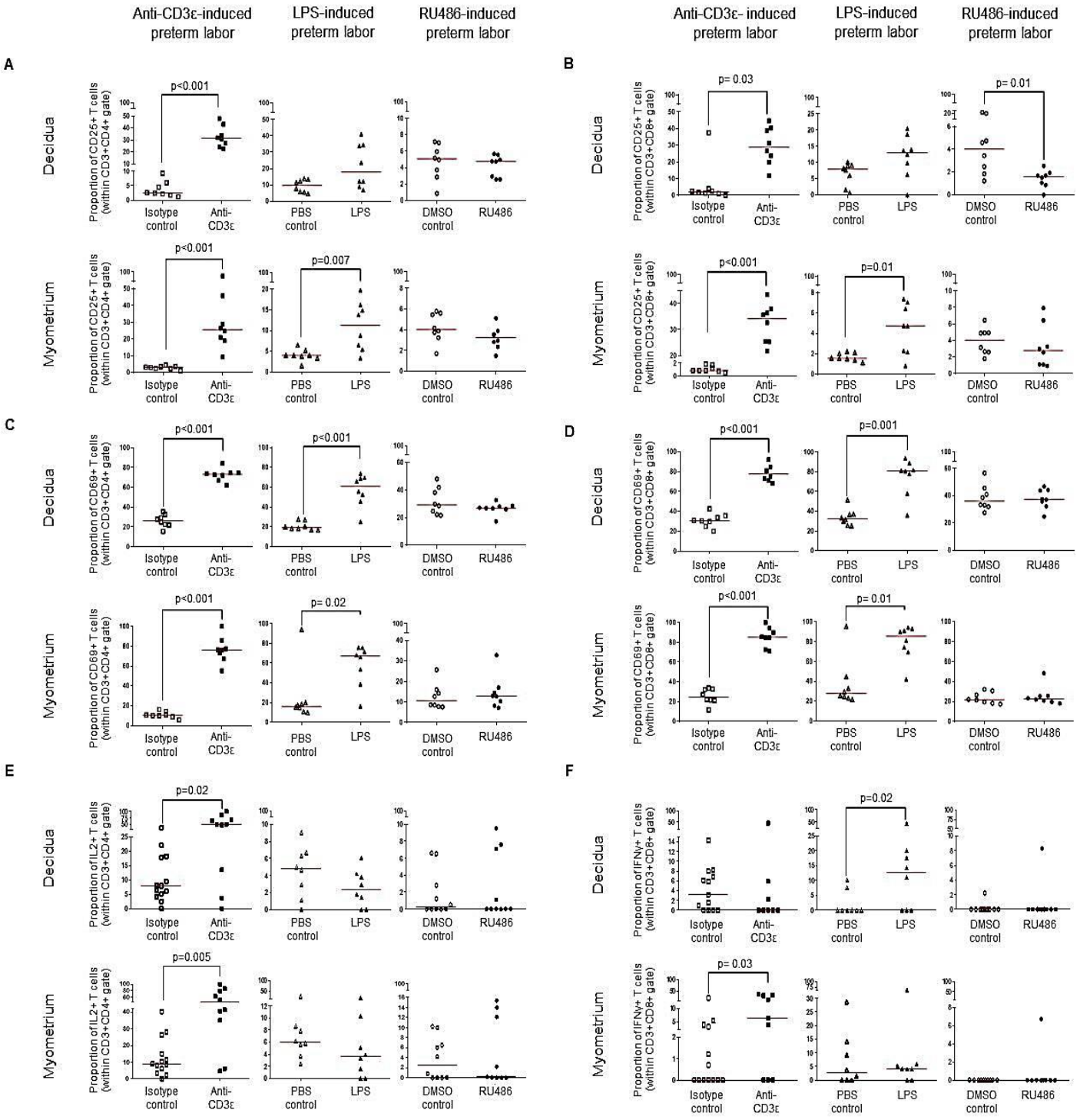


Figure 7. Activation of T at the maternal-fetal interface and in the myometrium prior to preterm birth. Proportions of **(A)** CD4+ and (**B**) CD8+ T cells expressing CD25 in decidual and myometrial tissues from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 8 each). Proportions of (**C**) CD4+ and (**D**) CD8+ T cells expressing CD69 in decidual and myometrial tissues from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 8 each). (**E**) Proportion of CD4+ T cells expressing IL-2 in decidual and myometrial tissues from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 8–13 each). (**F**) Proportion of CD8+ T cells expressing IFN-γ in decidual and myometrial tissues from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 8–13).

Dams injected with anti-CD3ε delivered preterm (Figure 8A), and neonatal mortality at birth (Figure 8D) as previously reported [97]. In order to understand the pathophysiology of preterm birth induced by T-cell activation, we compared this model to two well-established models of preterm birth: lipopolysaccharide (LPS)-induced [98, 121] (Figure 8B&E) and RU486-induced [99] (Figure 8C&F).

Collectively, these data show that anti-CD3ε causes preterm labor and birth by activating T cells at the maternal-fetal interface, myometrium, and systemically; yet, decidual and myometrial T-cell responses are distinct from those observed in lymphatic tissues.

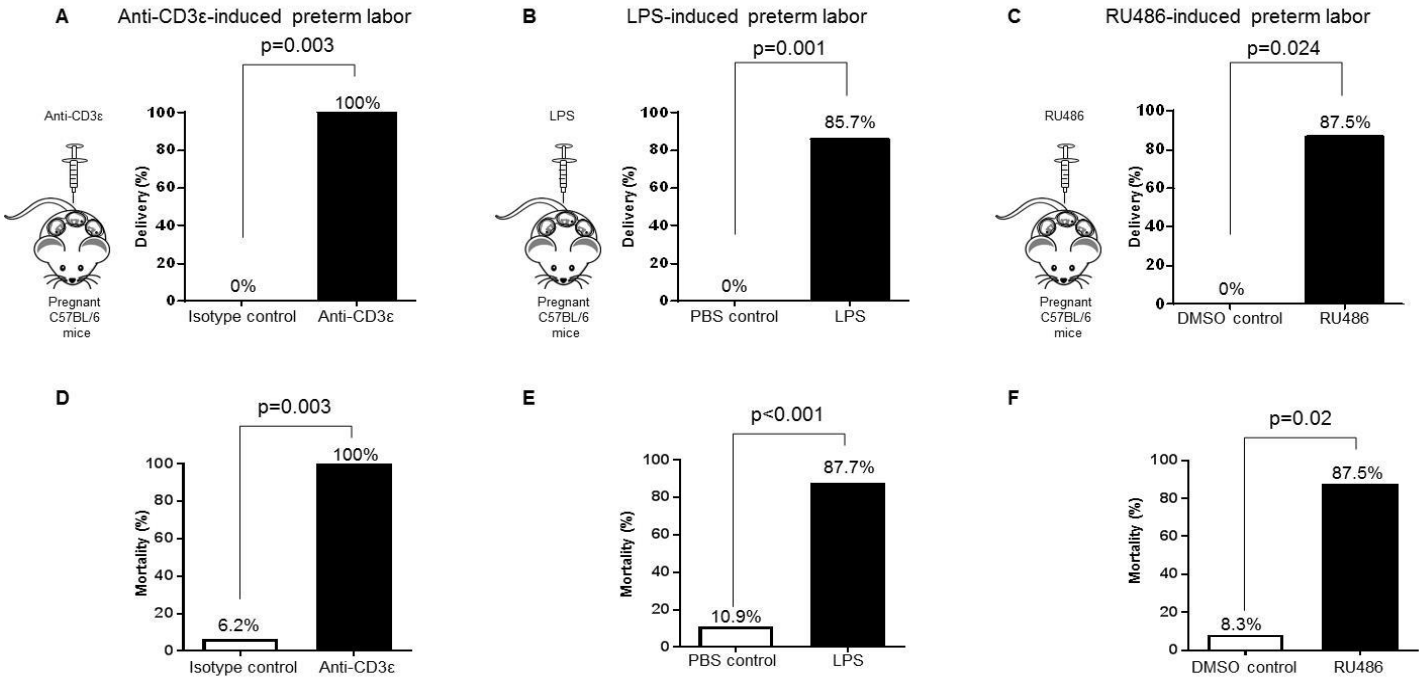


Figure 8. Rate of preterm birth and neonatal mortality at birth of dams injected with (**A&D**) anti-CD3ε (or isotype control), (**B&E**) LPS (or PBS control), or (**C&F**) RU486 (or DMSO control); n = 3–8 each.

7.3. In vivo T-cell activation induces fetal bradycardia and increased umbilical artery pulsatility index

In vivo T-cell activation-induced preterm birth also results in a high mortality at birth (>80% of mortality at birth). We then investigated whether these pups were dying in the uterus (fetal death), since abnormalities in the fetal heart rate and umbilical artery pulsatility are associated with fetal compromise [122-125]. We found that intraperitoneal injection of anti-CD3ε is associated with a decrease in fetal heart rate [anti-CD3ε 104.33 ± 4.11 bpm (n=88) versus isotype 154.69 ± 3.54 bpm (n=82); p<0.0001; Figure 9A]. In addition, anti-CD3ε intraperitoneal injection increased the umbilical artery pulsatility index [anti-CD3ε 1.831 ± 0.018 (n=87) versus isotype 1.744 ± 0.015 pulsatility index (n=82); p=0.041; Figure 9B].

Together, these data demonstrated that, although pups from dams injected with a monoclonal anti-CD3ε antibody did not die in the uterus, their health was compromised before birth.

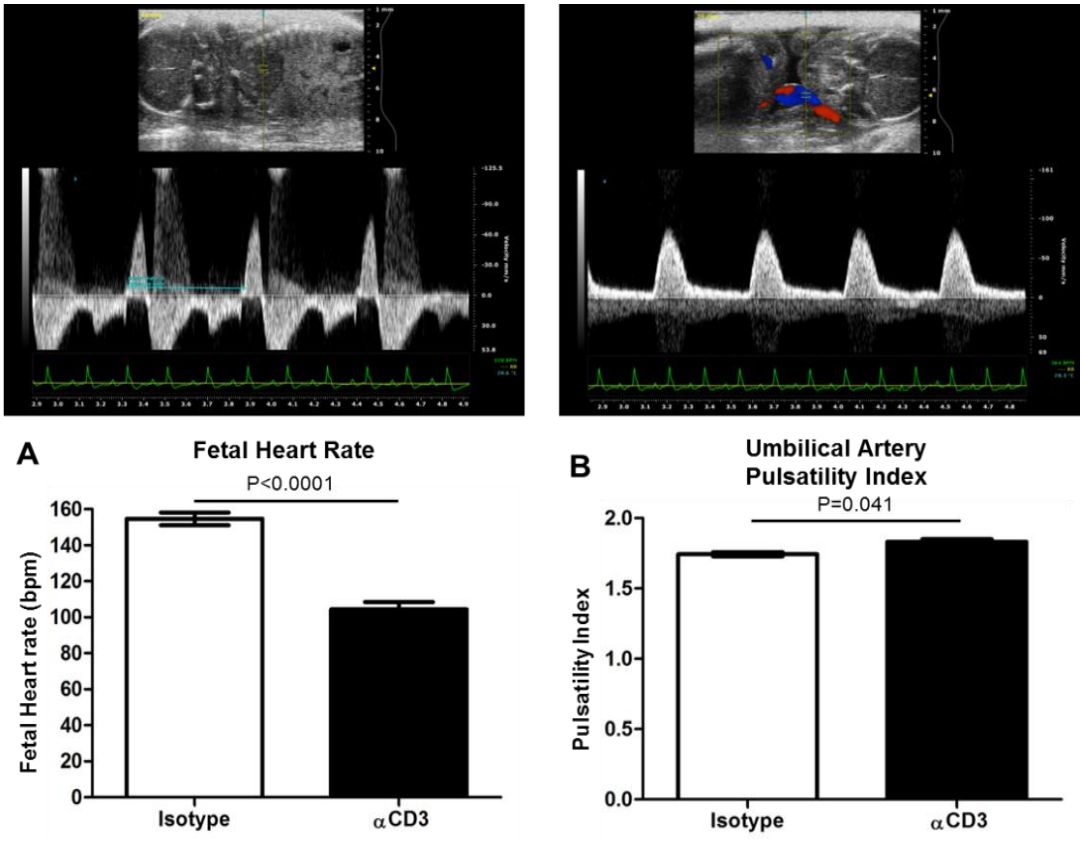


Figure 9. *In vivo* imaging by Ultrasound. Doppler ultrasound was performed on fetuses after intraperitoneal injection of 10µg/200µL of anti-CD3ε or isotype control in dams at 16.5 days *post coitum* (dpc). (**A**) Fetal heart rate, and (**B**) umbilical artery pulsatility index.

7.4. In vivo T-cell activation induces local and systemic pro-inflammatory responses prior to preterm birth

Preterm labor is characterized by the upregulation of inflammatory mediators in the decidua [40, 126-129], myometrium [40, 130-132], and cervix [133]. Such inflammatory mediators include cytokines [40, 129], chemokines [129, 134, 135], adhesion molecules [136, 137], and inflammasome components [138, 139]. Quantitative RT-PCR profiling revealed that both anti-CD3ε and LPS caused the upregulation of several inflammatory mediators in the decidua, myometrium, and cervix (Figures 10A-B, 11A-B & 12A-B). However, there were subtle differences between the genes upregulated by anti-CD3ε and LPS (Figures 10B, 11B & 12B). For example, whereas chemokines (*Ccl2*, *Ccl5*, *Ccl17*, *Ccl22*, *Cxcl9*, and *Cxcl10*), cytokines (*Il6* and *Ifng*), T-cell activation molecules (*Ctla4*, *Pdcd1* and *Sell*) and inflammasome components (*Pycard*, *Nod1*, and *Casp1*) were upregulated in both the anti-CD3ε and LPS models, *Il1b* was only upregulated in the LPS model in the decidual tissues (Figure 10B). In the myometrial tissues, both anti-CD3ε and LPS caused the upregulation of *Ccl2*, *Ccl5*, *Ccl17*, *Ccl22*, *Cxcl9*, *Cxcl10*, *Ifng*, *Pdcd1*, *Nod1*, and *Casp-1* but only LPS induced the upregulation of *Il6* (Figure 11B). In the cervical tissues, anti-CD3ε and LPS caused the upregulation of *Ccl5*, *Ccl17*, *Cxcl9*, *Cxcl10*, *Il6,Sell*, and *Casp-1* but only anti-CD3ε induced the upregulation of *Ifng, Ctla4, Pdcd1* and *Nod1* (Figure 12B). Most of the inflammatory genes in the decidua, myometrium, and cervix were unchanged upon RU486 injection (Figure 10B, 11B & 12B), confirming that this is a non-inflammatory model of preterm birth [140].

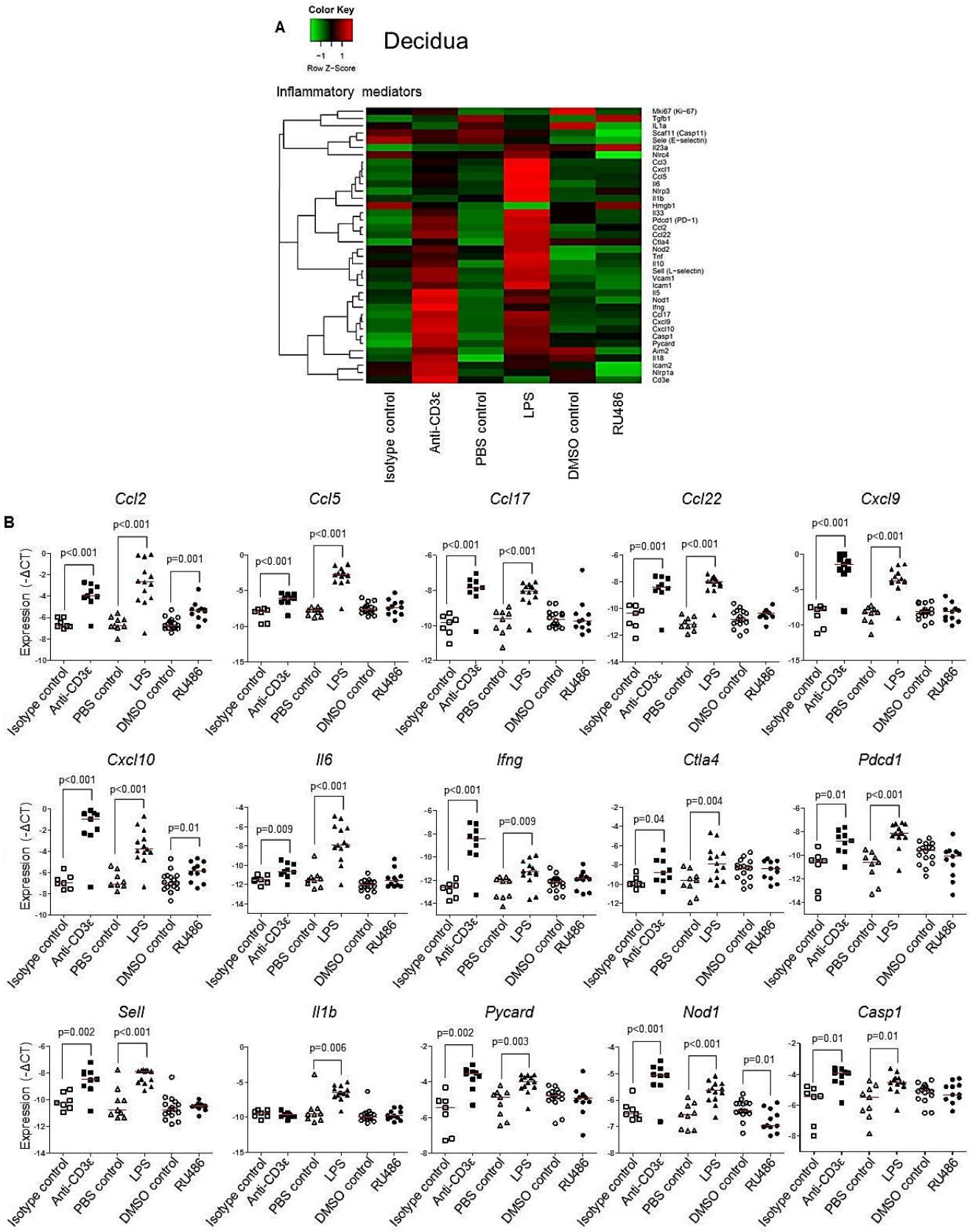


Figure 10. Inflammatory gene expression at the maternal-fetal interface prior to preterm birth. Decidual tissues from dams injected with anti-CD3ε (or isotype control), LPS (or PBS control), or RU486 (or DMSO control). (**A**) Heat map visualization of inflammatory gene expression in the decidua. (**B**) mRNA expression of selected genes in decidual tissues. Negative (-)ΔCt values were calculated using *Actb, Gusb, Gapdh,* and *Hsp90ab1* as reference genes. Data are from individual dams (n = 7–16 each).

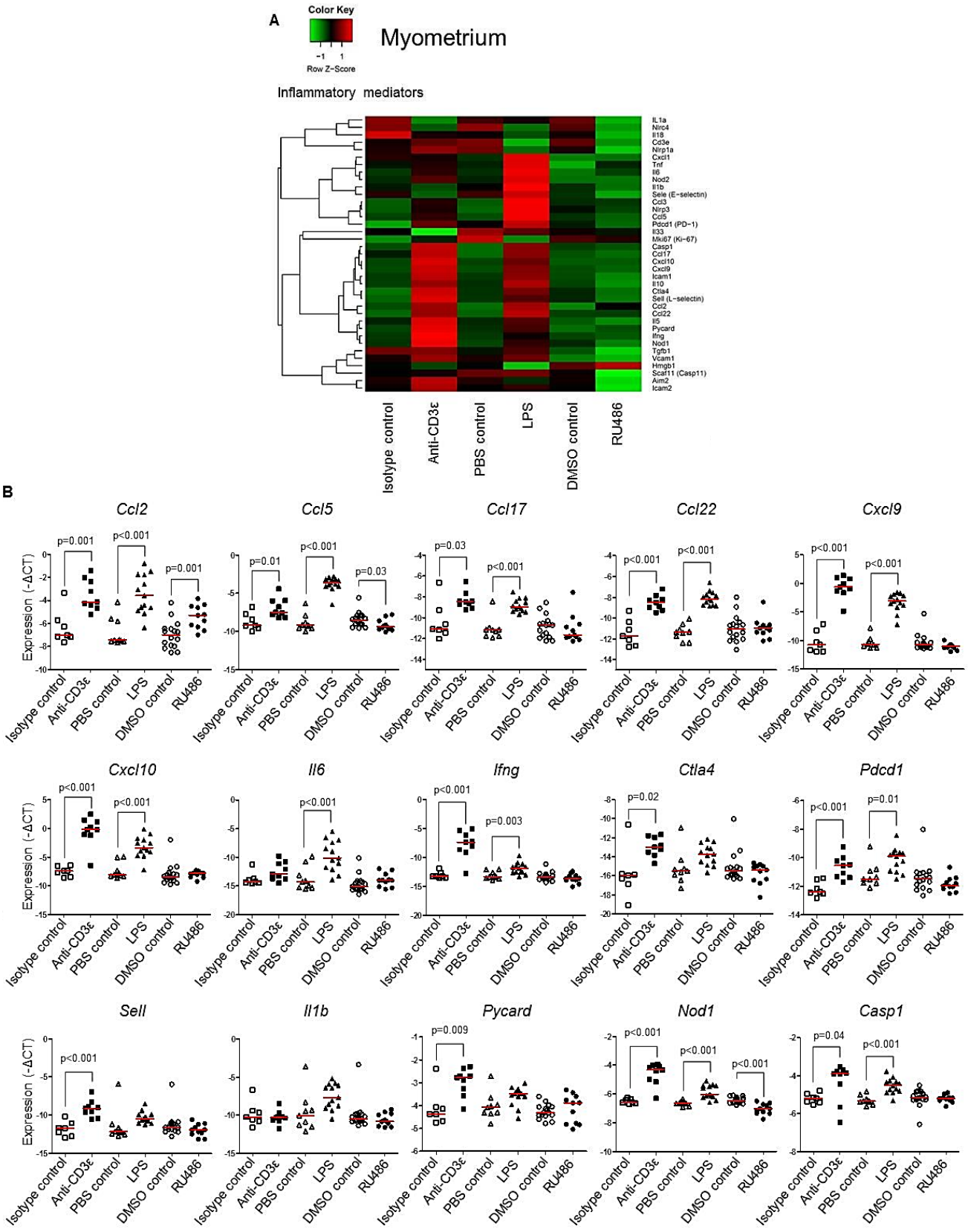


Figure 11. Inflammatory gene expression in the myometrium prior to preterm birth. Myometrial tissues from dams injected with anti-CD3ε (or isotype control), LPS (or PBS control), or RU486 (or DMSO control). (**A**) Heat map visualization of inflammatory gene expression in the decidua. (**B**) mRNA expression of selected genes in decidual tissues. Negative (-)ΔCt values were calculated using *Actb, Gusb, Gapdh,* and *Hsp90ab1* as reference genes. Data are from individual dams (n = 7–16 each).

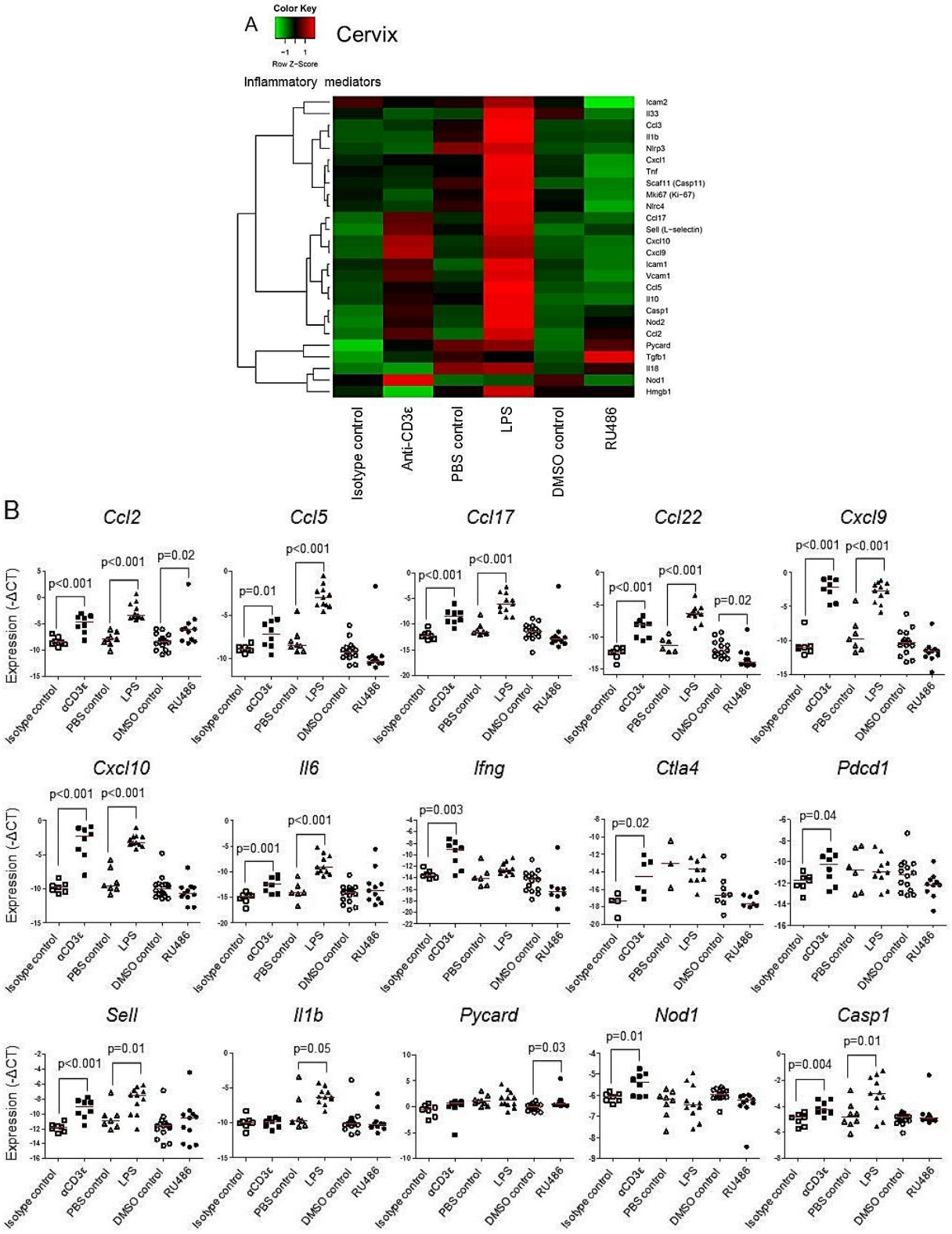


Figure 12. Inflammatory gene expression in the cervix prior to preterm birth. Cervical tissues from dams injected with anti-CD3ε (or isotype control), LPS (or PBS control), or RU486 (or DMSO control). (**A**) Heat map visualization of inflammatory and contractility-related gene expression in the myometrial tissues. (**B**) mRNA expression of selected genes in myometrial tissues. Negative (-)ΔCt values were calculated using *Actb, Gusb, Gapdh,* and *Hsp90ab1* as reference genes. Data are from individual dams (n = 7–16 each).

A systemic inflammatory response is associated with preterm labor in the context of clinical chorioamnionitis [141, 142] and acute pyelonephritis [143, 144]. Hence, systemic inflammatory responses were determined by measuring inflammatory mediators in the maternal serum from each of the preterm birth models. Anti-CD3ε and LPS induced a systemic maternal inflammatory response as evidenced by the upregulation of IL-6, IL-18, IL-17A, IL-4, IL-5, CCL5, CXCL10, G-CSF, and IFNγ in the maternal serum (Supplementary Figure 2). However, RU486 did not induce such an effect (Supplementary Figure 2).

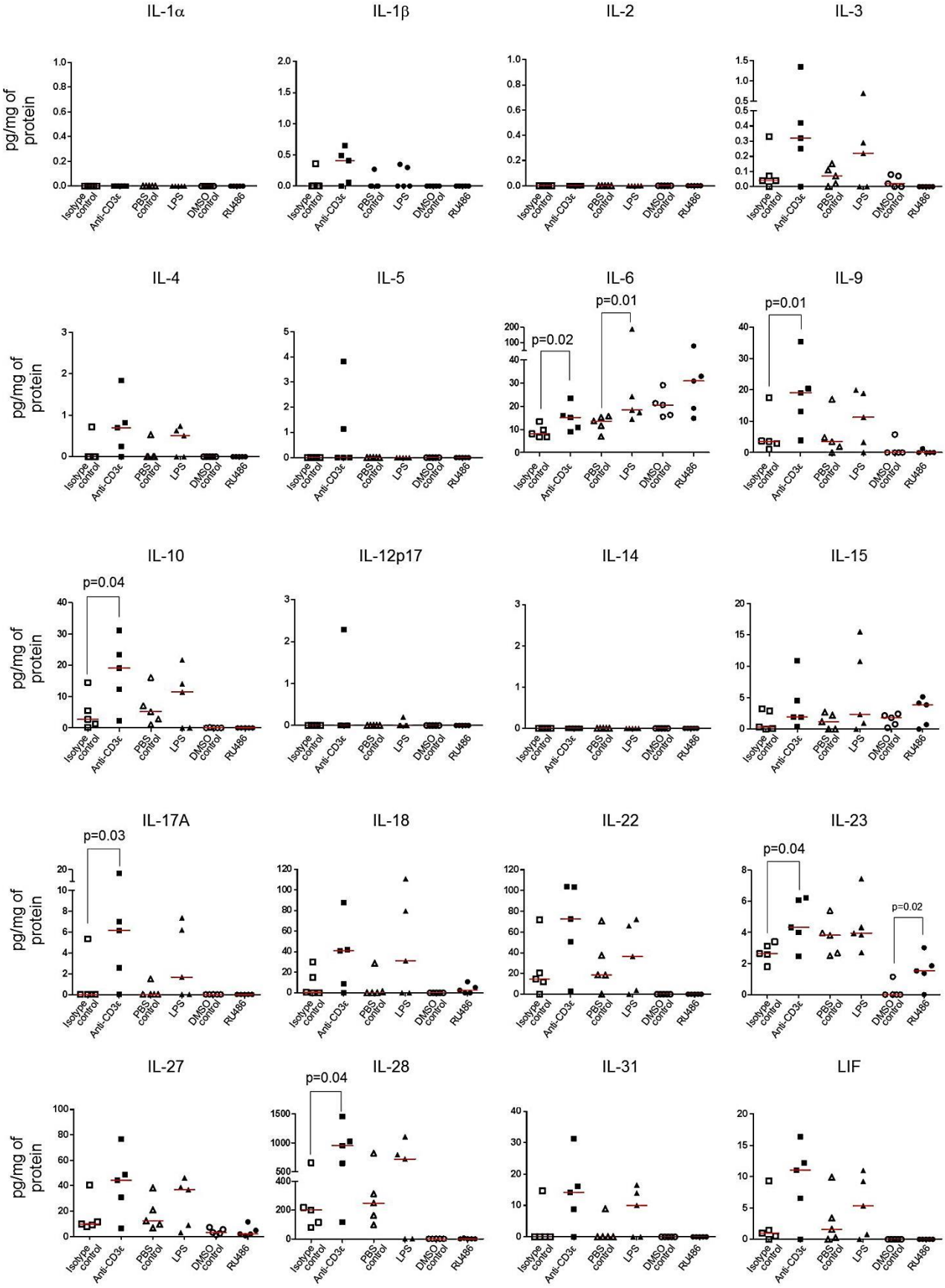
These findings show that *in vivo* T-cell activation induces local and systemic pro-inflammatory responses prior to preterm birth, resembling those observed in the microbial inflammation model.

7.5. In vivo T-cell activation induces intra-amniotic inflammation prior to preterm birth

It is well documented that preterm labor, either in the context of infection or sterile inflammation [15, 145, 146], is accompanied by increased amniotic fluid concentrations of pro-inflammatory cytokines and chemokines [147].

We therefore evaluated cytokine and chemokine concentrations in amniotic fluid prior to preterm birth. Anti-CD3ε induced a massive pro-inflammatory response in the amniotic cavity, which was even more severe than that generated by LPS (Figure 13). For example, anti-CD3ε but not LPS induced the upregulation of IL-9, IL-10, IL-17A, IL-23, and IL-28 (Figure 13). Yet, both anti-CD3ε and LPS caused the upregulation of IL-6, CCL3, CCL5, CXCL5, and G-CSF (Figure 13). RU486-induced preterm labor/birth, however, occurred in the absence of elevated amniotic fluid cytokines, except for IL-23 and CCL4 (Figure 13). These data indicate that in vivo T-cell activation induces a distinct intra-amniotic inflammatory response, and which is stronger than that induced by a microbial product.

As a whole, these findings show that in vivo T-cell activation induces an intra-amniotic pro-inflammatory response that can negatively impact the fetus, causing fetal compromise and extends into the neonatal period.


IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12p17
IL-14
IL-15
IL-17A
IL-18
IL-22
IL-23
IL-27
IL-28
IL-31
LIF
pg/mg of protein
p=0.01
p=0.02
p=0.04
p=0.03
Isotype control
Anti-CD3ε
PBS control
LPS
DMSO control
RU486

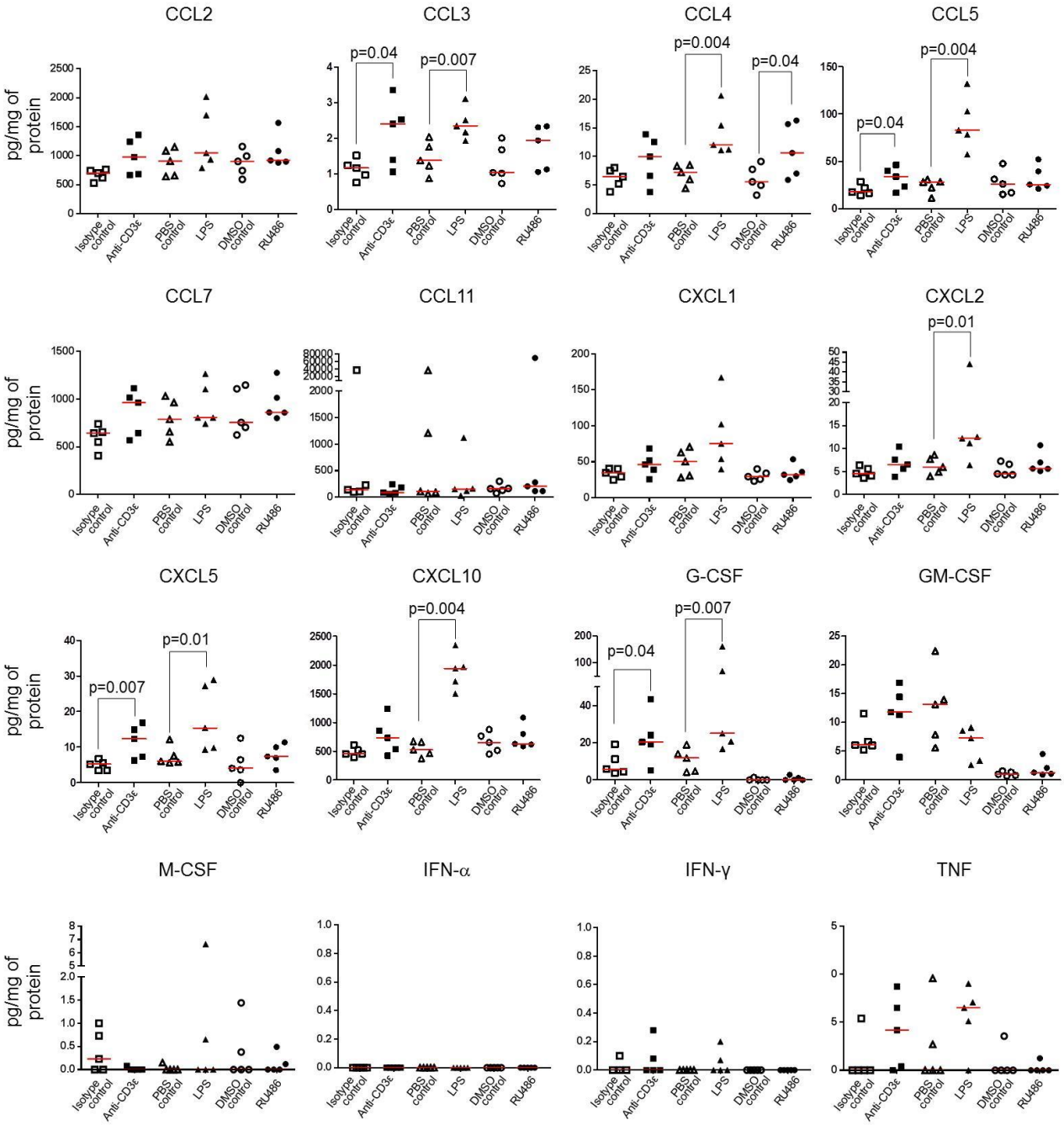


Figure 13. The fetal inflammatory response prior to preterm birth. Dams were injected with anti-CD3ε (or isotype control), LPS (or PBS control), or RU486 (or DMSO control). Concentrations of cytokines and chemokines in amniotic fluid were determined using a multiplex assay (n = 5 each).

7.6. In vivo T-cell activation induces pro-inflammatory macrophage polarization but does not lead to an increased neutrophil influx into the maternal-fetal interface and myometrium

Activation of T cells can induce macrophage polarization [148, 149]. Indeed, an M1-like (i.e. pro-inflammatory) macrophage polarization has been implicated in the mechanisms that lead to spontaneous preterm labor and birth [39]. Hence, we evaluated whether prior to preterm birth, anti-CD3ε could induce macrophage activation and polarization towards M1-like and M2-like phenotypes at the maternal-fetal interface. First, flow cytometry was performed to identify M1-like (CD11b+F4/80+iNOS+ cells) and M2-like (CD11b+F4/80+Arg1+ cells) macrophages in the decidua and myometrium. Anti-CD3ε increased the proportion of M1-like macrophages in the decidua and myometrium (Figure 14). However, neither LPS nor RU486 altered the proportion of M1-like macrophages at the maternal-fetal interface or the myometrium (data not shown). Although M2-like macrophages were detected at the maternal-fetal interface and myometrium, their proportions were unchanged upon anti-CD3ε, LPS, or RU486 injection (data not shown). In order to confirm that T-cell activation induced the polarization of M1-like macrophages, we determined the mRNA expression of multiple established M1 and M2 macrophage markers [150] in sorted decidual and myometrial macrophages (Figure 15). Anti-CD3ε caused the upregulation of some M1 markers in macrophages from both the decidua and myometrium (Figure 15). LPS also induced an increase in the expression of some M1 markers in the decidual macrophages, yet this effect was minimal in myometrial macrophages (Figure 16A). However, RU486 did not dramatically affect the expression of M1 markers by decidual or myometrial macrophages (Figure 16A). Furthermore, we found that anti-CD3ε induced the upregulation of some M2 markers by decidual and myometrial macrophages (Figure 16B). LPS had a similar effect, but RU486 did not drastically affect the expression of M2 macrophage markers in the decidua and myometrium (Figure 16B). Together, these results show that T-cell activation induces the upregulation of different M1 and M2 macrophage markers in the decidua and myometrium, yet favors their pro-inflammatory phenotype at the maternal-fetal interface.

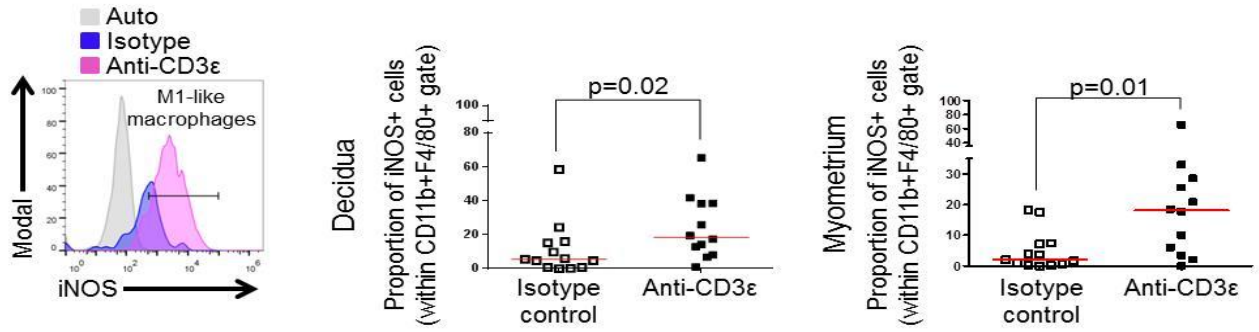


Figure 14. M1-like macrophages are enriched at the maternal-fetal interface and myometrium prior to *in vivo* T cell activation–induced preterm birth. Gating strategy used to identify M1-like (CD11b+ F4/80+ iNOS+ cells) macrophages at the maternal-fetal interface. Gray histograms represent autofluorescence controls and colored histograms represent the expression of iNOS at the maternal-fetal interface of dams injected with isotype control or anti-CD3ε, respectively. Proportions of M1-like macrophages in the decidual and myometrial tissues from dams injected with anti-CD3ε or isotype (n = 12–13 each).

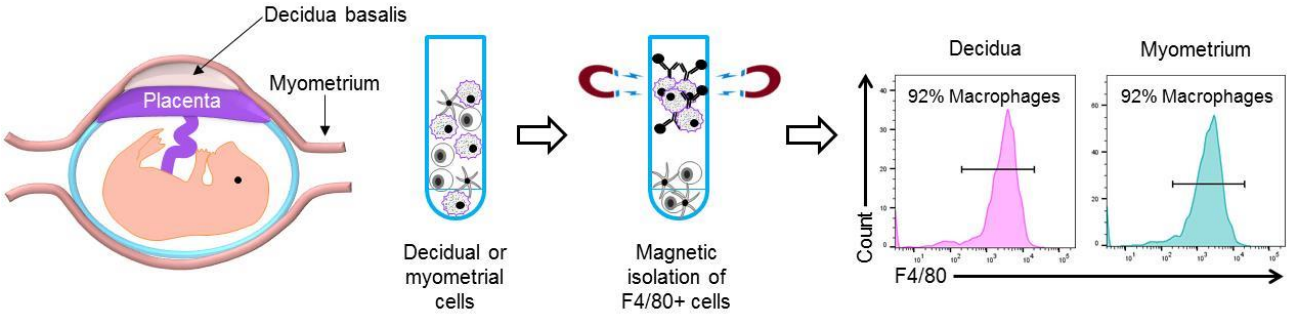


Figure 15. Macrophage isolation from the maternal-fetal interface and myometrium prior to *in vivo* T cell activation–induced preterm birth. Left to right: spatial localization of the murine decidua and myometrium. Workflow showing the magnetic isolation of macrophages from decidual and myometrial cells; macrophage purity (F4/80+ cells; >92%) was determined by flow cytometry.

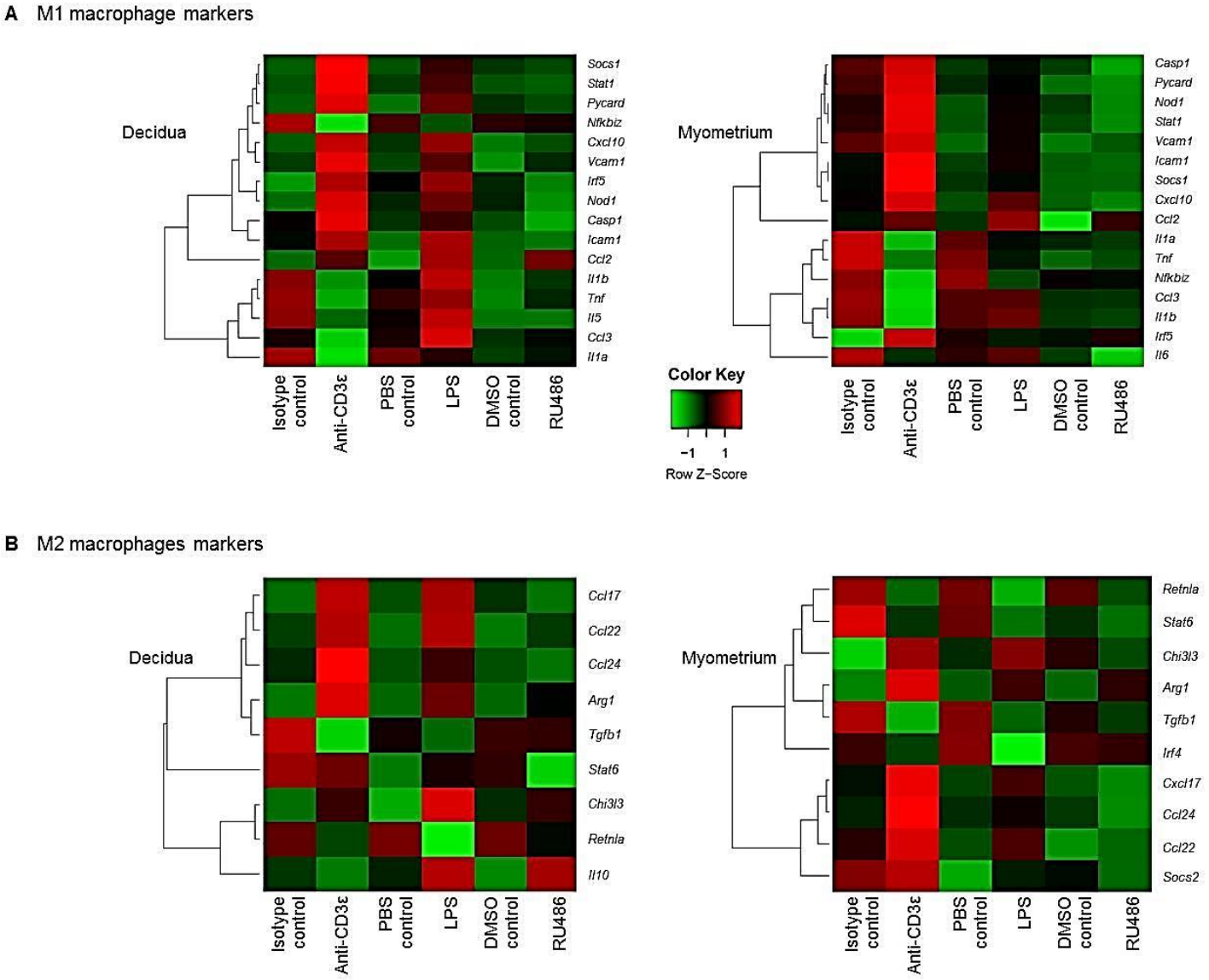


Figure 16. Proinflammatory macrophages at the maternal-fetal interface and myometrium prior to *in vivo* T cell activation–induced preterm birth. (**A**) Heat map visualization of the expression of M1 macrophage markers by F4/80+ cells isolated from the decidual and myometrial tissues of dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 6–8 each). (**B**) Heat map visualization of the expression of M2 macrophage markers by F4/80+ cells isolated from the decidual and myometrial tissues of dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 6–8 each). Negative (-)ΔCt values were calculated using *Actb, Gusb, Gapdh*, and H*sp90ab1* as reference genes.

Neutrophils are central players in the acute innate immune responses related to preterm labor associated with intra-amniotic inflammation/infection [151-156]. Therefore, we performed the immunophenotyping of neutrophils as well

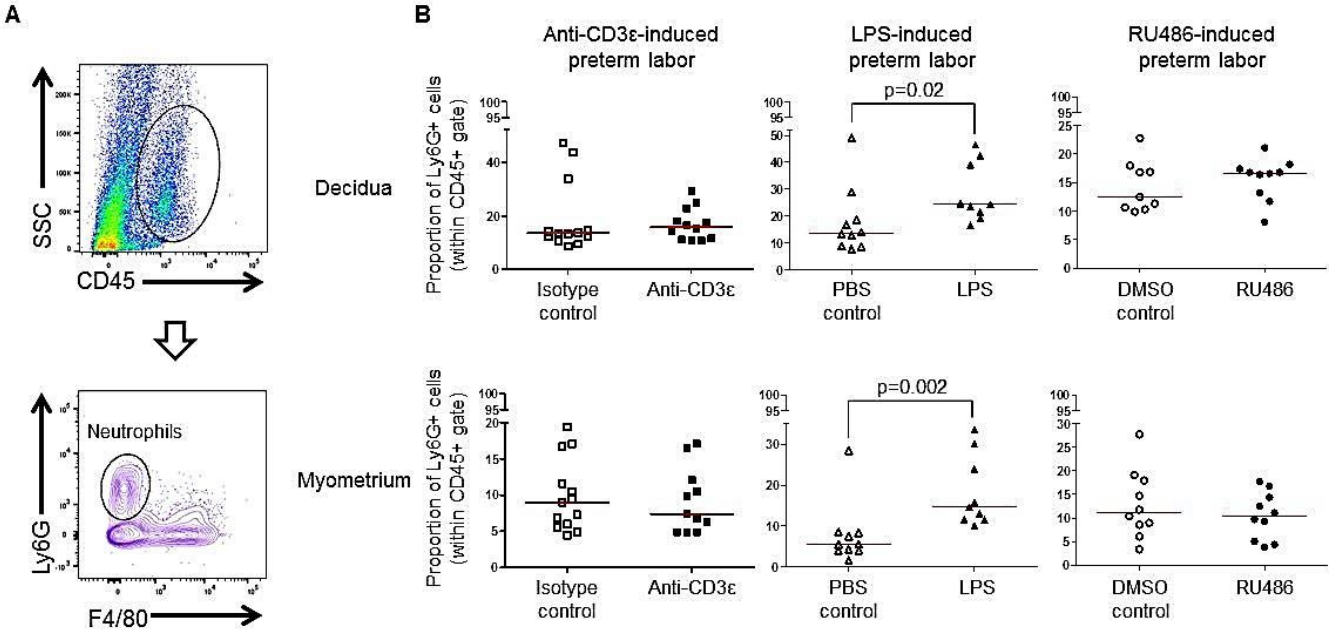


Figure 17. Lack of a neutrophilic influx at the maternal-fetal interface and myometrium prior to *in vivo* T cell activation–induced preterm birth. (**A**) Gating strategy used to identify neutrophils (CD45+ Ly6G+ cells) at the maternal-fetal interface. (**B**) Proportion of neutrophils in decidual and myometrial tissues from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n = 9–13 each).

(Figure 17A). Indeed, an influx of neutrophils in the decidua and myometrium is observed prior to LPS-induced preterm labor/birth [38, 157]. Consistently, we showed that LPS caused increased neutrophil infiltration in the decidua and myometrium (Figure 17B). However, such a neutrophilic response was not observed upon anti-CD3ε or RU486 injection (Figure 17B). These data are relevant since they suggest that *in vivo* T-cell activation induces preterm labor in the absence of an augmented neutrophil infiltration at the maternal-fetal interface and myometrium, indicating that it is a distinct inflammatory process from that induced by bacteria.

Taken together, these data demonstrate that *in vivo* T-cell activation induces preterm labor and birth by inducing an increased in M1-like macrophages; yet, these inflammatory responses are independent of an increased neutrophil infiltration at the maternal-fetal interface and myometrium.

7.7. In vivo T-cell activation-induced preterm labor and birth is prevented by treatment with progesterone

Parturition in animals [158], and likely in humans [159], is associated with a functional progesterone (P4) withdrawal. Thus, the administration of this steroid hormone is clinically used to prevent preterm birth [160-168]. Therefore, we tested whether *in vivo* T-cell activation could induce a systemic withdrawal of P4 and whether its administration could prevent preterm labor/birth. Anti-CD3ε induced a drop in the systemic concentration of P4 (Figure 18A). Thus, dams received P4 prior to T-cell activation, as shown in the treatment diagram (Figure 18B). Strikingly, T-cell activation-induced preterm labor/birth was entirely prevented by treatment with P4, which was translated to a longer gestational length (Figure 18C&D). Importantly, neonates born to dams injected with anti-CD3ε and treated with P4 had reduced mortality at birth compared to those injected with anti-CD3ε and the vehicle (sesame oil or SO) (Figure 18E). Representative images showed that neonates born to dams injected with anti-CD3ε and treated with P4 are comparable in size to controls (SO + isotype and P4 + isotype) (Figure 18F). Indeed, neonates born to dams injected with anti-CD3ε and treated with P4 thrived as indicated by the presence of the milk band which was not observed in those pups born to untreated dams who died shortly after delivery (Figure 18G, rectangles).

It is well established that progesterone prevents preterm birth by exhibiting anti-inflammatory effects at the maternal-fetal interface, myometrium, and the cervix [169-171]. Therefore, we next evaluated the global anti-inflammatory effect of progesterone in dams injected with anti-CD3ε. Targeted qRT-PCR profiling showed that treatment with P4 downregulated the expression of several inflammatory mediators induced by anti-CD3ε in the decidua and cervix, but such an effect was not as strong in the myometrium (Figure 19A-C). For example, dams treated with P4 + anti-CD3ε had reduced expression of *Casp11*, *Ccl22*, *Icam1, Ctla4, Nod1,* and *Ccl5* in the decidua compared to dams injected with SO + anti-CD3ε (Figure 20A). In the myometrium, only *Il33* was downregulated upon P4 + anti-CD3ε treatment (Figure 19B). In addition, dams treated with P4 + anti-CD3ε had reduced expression of *Il33*, *Il6*, *Il12b, Il1a*, *Pycard*, and *Il4* in the cervical tissues compared to those injected with SO + anti-CD3ε (Figure 20B).

Collectively, these findings provide further evidence that progesterone attenuates local inflammatory responses at the maternal-fetal interface and in the cervix, preventing T-cell activation-induced preterm labor and birth, which translates to reduced adverse neonatal outcomes.

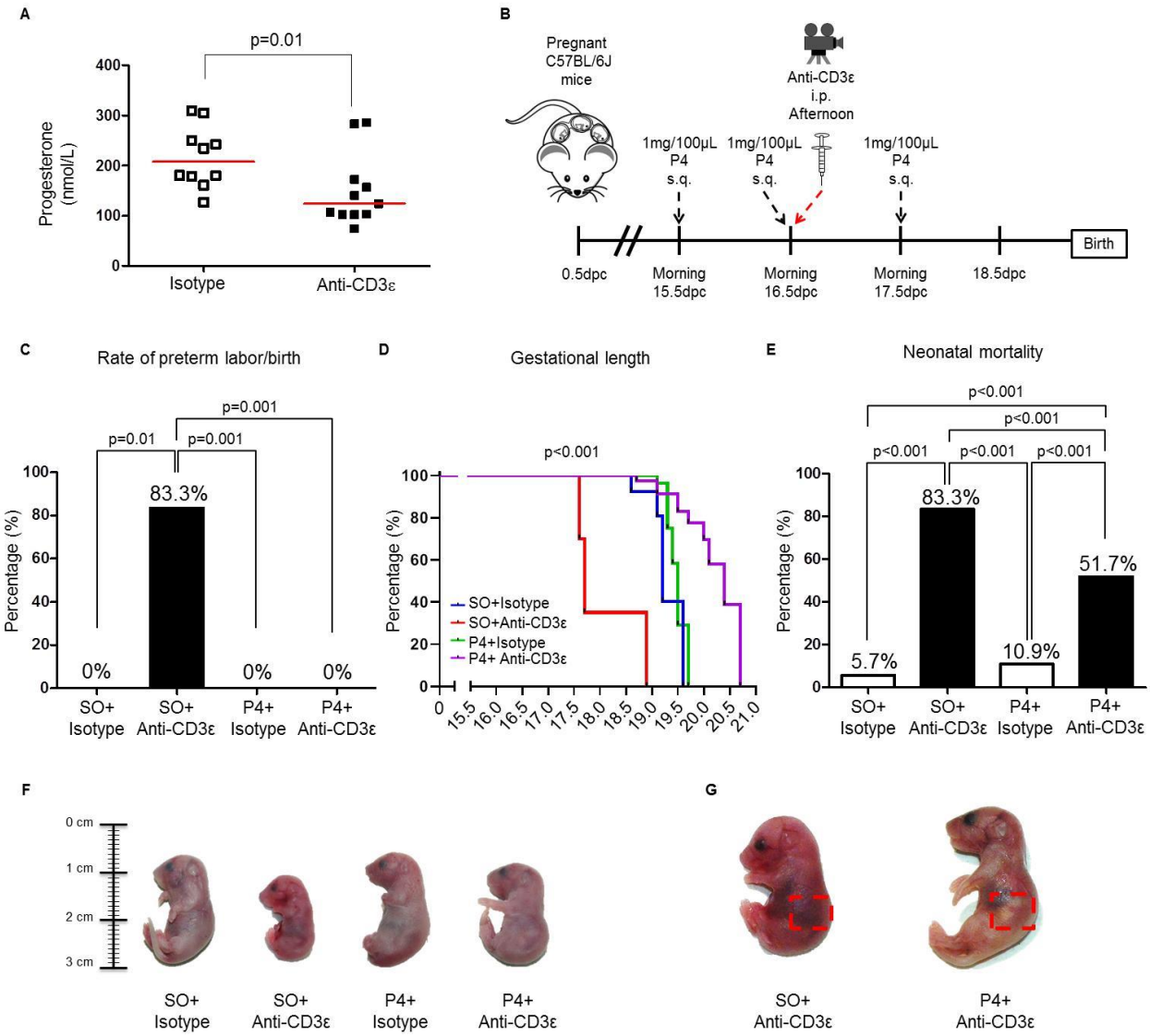
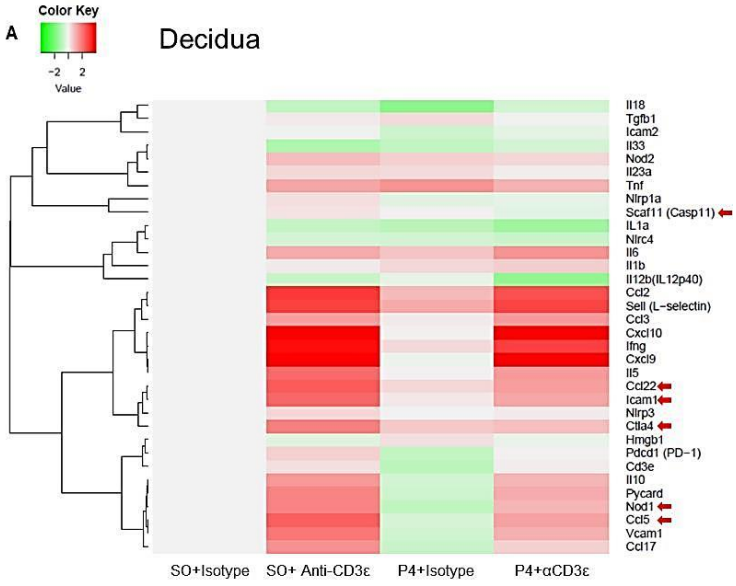
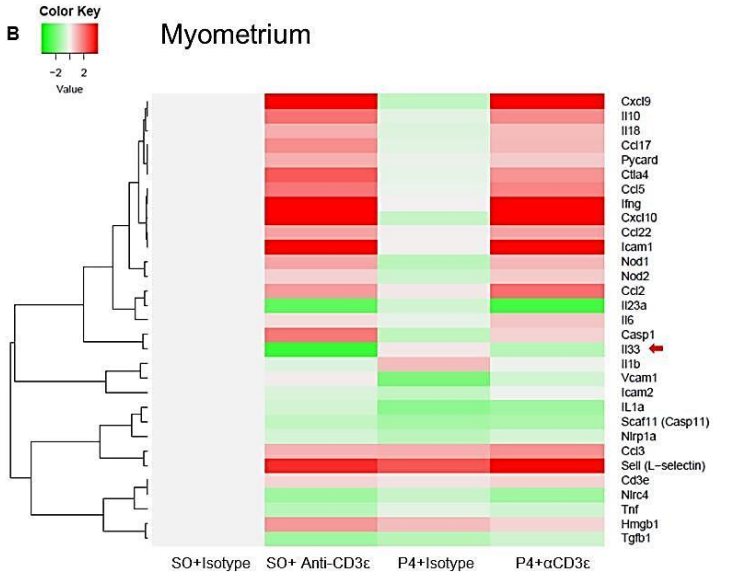


Figure 18. Progesterone prevents *in vivo* T cell activation–induced preterm labor/birth and reduces adverse neonatal outcomes. (**A**) Systemic P4 concentration in dams injected with anti-CD3ε (or isotype control) (n = 10–11 each). The p value was determined by two-tailed Mann-Whitney U test. (**B**) Scheme of treatment with P4: dams were treated with either P4 or SO, injected with either anti-CD3ε or isotype control, and video monitored until delivery (n = 5–10 each). (**C**) The rate of preterm birth in dams injected with SO + isotype, SO + anti-CD3ε, P4 + isotype, or P4 + anti-CD3ε (n = 5–10 each). (**D**) Gestational length and (**E**) the rate of neonatal mortality from pups born to dams injected with SO + isotype, SO + anti-CD3ε, P4 + isotype, or P4 + anti-CD3ε (n = 5–10 each). (**F**) Representative images of neonates born to dams injected with SO + isotype, SO + anti-CD3ε, P4 + isotype, or P4 + anti-CD3ε (n = 3 each). (**G**) Representative images of neonates born to dams injected with SO + anti-CD3ε (left) or P4 + anti-CD3ε (right). Red dashed rectangle indicates the location of the milk band (n = 3 each).





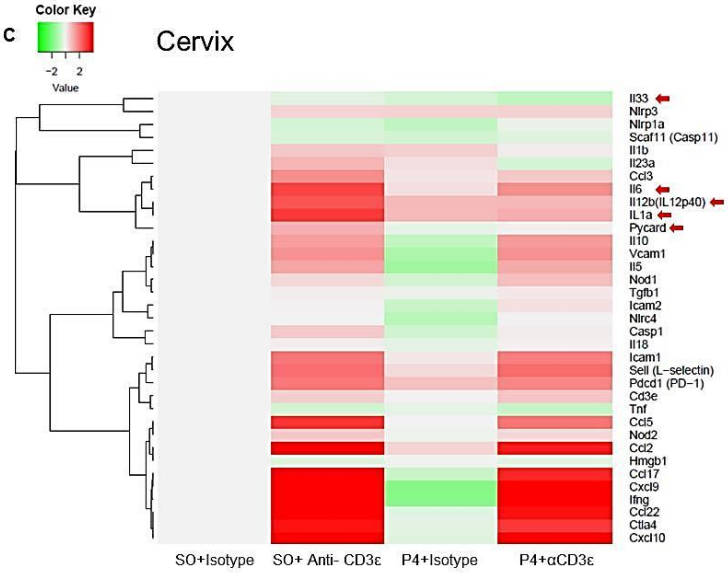


Figure 19. Progesterone prevents *in vivo* T cell activation–induced preterm labor/birth by downregulating inflammatory gene expression at the maternal-fetal interface and in the cervix. Decidual, myometrial, and cervical tissues from dams injected with SO + isotype, SO + anti-CD3ε, P4 + isotype, or P4 + anti-CD3ε (n = 5 each). Heat map visualization of inflammatory gene expression in the (**A**) decidual, (**B**) myometrial, and (**C**) cervical tissues. Red arrows alongside the heat maps indicate the genes chosen for plotting. Data are from individual dams (n = 5 each).

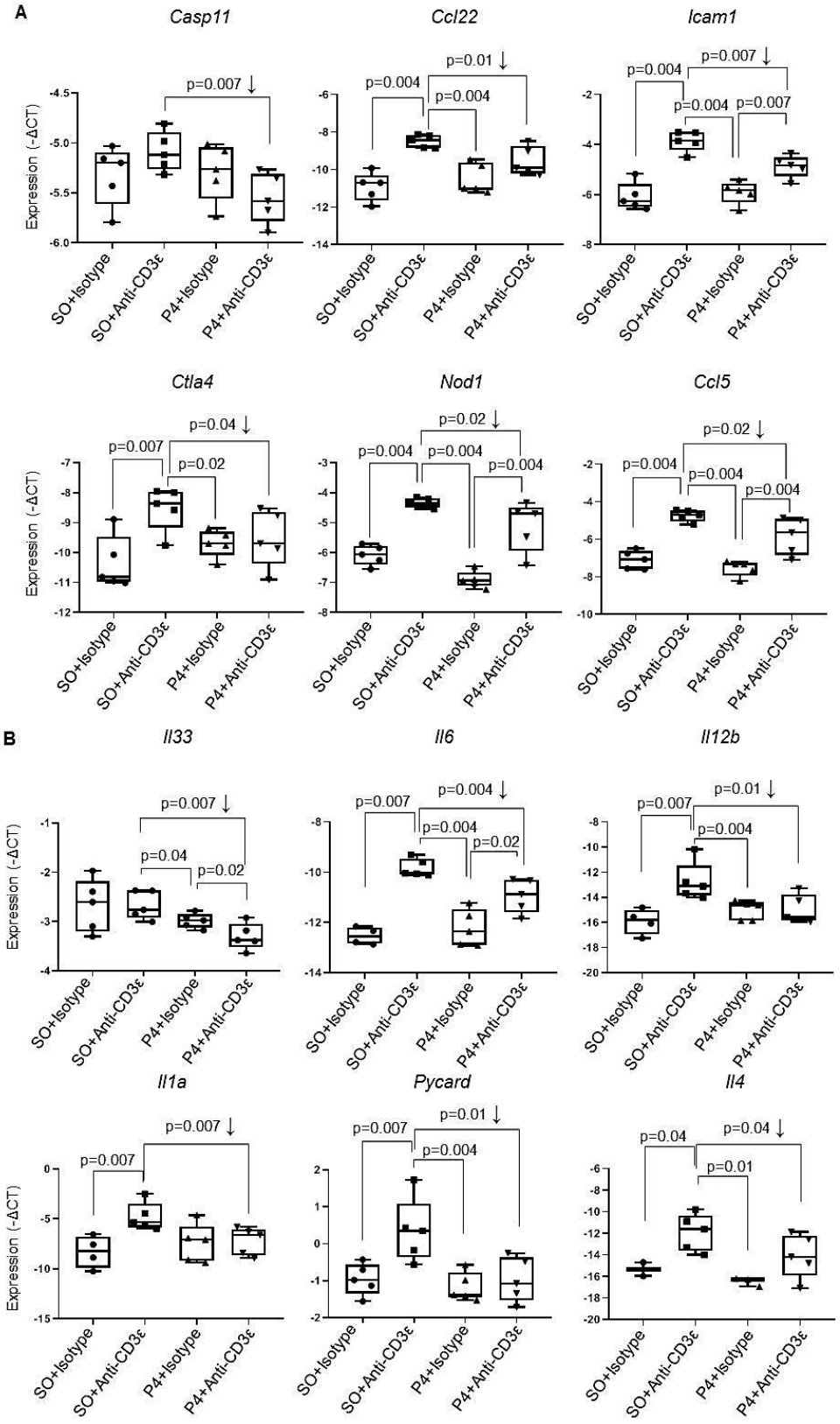


Figure 20. Progesterone prevents *in vivo* T cell activation–induced preterm labor/birth by downregulating inflammatory gene expression at the maternal-fetal interface and in the cervix. The -ΔCt values of each group were centered on the -Δ Ct value of the control group treated with SO + isotype. (**A** and **B**) mRNA expression of selected genes in decidual and cervical tissues. Negative (-)ΔCt values were calculated using *Actb, Gusb, Gapdh*, and *Hsp90ab1* as reference genes. Data are from individual dams (n = 5 each).

8. DISCUSSION

The study herein presents evidence that effector/activated maternal T cells lead to pathological inflammation and, subsequently, preterm labor/birth and neonatal mortality. We showed that effector memory and activated maternal T cells expressing granzyme B and perforin are enriched at the maternal-fetal interface of women with spontaneous preterm labor and birth. Next, using a murine model, we reported that, prior to inducing preterm birth, *in vivo* T-cell activation induces an increased in M1-like macrophages, and the upregulation of inflammatory mediators at the maternal-fetal interface and myometrium, in the absence of an increased influx of neutrophils. Moreover, we showed that *in vivo* T-cell activation triggers an intra-amniotic inflammatory response causing fetal compromise *in utero* prior to preterm birth. We then provided evidence that treatment with progesterone could serve as an anti-inflammatory strategy to prevent preterm birth and adverse neonatal outcomes induced by T-cell activation (Figure 21).

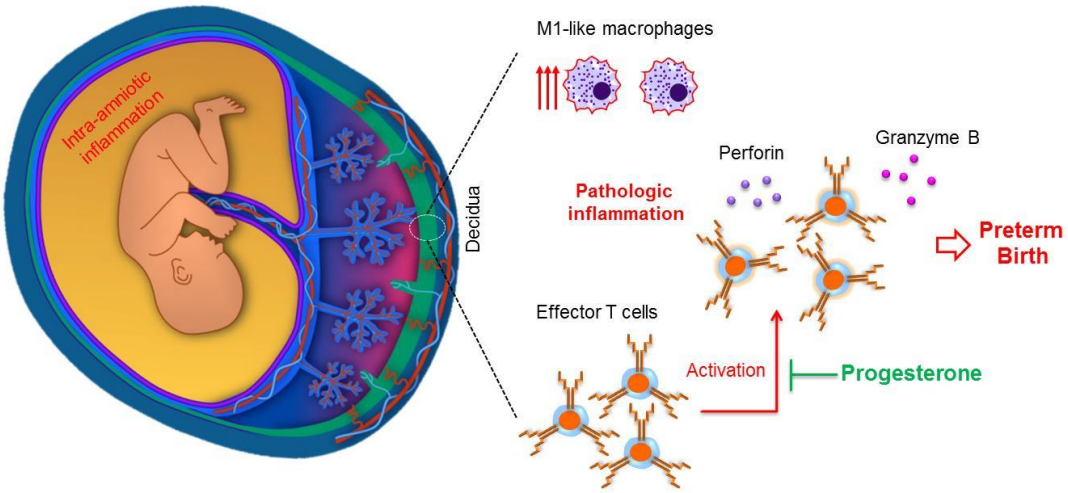


Figure 21. Maternal effector and activated T cells expressing granzyme B and perforin can induce pathologic inflammation by initiating local immune responses at the maternal-fetal interface (decidua) (i.e., an increased in M1-like macrophages without an increased influx of neutrophils) which, in turns, leads to preterm labor and birth. Activation of T cells also induces inflammatory responses in the maternal circulation and the amniotic cavity, inducing fetal compromise prior to preterm labor and birth. These effects can be abrogated by treatment with the anti-inflammatory and clinically approved strategy, progesterone.

We and others have shown that effector memory and/or activated T cells can be recruited by [70, 91], and are present at [48, 49, 71, 78, 80, 172-176], the human maternal-fetal interface in term pregnancy. However, providing a role for these T cells in the pathogenesis of pregnancy complications has been challenging because of the absence of the disease (e.g. preterm labor). Herein, we report that effector memory maternal T cells are enriched in the decidual tissues of women with spontaneous preterm labor, who delivered preterm. Such an increase was not observed in patients who underwent the physiological process of labor at term, indicating that these effector cells contribute solely to the pathological process of premature labor. Such T-cell responses could be antigen-dependent or antigen-independent; the latter could be driven by cytokines [106, 177]. The conventional belief is that effector memory T cells recognized placental-fetal antigens [60, 63, 175, 178]; yet, we suggest that both antigen-dependent and antigen-independent processes may occur at the human maternal-fetal interface during spontaneous preterm labor. In line with this concept, CD8+ T_{EMRA} cells, lymphocytes with high cytolytic activity in the absence of *in vitro* pre-stimulation and that can proliferate in an antigen-independent manner [105, 179], were more abundant in the decidual tissues of women with spontaneous preterm labor compared to those who delivered preterm in the absence of labor.

Effector CD4+ and CD8+ T cells express high levels of granzyme B and perforin, which are stored in cytolytic granules and upon activation are released towards the target cell [180-183]. Recently, it was shown that term-isolated decidual CD8+ T cells can degranulate, proliferate and produce inflammatory mediators upon *in vitro* stimulation [176], suggesting a role for these cells in pregnancy complications. Herein, we show that both decidual CD4+ and CD8+ T cells can express increased levels of granzyme B and perforin in women with spontaneous preterm labor, providing conclusive evidence that decidual T cells exhibit an effector and pro-inflammatory phenotype during the pathological process of premature parturition.

To investigate the mechanisms whereby effector T cells lead to spontaneous preterm labor, we used a murine model of transient *in vivo* T-cell activation, the injection of the hamster anti-CD3 monoclonal antibody [97, 184]. *In vivo* T-cell activation at the maternal-fetal interface and myometrium was proven by the upregulation of activation markers (e.g. CD25 [116] and CD69 [117]) in CD4+ and CD8+ T cells, and increased proportions of CD4+ T cells expressing IL-2 [185]. *In vivo* T-cell activation also induced an increase in the proportion of IFNγ-expressing CD8+ T cells in the myometrium but not in the decidua. This is consistent with *in vitro* studies showing that CD3 stimulation triggers the expression of IFNγ by CD8+ T cells [119]. IFNγ-expressing CD8+ T cells were also increased upon LPS injection in the decidua but not in the myometrium, suggesting that T cells respond differently in each anatomical compartment. The abundance of IFNγ-expressing CD8+ T cells in the myometrium, but not in the decidua, could be explained by the fact that this cytokine is implicated in myometrial function, including contractility [186, 187]. These data show that the *in vivo* activation of T cells induces preterm birth by initiating local pathophysiological processes, which partially resemble those induced by microbial products but are different from those caused by the blockage of progesterone action.

Yet, *in vivo* T-cell activation-induced preterm labor/birth was accompanied by the upregulation of inflammatory mediators at the maternal-fetal interface and myometrium as well as in the amniotic cavity. These local [13, 127, 128, 142, 147, 188] immune responses have been frequently observed in women who undergo spontaneous preterm labor with intra-amniotic infection and/or inflammation. Therefore, the *in vivo* T-cell activation-induced preterm labor/birth model shares the local inflammatory responses with the microbial-induced preterm labor/birth model. In contrast, the RU486-induced preterm labor/birth model is characterized by the absence of local inflammation [189]. The severe inflammatory responses in the amniotic cavity explained the deleterious effect (i.e. bradycardia) observed in fetuses/neonates from dams injected with anti-CD3.

In vivo T-cell activation also had unique effects on innate immune cells at the maternal-fetal interface and myometrium. Previous studies have shown that macrophage activation is complex and does not tally with the M1/M2 polarization model [190]. Similarly, at the human maternal-fetal interface, macrophage phenotypes do not entirely fit the M1/M2 polarization model [191, 192]; yet, these cells possess a pro-inflammatory phenotype (i.e. M1-like) in women who underwent spontaneous preterm labor [39]. Consistently, we found that, prior to *in vivo* T-cell activation-induced preterm birth, a predominantly pro-inflammatory (M1-like) macrophage population was observed at the murine maternal-fetal interface and myometrium. In this context, a pro-inflammatory macrophage phenotype could have been driven by cytokines released upon T-cell activation [193]. Importantly, *in vivo* T-cell activation did not cause an increase in the infiltration of neutrophils in the decidua and myometrium prior to preterm birth. Such an increase in neutrophils has been consistently observed in the context of preterm labor/birth associated with intra-amniotic infection [38, 157, 194-196] but not in the anti-progestin model [194, 195]. Thus, we surmise that *in vivo* T-cell activation induces preterm birth by initiating immune responses that are partially different from those triggered by microbes and distinct from those caused by anti-progestins. In addition, these results indicate that the model of *in vivo* T-cell activation-induced preterm birth occurs in the absence of neutrophilic infiltration, which is distinct from the most commonly studied cause of preterm labor/birth, intra-amniotic infection [11, 197, 198].

In vivo T-cell activation-induced preterm labor/birth was prevented by treatment with progesterone. Importantly, the adverse neonatal outcomes induced upon T-cell activation were also ameliorated. Indeed, most of the neonates born to dams injected with anti-CD3 and treated with progesterone thrived. The protective effects of progesterone during pregnancy have been previously shown to be mediated, at least in part, by modulating T-cell responses [199-201]. Indeed, we have previously shown that progesterone prevents endotoxin-induced preterm birth by fostering an anti-inflammatory response at the maternal-fetal interface, characterized by fewer effector T cells, activated macrophages, and neutrophils, as

well as increased regulatory T cells [169]. The anti-inflammatory effects of progesterone were reflected in the cervical tissues as well [169]. Progesterone also has anti-inflammatory effects in the systemic circulation and myometrium [169, 171]. Herein, we provide further evidence of the anti-inflammatory effects of progesterone. Specifically, we show that progesterone attenuated the local pro-inflammatory responses at the maternal-fetal interface and the cervix. Of interest, progesterone dampened genes related to the inflammasome pathway (e.g. *Casp11*, *Pycard*, *Nod1*), which has been strongly associated with the mechanisms initiating the pathological process of preterm labor [26, 138, 139, 202-205]. Collectively, these results demonstrate that preterm labor/birth and adverse neonatal outcomes induced by T-cell activation can be treated by using the anti-inflammatory effects of progesterone, a clinically approved therapy.

It is worth mentioning that, following T-cell activation, progesterone did not strongly impact the expression of inflammatory genes in the myometrium, except for upregulating the expression of *Il33*. The expression of this cytokine, however, was attenuated in the cervix, indicating that each anatomical compartment is differentially regulated by progesterone. This concept is in line with our initial findings indicating that T-cell activation and LPS trigger different responses in the decidua and myometrium. This last observation provides an example of the complexity of immune interactions at the maternal-fetal interface and in the reproductive tissues: each compartment must regulate specific mediators which contribute to the complex phenomenon of parturition.

A central question that remains unanswered is what causes T-cell activation leading to spontaneous preterm labor and birth in women. The most tempting explanation is that placental-fetal antigens induce the activation of maternal T cells in the systemic circulation and/or at the maternal-fetal interface. These antigens could have been presented by APCs in the spleen and uterine-draining lymph nodes and/or at the maternal-fetal interface [79, 94]. However, such a hypothesis is difficult to prove in humans [94]. A second possibility is that effector memory T cells are not specific for placental-fetal antigens, but instead they recognize microbe-derived peptides which are normally present in the basal plate of the placenta [206]

and/or the endometrium [207]. Such T cells can then proliferate in an antigen-independent manner by pro-inflammatory mediators [177] induced by dangers signals or alarmins in the intra-amniotic space (i.e. sterile intra-amniotic inflammation) [147]. This concept is supported by the fact that most cases of preterm labor take place in the setting of sterile intra-amniotic inflammation [15, 145], which can occur in the absence of acute histologic chorioamnionitis [neutrophil infiltration in the chorioamniotic membranes [208]] [145, 205]. A third possibility is that effector memory T cells at the maternal-fetal interface are virus-specific; however, a clear association between viral infections and human preterm labor requires further epidemiological studies. In this latter context, it has been suggested that maternal-fetal tolerance is compromised by the virus and polymicrobial infections can take place, inducing preterm labor and birth [27, 209-211]. Further research is required to investigate the antigen specificity and clonality of effector T cells at the maternal-fetal interface in the context of preterm labor and birth.

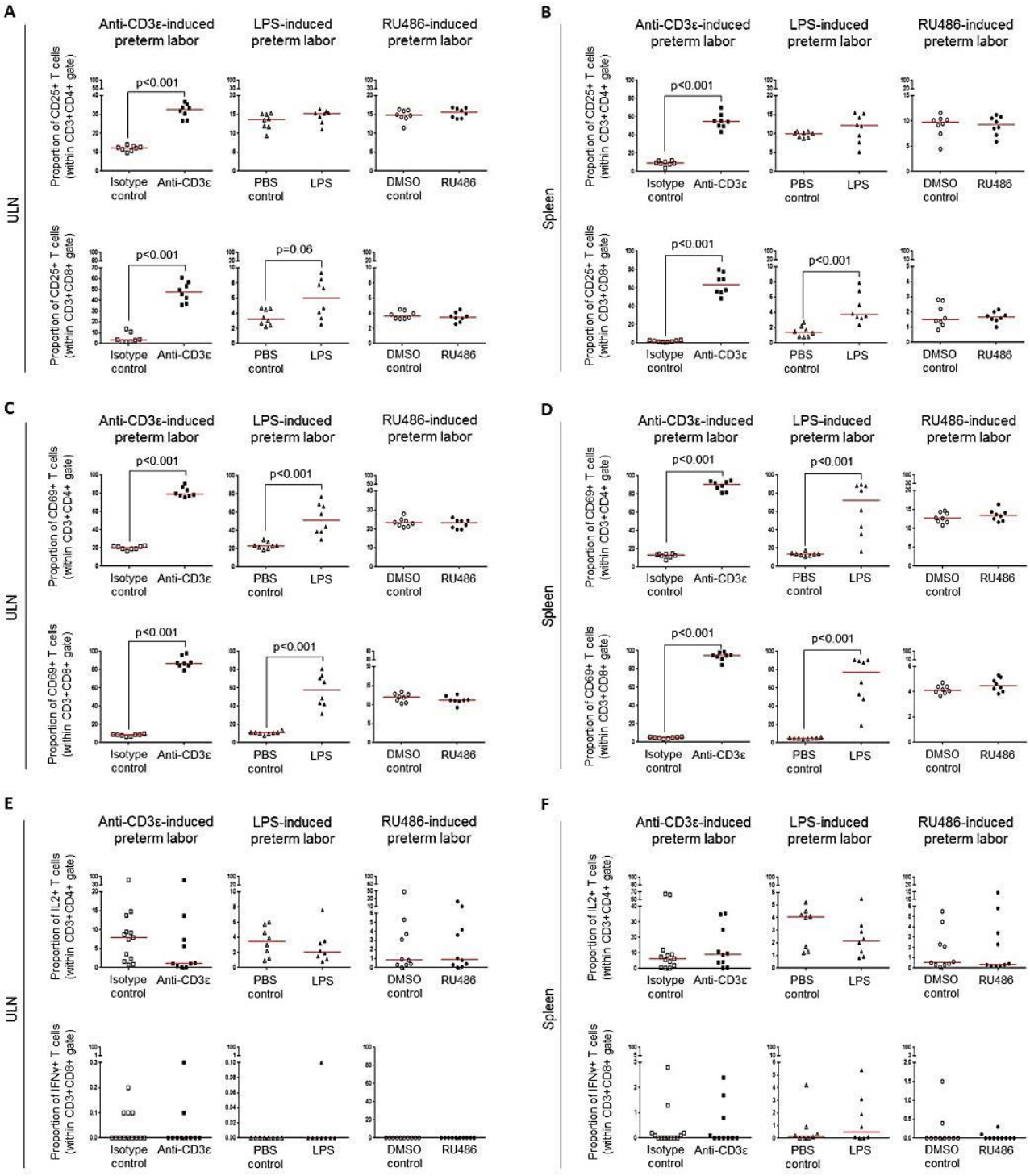
9. CONCLUSIONS

1. Effector and activated maternal T cells expressing granzyme B and perforin are enriched at the maternal-fetal interface of women with spontaneous preterm labor and birth
2. Anti-CD3ε antibody induced the activation of T cells at the maternal-fetal interface and myometrium, as well as in the lymphatic tissues
3. The *in vivo* activation of T cells using an anti-CD3ε antibody, induced preterm labor/birth and adverse neonatal outcomes
4. The *in vivo* activation of T cells using an anti-CD3ε antibody, caused fetal compromise *in utero*
5. *In vivo* T-cell activation induced the upregulation of inflammatory mediators at the maternal-fetal interface and in the myometrium, as well as intra-amniotic inflammatory responses prior to preterm birth
6. *In vivo* T-cell activation caused an increased proportion of M1-like macrophages in the absence of an increased influx of neutrophils at the maternal-fetal interface prior to preterm birth
7. *In vivo* T-cell activation induces preterm birth by initiating immune responses that are partially different from those triggered by microbes (LPS) and distinct from those caused by anti-progestins (RU486)
8. Treatment with progesterone could serve as an anti-inflammatory strategy to prevent preterm birth and reduce adverse neonatal outcomes induced by T-cell activation

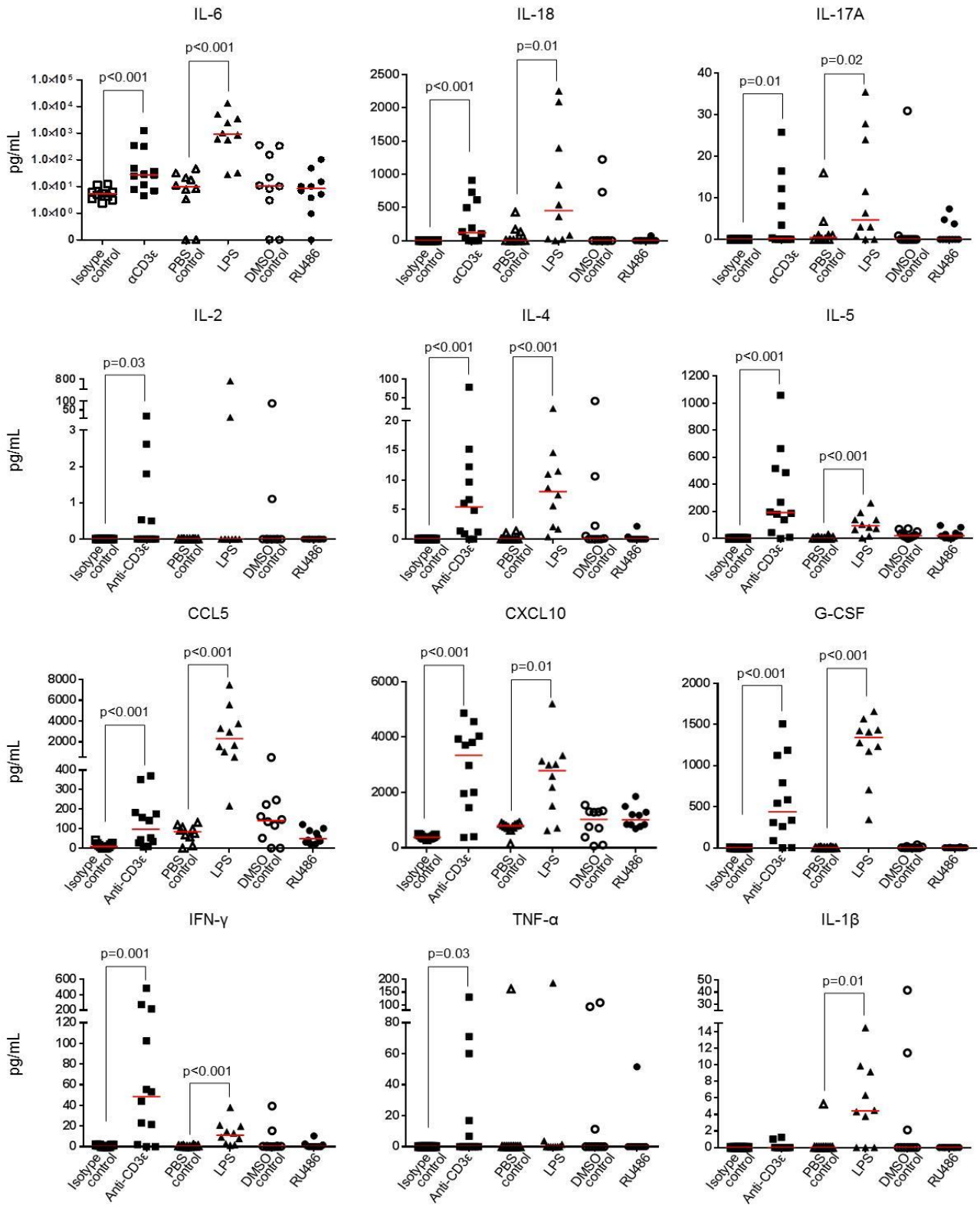
10. PERSPECTIVES

- To investigate the antigen specificity and clonality of the effector and activated T cells at the maternal-fetal interface in the context of preterm labor and birth
- To determine whether other effector T cells are enriched at the maternal-fetal interface of women that underwent spontaneous preterm labor and birth
- To create an animal model to induce a local (i.e. maternal-fetal interface) inflammatory T cell responses
- To determine whether a local pro-inflammatory T cell response induce preterm labor/birth and adverse neonatal outcomes

11. SUPPLEMENTARY DATA



Supplementary Figure 1. Activation of T cells in lymphatic tissues. Proportions of CD4+ and CD8+ T cells expressing CD25 in the (**A**) ULN and (**B**) spleen from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n=8 each). Proportions of CD4+ and CD8+ T cells expressing CD69 in the (**C**) ULN and (**D**) spleen from dams injected with αCD3ε, isotype, LPS, PBS, RU486, or DMSO (n=8 each). Proportion of CD4+ T cells expressing IL2 and CD8+ T cells expressing IFN-γ in the (**E**) ULN and (**F**) spleen from dams injected with anti-CD3ε, isotype, LPS, PBS, RU486, or DMSO (n=8-13 each).



Supplementary Figure 2. The maternal inflammatory response prior to preterm birth. Dams were injected with anti-CD3ε (or isotype control), LPS (or PBS control), or RU486 (or DMSO control). Concentrations of IL-6, IL-18, IL-17A, IL-2, IL-4, IL-5, CCL5, CXCL10, G-CSF, IFN-γ, TNF-α, and IL-1β in the maternal serum were determined using a cytokine multiplex assay (n=10-12 each).

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