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RESEARCH ARTICLE

An imbalance between innate and adaptive immune cells at the maternal–fetal interface occurs prior to endotoxin-induced preterm birth

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Preterm birth (PTB) is the leading cause of neonatal morbidity and mortality worldwide. A transition from an anti-inflammatory state to a pro-inflammatory state in the mother and at the maternal–fetal interface has been implicated in the pathophysiology of microbial-induced preterm labor. However, it is unclear which immune cells mediate this transition. We hypothesized that an imbalance between innate and adaptive immune cells at the maternal–fetal interface will occur prior to microbial-induced preterm labor. Using an established murine model of endotoxin-induced PTB, our results demonstrate that prior to delivery there is a reduction of CD4+ regulatory T cells (Tregs) in the uterine tissues. This reduction is neither linked to a diminished number of Tregs in the spleen, nor to an impaired production of IL10, CCL17, or CCL22 by the uterine tissues. Endotoxin administration to pregnant mice does not alter effector CD4+ T cells at the maternal–fetal interface. However, it causes an imbalance between Tregs (CD4+ and CD8+), effector CD8+ T cells, and Th17 cells in the spleen. In addition, endotoxin administration to pregnant mice leads to an excessive production of CCL2, CCL3, CCL17, and CCL22 by the uterine tissues as well as abundant neutrophils. This imbalance in the uterine microenvironment is accompanied by scarce APC-like cells such as macrophages and MHC II+ neutrophils. Collectively, these results demonstrate that endotoxin administration to pregnant mice causes an imbalance between innate and adaptive immune cells at the maternal–fetal interface.
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INTRODUCTION

Preterm birth (PTB), defined as delivery before 37 weeks of gestation, is the leading cause of neonatal morbidity and mortality.[1,2] Premature neonates are at high risk for short-term complications such as respiratory distress syndrome, interventricular hemorrhage, neonatal sepsis, and necrotizing enterocolitis. Long-term complications include neurodevelopmental disorders such as cerebral palsy, chronic lung disease, blindness, and deafness.[3] In the USA, PTBs accounted for 11.55% of all documented births in 2012.[1] Therefore, it is essential to determine the mechanisms that lead to preterm labor in order to understand the pathophysiology of PTB.

Preterm labor is a syndrome caused by multiple pathological processes.[4] Of all these putative causes, intra-amniotic infection is the only process for which a causal link to PTB has been established.[5,6] Indeed, infection accounts for 30% of PTBs.[7] It is well accepted that infection causes a transition from an anti-inflammatory state to a pro-inflammatory state in the mother, which involves infiltration of innate immune cells and increased expression of inflammatory mediators at the maternal–fetal

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interface, triggering preterm parturition.[6,8] Therefore, the phenotypic characterization of innate and adaptive immune cells and their mediators may provide new insight into the mechanisms that lead to infection-induced PTB.

Animal models used to investigate the mechanisms whereby infection causes PTB have included the mouse,[9–12] rat,[13,14] rabbit,[15] sheep,[16] guinea pig,[17,18] and monkey.[19,20] Infection-induced PTB can be caused by systemic or local administration of a bacterial product such as lipopolysaccharide (LPS) or endotoxin.[21] Although there is a direct association between an infection-induced inflammatory response and PTB, it is unclear which innate and adaptive immune cells participate in this process.

Endotoxin-induced PTB is characterized by an increased number of neutrophils at the maternal–fetal interface (decidual and uterine tissues).[22] However, the role of neutrophils remains unclear because their depletion does not prevent PTB in mice treated with endotoxin.[23] Macrophages also participate in the immunological mechanisms that lead to endotoxin-induced PTB as their depletion restores parturition on time.[24] Both macrophages and neutrophils are classically known as sources of pro-inflammatory cytokines at the maternal–fetal interface during labor at term and preterm stages.[22,25–29] However, these innate cells can also have anti-inflammatory functions [30–32] or act as antigen-presenting cells (APCs) that initiate T-cell responses as they express MHC class II and co-stimulatory molecules.[33,34]

T-cell responses may also be implicated in the mechanisms that lead to PTB since CD8+ T cells are present at the maternal–fetal interface of women with chronic chorioamnionitis,[35] a placental lesion associated with spontaneous PTB.[36] Memory-like CD4+ T cells are also abundant at the human maternal–fetal interface during spontaneous labor at term.[37] Pregnancy-driven CD4+ T cells can exhibit effector or regulatory functions.[8] Regulatory T cells (CD4+CD25+Foxp3+ T cells; Tregs) may contribute to the establishment of immune privilege at the maternal–fetal interface.[38–41] A reduction in the proportion or function of circulating Tregs is associated with both term and preterm labor.[42,43] However, the role of Tregs in acute inflammation that leads to PTB is poorly understood. Effector T cells have also been implicated in the mechanisms that lead to PTB. For example, Th17 cells infiltrate at the maternal–fetal interface, suggesting a role for these cells in the pathophysiology of acute chorioamnionitis,[44] a major cause of spontaneous preterm labor.[6] Indeed, an imbalance between Tregs and Th17 cells has been implicated in the pathophysiology of pregnancy-related complications such as implantation failure, miscarriage, pre-eclampsia, and preterm labor.[45,46]

Using a murine model of endotoxin-induced PTB, the aims of this study were to (1) investigate whether Tregs are altered prior to PTB, (2) determine whether Treg alterations are related to the concentrations of IL10 and/or Treg chemokines (CCL17 and CCL22) in the uterine tissues, (3) determine whether these Treg alterations are linked to changes in effector CD4+ T cells (Th1/Th2/Th9/Th17) and CD8+ T cells, and (4) evaluate whether these events are associated with changes in the phenotype of neutrophils and/or macrophages, and in macrophage chemokines (CCL2 and CCL3), at the maternal–fetal interface. The phenotypic characterization of the adaptive and innate immune cells at the maternal–fetal interface prior to endotoxin-induced PTB may provide insight into the mechanisms that lead to microbial-induced preterm labor.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in the animal facility at the C. S. Mott Center for Human Growth and Development (Wayne State University, Detroit, MI, USA). Mice were housed under a circadian cycle (light:dark = 12:12h). Females of 8–12 weeks old were mated with male mice of proven fertility. Females were examined daily for the presence of a vaginal plug, which indicates the 0.5-day post-coitum (dpc). Pregnant mice were injected intraperitoneally on 16.5 dpc with either 15 μg of LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA) in 200 μL of 1× phosphate-buffered saline (PBS) or 200 μL of PBS as a control. Administration of LPS caused 100% of PTB (Table 1). Mice were euthanized 12 h post-injection, prior to PTB for the LPS-treated mice (Figure 1a). Uterine tissues at the implantation sites were collected from pregnant mice and snap-frozen and stored at –80°C until their use. Procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University, Detroit, MI, USA (Protocol Number A 09-08-12).

Leukocyte isolation

Immediately following euthanasia, the uterine and decidual tissues were collected and homogenized with scissors while suspended in StemPro® Accutase® Cell Dissociation Reagent (Life Technologies, Grand Island, NY, USA). Homogenates were incubated for 35 min at 37°C with gentle shaking, and

Table 1 Observational data from pregnant mice injected with PBS or LPS.

	PBS (200 μL)	LPS (15 μg/200 μL)	*P*-value
Number of mice	10	7	–
Gestational length (days)[a]	19.203 ± 0.07	17.77 ± 0.01	<0.0001
Delivery length (hours)[b]	1.41 ± 0.18	1.36 ± 0.27	NS
Stillbirth (%)[c]	8.19 ± 3.14	100	<0.0001
Preterm birth (%)[d]	0	100	–

[a] Days elapsed from the visualization of the vaginal plug (0.5-day post-coitum) to the delivery of the first pup. Pregnant mice were monitored via video camera following an injection of PBS or LPS;
[b] Hours elapsed from the birth of the first pup to the last pup;
[c] Number of dead pups/number of live pups at birth × 100;
[d] Number of dams delivered at preterm gestation (<18.0-day post-coitum)/number of dams delivered at term gestation (>18.0-day post-coitum) × 100.

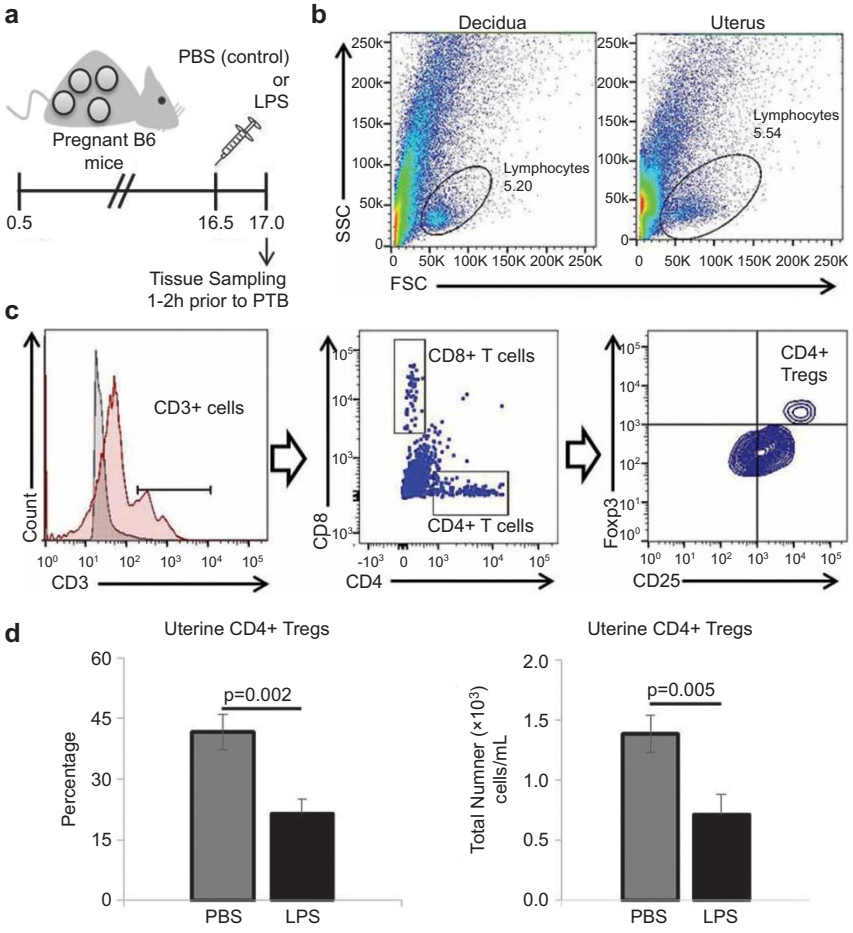

Figure 1 A reduction of uterine CD4+ Tregs prior to endotoxin-induced PTB. (**a**) LPS-induced PTB model. (**b**) Representative example of lymphocyte populations in the decidual and uterine tissues. (**c**) Gating strategy used to identify CD4+ Tregs in the uterine tissues. CD3+ (T) cells were gated within the lymphocyte gate (FSC vs. SSC), CD4+ and CD8+ T cells within the CD3+ gate, and CD4+ or CD8+ Tregs within the CD4+ or CD8+ gate, respectively. The gray histogram represents the auto-fluorescence control. (**d**) Proportion and number of uterine CD4+ Tregs in pregnant mice injected with PBS or LPS (n = 7–8 each). Data shown are means ± standard error of the mean.

then filtered using a 100 μm cell strainer (Fisher Scientific, Pittsburgh, PA, USA). Finally, cell suspensions were washed with fluorescence-activated cell sorting (FACS) buffer (bovine serum albumin 0.1%, sodium azide 0.05%, 1× PBS; BD Biosciences, San Jose, CA, USA), and the resulting cell pellet was immediately processed for immunophenotyping.[47]

The spleen and uterine-draining lymph nodes were collected and manually dissociated using glass slides and FACS buffer in a Petri dish. Released lymphocytes were then washed with FACS buffer, and the cell pellets were used for immunophenotyping.

Immunophenotyping

Cell suspensions were incubated with a mouse CD16/CD32 (Fcγ III/II Receptor; BD Biosciences) monoclonal antibody for 10 min at 4°C. Cell suspensions were then incubated for 30 min at 4°C with the appropriate extracellular and/or intracellular fluorochrome-conjugated antibodies as described in Supplementary Table 1.

Three different groups of pregnant mice were injected with either PBS or LPS as previously described, and tissues were processed for immunophenotyping. In the first group of mice, we determined the proportion and number of CD4+ and CD8+ Tregs in the uterine and decidual tissues using the extracellular markers CD3, CD4, CD8, and CD25, and the transcriptional factor Foxp3.

In the second group of mice, we determined the proportion and number of CD4+ and CD8+ Tregs in the spleen and uterine-draining lymph nodes, since these are sites of expansion of Tregs during pregnancy.[38] In this group, we also determined the proportion and number of effector CD4+ and CD8+ T cells using the extracellular markers CD3, CD4, CD8, and CD25, the intracellular cytokines IFNγ, IL9, IL10, and IL17A, and the transcriptional factor Foxp3.

In the third group of mice, we determined the proportion and number of effector CD4+ and CD8+ T cells in the decidual and uterine cell suspensions using the extracellular markers CD3, CD4, and CD8, the intracellular cytokines IFNγ, IL9, and IL17A, and the transcriptional factor Tbet. Additionally, we determined the proportion and number of neutrophils and macrophages and their subsets in the decidual and uterine

tissues using the extracellular markers CD11b, F4/80, Ly6G, and MHC II, and the intracellular cytokines IFN-γ and IL4.

For Foxp3 staining, the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) was used. For cytokine staining, the Cytofix/Cytoperm fixation and permeabilization solution (BD Biosciences) was used. The phenotypes of T cells and macrophages/neutrophils were analyzed within the CD3+ or CD11b+ region, respectively. The total number of splenic T cells was determined using CountBright Absolute Counting Beads (Molecular Probes, Invitrogen, Eugene, OR, USA). As a control for cellular auto-fluorescence, unstained cells were also treated in this same manner. Stained and unstained cell suspensions were re-suspended in 0.5 mL of FACS buffer (BD Biosciences), and acquired using the LSRFortessa Flow Cytometer. Flow cytometry analysis was performed using the BD FACSDiva Software Version 7.0 (BD Biosciences). The figures were prepared using FlowJo Software Version 10 (FlowJo, LLC, Ashland, OR, USA).

Uterine tissue protein extracts

The uterine tissues snap-frozen in liquid nitrogen were gradually defrosted and placed in a Falcon 12-well plate (Corning, Tewksbury, MA, USA). About 1 mL of Dulbecco's modified Eagle's medium supplemented with 1% of Gibco Antibiotic-Antimycotic solution (Life Technologies) was added into each well. Tissues were incubated at 37°C in 5% CO_2 for 24 h. Following incubation, tissues were homogenized using a Tissue-Tearor (Model No. 985370-395, BioSpec Products, Inc., Bartlesville, OK, USA) and centrifuged at 4°C, 21000g for 30 min to obtain a cell-free supernatant. The uterine protein extracts were then stored at –20°C until use.

ELISAs

ELISAs (Mouse/Rat CCL2/JE/MCP-1, Mouse CCL3/MIP-1α, Mouse CCL17/TARC, Mouse CCL22/MDC, and Mouse IL10; R&D Systems, Minneapolis, MN, USA) were used to quantify the concentrations of IL10, CCL2, CCL3, CCL17, and CCL22 in the uterine protein extracts, according to the manufacturer's instructions. An initial assay validation was performed in our laboratory prior to measurement. Briefly, recombinant mouse standards and the samples were incubated in duplicate wells of the 96-well microplates pre-coated with polyclonal/monoclonal antibodies specific to target analytes. During incubation, immobilized antibodies in the microplates bound target proteins that were present in the standard and sample groups. After washing unbound substances, enzyme-conjugated polyclonal antibodies specific to the target proteins were added to the wells. Upon completion of incubation, assay plates were washed to remove unbound antibodies. This step was then followed by the addition of a substrate solution which developed color that was proportional to the amount of target protein bound in the initial step. Finally, the color development was stopped by the addition of a hydrochloric acid solution, and the microplates were read using a programmable spectrophotometer (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The sensitivities of the assays were <2 pg/mL (CCL2), <1.5 pg/mL (CCL3), <5 pg/mL (CCL17), 1.2 pg/mL (CCL22), and 0.625–5.22 pg/mL (IL10), according to the manufacturer's instructions. The total protein concentration in the uterine protein extracts was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA); however, no differences were observed between mice injected with PBS or LPS (Supplementary Figure 1).

Statistical analysis

Analyses were performed using SPSS Version 19.0 for 2010 (IBM Corporation, Armonk, NY, USA). A Shapiro–Wilk test was performed to determine whether the data were normally distributed. When this was the case, the *t*-test was used. The Mann–Whitney *U* test was used when the data were not normally distributed. For clarity of presentation, we have presented the data in graphical form as mean ± standard error of the mean. A *P*-value of $\leqslant 0.05$ was considered statistically significant.

RESULTS

LPS administration to pregnant mice causes a reduction of uterine CD4+ Tregs

We first investigated whether the administration of LPS, which causes 100% of PTB (Table 1), alters the proportion of CD4+ and CD8+ Tregs (CD25+Foxp3+ cells) at the maternal–fetal interface. The gating strategy used to determine CD4+ Tregs in the decidual/uterine tissues is shown in Figure 1b and c. Pregnant mice injected with LPS had a reduced proportion and number of uterine CD4+ Tregs when compared to the control mice (Figure 1d). However, LPS injection did not reduce the proportion and number of CD4+ Tregs in the decidual tissues (Supplementary Figure 2). No changes were observed in the uterine CD8+ Tregs (data not shown). These results demonstrate that there is a reduction of uterine CD4+ Tregs prior to endotoxin-induced PTB.

LPS administration to pregnant mice causes an expansion of splenic CD4+ and CD8+ Tregs

The influx of CD4+ Tregs into the uterine tissues is controlled by chemotactic gradients.[48,49] These cells are recruited from secondary lymphatic organs and/or generated by a local microenvironment.[49] We next investigated whether the administration of LPS reduces the proportion and number of CD4+ and CD8+ Tregs in the spleen and uterine-draining lymph nodes, as it did in the uterine tissues. The gating strategy used to determine CD4+ and CD8+ Tregs in the spleen and uterine-draining lymph nodes is shown in Figure 2a. Contrary to our hypothesis, the proportion and number of splenic CD4+ and CD8+ Tregs were higher in the LPS-injected mice than in the control mice (Figure 2b). No differences were found in the number of CD4+ and CD8+ Tregs in the uterine-draining lymph nodes between mice injected with PBS or LPS (Supplementary Figure 3). We investigated whether the splenic Tregs had a functional phenotype. Since functional Tregs produce IL10 in order to control T-cell responses *in vitro* and *in vivo*,[50–52] we determined the expression of IL10 in splenic CD4+ and CD8+ Tregs. High proportions of splenic CD4+ (~80%)

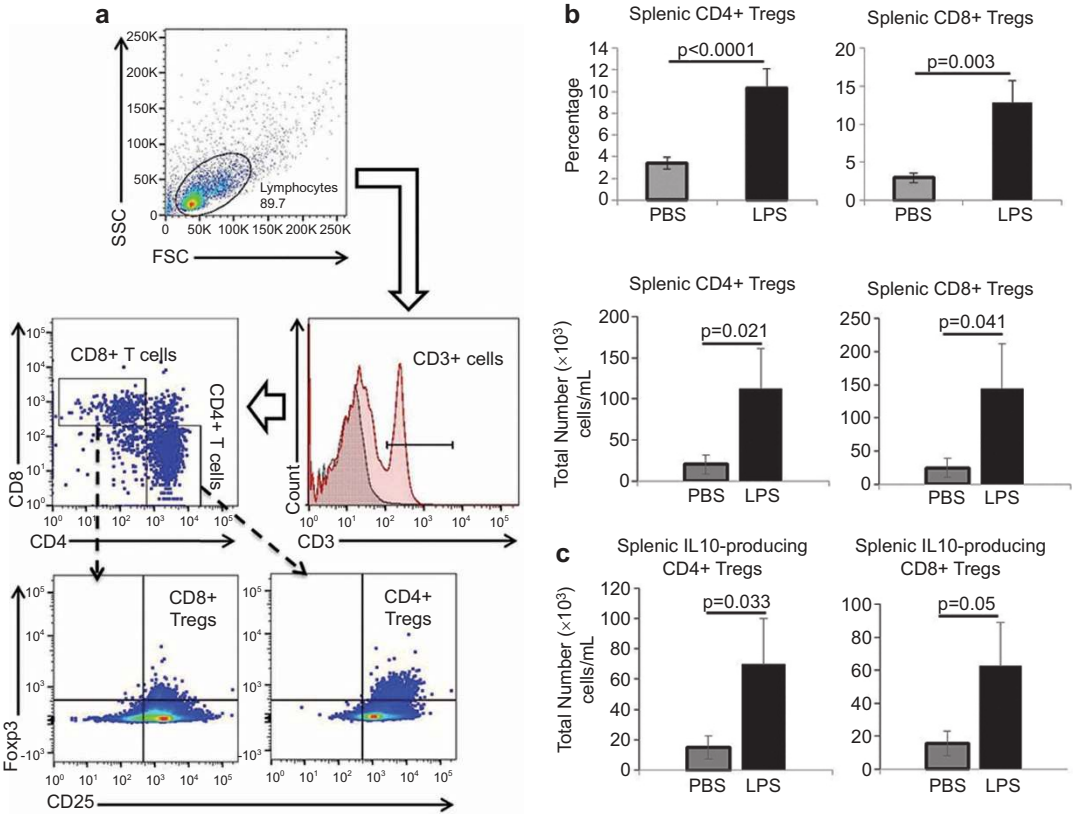

Figure 2 An expansion of splenic CD4+ and CD8+ Tregs prior to endotoxin-induced PTB. (**a**) Gating strategy used to identify CD4+ and CD8+ Tregs in the spleen- and uterine-draining lymph nodes. CD3+ (T) cells were gated within the lymphocyte gate (FSC vs. SSC), CD4+ and CD8+ T cells within the CD3+ gate, and CD4+ or CD8+ Tregs within the CD4+ or CD8+ gate, respectively. The gray histogram represents the autofluorescence control. (**b**) Proportion and number of splenic CD4+ and CD8+ Tregs in pregnant mice injected with PBS or LPS (n = 10–11 each). (**c**) Proportion and number of splenic IL10-producing CD4+ and CD8+ Tregs in pregnant mice injected with PBS or LPS (n = 8–11 each). Data shown are means ± standard error of the mean.

and CD8+ (~70%) Tregs were positive for IL10. Consequently, IL10-producing CD4+ and CD8+ Tregs were more abundant in the spleen of pregnant mice injected with LPS than in the control mice (Figure 2c). These results demonstrate that prior to endotoxin-induced PTB there is an expansion of splenic IL10-producing CD4+ and CD8+ Tregs, instead of a reduction.

LPS administration to pregnant mice does not alter the production of IL10 by the uterine tissues

We next investigated whether the reduction of uterine CD4+ Tregs was associated with reduced concentrations of IL10 in the uterine tissues. This anti-inflammatory cytokine regulates in a paracrine manner the expression of Foxp3,[53,54] an essential transcriptional factor for Treg generation and function.[55] IL10 concentrations in the uterine tissues were not different between mice injected with PBS or LPS (Figure 3a). This result demonstrates that the reduction of uterine CD4+ Tregs prior to endotoxin-induced PTB is not linked to the production of IL10 by uterine tissues.

LPS administration to pregnant mice increases the concentration of CCL17 and CCL22 in the uterine tissues

The infiltration of CD4+ Tregs into the uterine tissues is mediated by chemokines and their receptors.[48,49] CCL17 and CCL22 participate in the recruitment of Tregs into the site of inflammation.[56,57] Therefore, we investigated whether the reduction of CD4+ Tregs caused by endotoxin administration was due to a diminished production of CCL17 and CCL22 by the uterine tissues. Chemokine concentrations were quantified by ELISAs in uterine protein extracts from pregnant mice injected with PBS or LPS. LPS administration to pregnant mice increased the production of CCL17 and CCL22 by the uterine tissues (Figure 3b). This finding demonstrates that the reduction of uterine CD4+ Tregs prior to endotoxin-induced PTB is not linked to diminished production of Treg chemokines by the uterine tissues.

LPS administration to pregnant mice causes a reduction of Th17 cells and an expansion of effector IFNγ+CD8+ T cells in the spleen but not at the maternal–fetal interface

An imbalance between effector and Tregs (i.e., Th1/Th2/Th17/Tregs) may be implicated in the pathogenesis of PTB.[44,45] Therefore, we determined whether LPS administration to pregnant mice, which causes a reduction of uterine CD4+ Tregs, an expansion of splenic CD4+ and CD8+ Tregs, and PTB, alters effector CD4+ or CD8+ T cells at the maternal–fetal interface and in the spleen. Effector CD4+ T cells included Th1 (CD4+IFNγ+ or CD4+Tbet+), Th2 (CD4+IL10), Th9

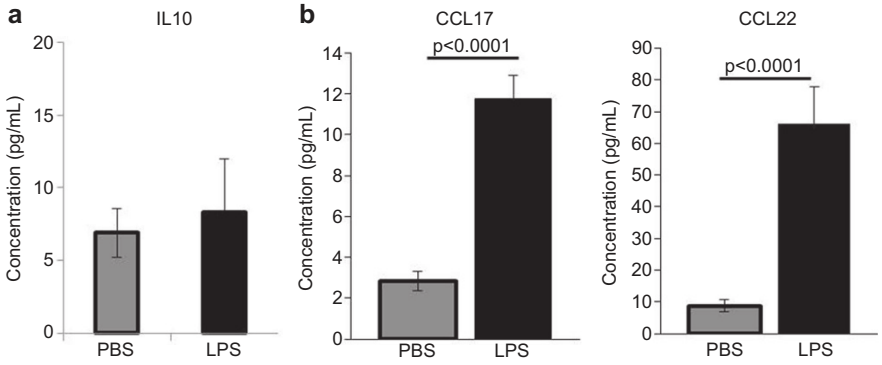

Figure 3 Concentrations of IL10, CCL17, and CCL22 in the uterine protein extracts prior to endotoxin-induced PTB. IL10 (a), CCL17, and CCL22 (b) concentrations in the uterine protein extracts from mice injected with PBS or LPS ($n = 8$–11 each) determined by ELISAs. Data shown are means ± standard error of the mean.

(CD4+IL9+), and Th17 (CD4+IL17A+). Effector CD8+ T cells included positive cells for IFNγ or Tbet. The gating strategy used to determine cytokine expression in CD4+ and CD8+ T cells is shown in Figure 4a. No differences were found in uterine Th cells between pregnant mice injected with PBS or LPS (Supplementary Figure 4a). However, mice injected with LPS had a lower proportion of Th17 cells and a higher proportion and number of IFNγ+CD8+ T cells in the spleen than the control mice (Figure 4b). No differences were observed between splenic Th1, Th2, and Th9 cells between LPS-injected mice and the control mice (Supplementary Figure 4b). Effector Tbet+ CD8+ T cells in the uterine tissues were not different between mice injected with PBS or LPS (Supplementary Figure 4c). These results demonstrate that LPS injection to pregnant mice does not alter the effector CD4+ T cells at the maternal–fetal interface, but it causes an imbalance between Th17 and effector CD8+ T cells in the spleen.

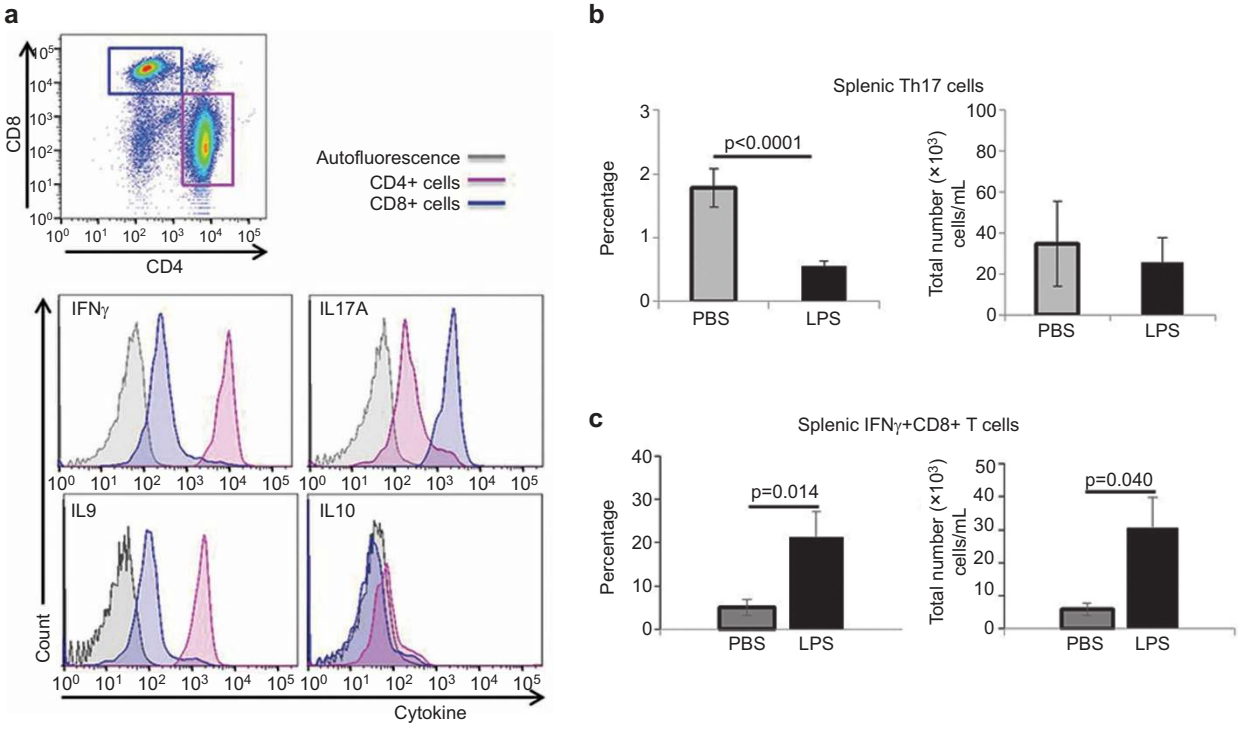

Figure 4 A reduction of Th17 cells and an expansion of effector CD8+ T cells in the spleen prior to endotoxin-induced PTB. (**a**) Gating strategy used to identify effector CD4+ and CD8+ T cells in the spleen. CD4+ and CD8+ T cells were gated within the CD3+ gate. The gray histogram represents the auto-fluorescence control; the blue histogram represents the fluorescence of CD8+ T cells; and the pink histogram represents the fluorescence of CD4+ T cells. (**b**) Proportion and number of splenic Th17 cells in pregnant mice injected with PBS or LPS ($n = 8$–11 each). (**c**) Proportion and number of splenic IFNγ+CD8+ T cells in pregnant mice injected with PBS or LPS ($n = 8$–11 each). Data shown are means ± standard error of the mean.

LPS administration to pregnant mice causes an increase of decidual and uterine neutrophils

To further characterize the immune microenvironment at the maternal–fetal interface prior to endotoxin-induced PTB, we analyzed neutrophil (CD11b+Ly6G+F4/80–) and macrophage (CD11b+F4/80+) populations in the decidual and uterine tissues of pregnant mice injected with PBS or LPS. The gating strategy used to determine neutrophil and macrophage populations in the decidual and uterine tissues is shown in Figure 5a. The proportion and number of decidual neutrophils were significantly greater in pregnant mice injected with LPS than in the control mice (Figure 5b). In addition, mice injected with LPS tended to have a higher proportion and number of uterine neutrophils than the control mice (Figure 5c). Consequently, LPS injections caused a reduction of the proportion of macrophages in the uterus, but a nonsignificant reduction in the number of these cells (Figure 5d). These results demonstrate that prior to endotoxin-induced PTB there is an imbalance between neutrophils and macrophages at the maternal–fetal interface.

LPS administration to pregnant mice increases the concentration of CCL2 and CCL3 in the uterine tissues

Recruitment of macrophages into the uterine tissues is mediated by CCL2 and CCL3.[58] We investigated whether the reduction of macrophages in the uterine tissues was associated with low concentrations of CCL2 and CCL3. LPS administration to pregnant mice increased the production of CCL2 and CCL3 by the uterine tissues (Figure 6). This finding demonstrates that the reduction of uterine macrophages prior to endotoxin-induced PTB is not linked to diminished production of macrophage chemokines by the uterine tissues.

LPS administration to pregnant mice causes a reduction of uterine MHC II+ neutrophils

Finally, we analyzed whether the cytokine production and MHC class II expression by macrophages and neutrophils were altered prior to endotoxin-induced PTB. The gating strategy used to determine the expression of MHC II, IL4, and IFNγ in the uterine neutrophils and macrophages is shown in Figure 7a. The administration of LPS to pregnant mice reduced the

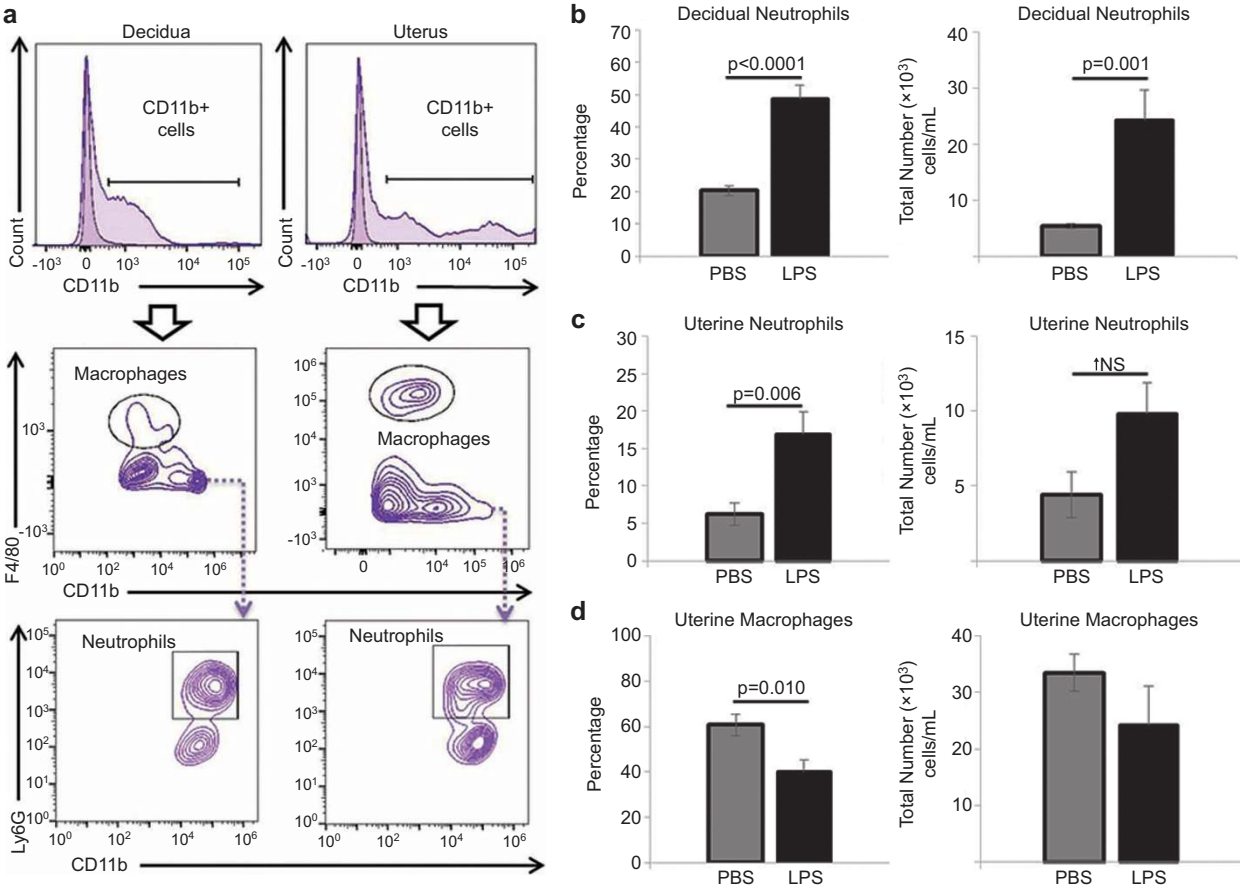

Figure 5 An increase of uterine and decidual neutrophils prior to endotoxin-induced PTB. (**a**) Gating strategy used to identify neutrophils and macrophages in the decidual and uterine tissues. CD11b+ cells were gated within the leukocyte gate (FSC vs. SSC), macrophages (F4/80+ cells) within the CD11b+ gate, and neutrophils within the CD11b+F4/80– gate. The gray histogram represents the auto-fluorescence control. (**b**) Proportion and number of decidual neutrophils in pregnant mice injected with PBS or LPS ($n = 11$ each). (**c**) Proportion and number of uterine neutrophils in pregnant mice injected with PBS or LPS ($n = 11$ each). (**d**) Proportion and number of uterine macrophages in pregnant mice injected with PBS or LPS ($n = 11$ each). Data shown are means ± standard error of the mean. NS, non-significant.

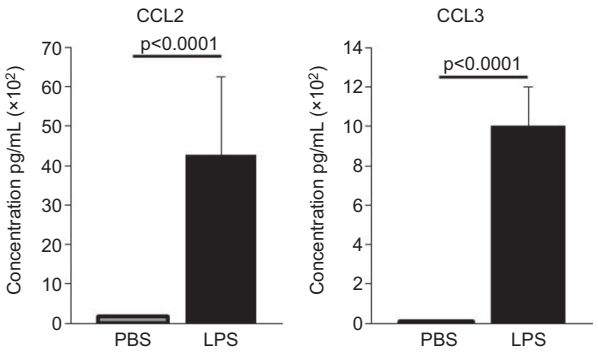

Figure 6 Concentrations of CCL2 and CCL3 in the uterine protein extracts prior to endotoxin-induced PTB. CCL2 and CCL3 concentrations in uterine protein extracts from mice injected with PBS or LPS (n = 10–11 each) determined by ELISAs. Data shown are means ± standard error of the mean.

proportion of uterine MHC II+ neutrophils (Figure 7b); however, it did not cause a reduction of MHC II+ macrophages (data not shown). No changes were observed in IL4+ and IFNγ+ neutrophils or macrophages between the groups of pregnant mice injected with PBS or LPS (data not shown). These results demonstrate that there is a reduction in MHC class II+ neutrophils in the uterine tissues prior to endotoxin-induced PTB.

DISCUSSION

Using an established murine model of endotoxin-induced PTB, our results demonstrate that prior to delivery there is a reduction of CD4+ Tregs in the uterine tissues. This reduction is neither linked to a diminished number of Tregs in the spleen, nor to an impaired production of IL10, CCL17 or CCL22 by the uterine tissues. Endotoxin administration to pregnant mice did not alter effector CD4+ T cells at the maternal–fetal interface. However, it causes an imbalance between Tregs (CD4+ and CD8+), effector CD8+ T cells, and Th17 cells in the spleen. In addition, endotoxin administration to pregnant mice leads to an excessive production of CCL2, CCL3, CCL17, and CCL22 by the uterine tissues, as well as to an abundance of neutrophils. This imbalance in the uterine microenvironment is accompanied by scarce APC-like cells such as macrophages and MHC II+ neutrophils. Collectively, these results demonstrate that endotoxin administration to pregnant mice causes an imbalance between innate and adaptive immune cells at the maternal–fetal interface.

Alterations in regulatory and effector T cells prior to endotoxin-induced PTB

Regulatory T cells. Pregnancy drives an expansion of CD4+ Tregs in the secondary lymphatic organs and peripheral blood, which promotes maternal immune tolerance against the paternally derived antigens of the semi-allograft fetus throughout gestation.[38,41,43,59,60] These cells are recruited into the maternal–fetal interface (decidual and uterine tissues)[61] by chemotactic gradients,[48,49] where they seem to regulate the local anti-inflammatory microenvironment that sustains pregnancy to full term.[8] A reduction in the proportion or function of CD4+ Tregs in the maternal circulation is associated with PTB.[42,43] In the study herein, endotoxin administration to pregnant mice causes a reduction in the number of CD4+ Tregs in the uterine tissues. These data suggest that CD4+ Tregs regulate the local microenvironment at the maternal–fetal interface through their anti-inflammatory functions; and when a microbial infection is present, this regulation is disrupted and leads to premature expulsion of the neonate.

Contrary to our initial hypothesis, administration of LPS to pregnant mice caused an expansion of splenic CD4+ Tregs. In acute inflammation models (i.e., viral infection), an expansion of splenic pathogen-specific CD4+ Tregs has been observed.[62] Thus, it is likely that endotoxin administration causes an expansion of CD4+ Tregs in pregnant mice since pregnancy generates paternal–fetal-specific T cells.[60] Antigen-specific CD4+ Tregs play a major role in mediating the resolution of acute inflammation by releasing IL10, which regulates neutrophil recruitment and T-cell responses.[62–64] Our results suggest that rather than to cause a reduction of splenic Tregs, endotoxin administration causes an expansion of splenic IL10-producing CD4+ Tregs in order to control the strong systemic pro-inflammatory response that leads to PTB.

Lymphatic CD8+ Tregs also exhibit suppressive functions *in vivo*,[65] suggesting that these Tregs may participate in maternal immune tolerance during pregnancy.[46] LPS administration to pregnant mice resulted in the expansion of splenic IL10-producing CD8+ Tregs, which demonstrates for the first time that these Tregs are also implicated in the mechanisms that lead to infection-induced PTB. Further research is required to investigate the functional properties between splenic CD4+ and CD8+ Tregs during late gestation and their respective roles in endotoxin-induced PTB.

As the reduction of uterine CD4+ Tregs prior to endotoxin-induced PTB was not due to a diminished proliferation of these cells in the secondary lymphatic organs, we next investigated whether endotoxin administration was disrupting Treg-differentiating factors, such as IL10,[54] in the uterine microenvironment. IL10 concentration in the uterine protein extracts was not diminished prior to endotoxin-induced PTB. These results demonstrate that the reduced number of uterine CD4+ Tregs is not linked to an impaired production of IL10 by the uterine tissues. Further research is needed in order to investigate whether uterine CD4+ Tregs produce or require IL10 for differentiation and/or proliferation, and whether these cells exhibit a suppressive function *in vitro*.

Up to this point, our results demonstrated that the reduction of uterine CD4+ Tregs prior to endotoxin-induced PTB is neither linked to a lack of proliferation of these cells in the secondary lymphatic organs nor to a diminished production of IL10 by uterine tissues. Another possibility is that endotoxin administration would inhibit the secretion of chemokines that recruit Tregs into the uterine tissues. Chemokines are small cytokines that attract immune cells to the site of inflammation.

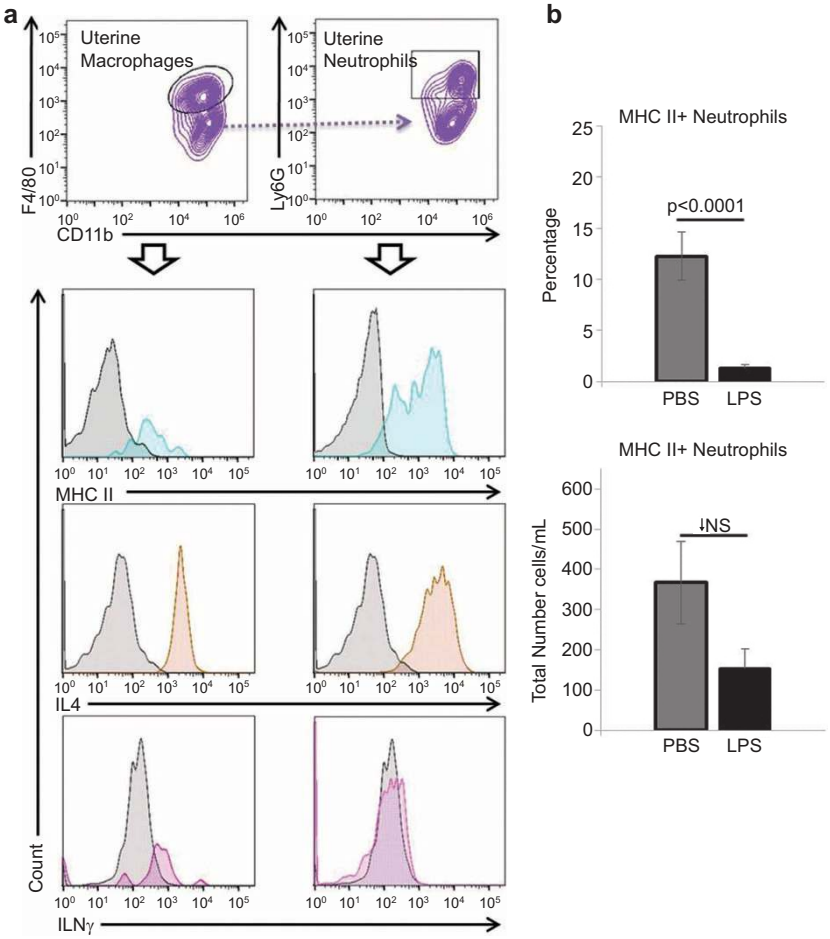

Figure 7 A reduction of uterine MHC II+ neutrophils prior to endotoxin-induced PTB. (**a**) Gating strategy used to identify neutrophils and macrophages that express MHC II, IL4, and IFNγ in the decidual and uterine tissues. Macrophages (F4/80+ cells) were gated within the CD11b+ gate and neutrophils were gated within the CD11b+F4/80– gate. The gray histogram represents the auto-fluorescence control. Expression of MHC II, IL4, and IFNγ is shown in the turquoise, orange, and pink histograms. (**b**) Proportion and total number of uterine MHC II+ neutrophils in pregnant mice injected with PBS or LPS ($n = 11$ each). Data shown are means ± standard error of the mean. NS, non-significant.

The cysteine–cysteine chemokines CCL17 and CCL22 participate in the recruitment and homing of CD4+ Tregs to the site of inflammation.[56,57] Endotoxin administration to pregnant mice did not reduce the production of CCL17 or CCL22; instead, it increased the concentration of these chemokines in the uterine tissues. These data demonstrate that the reduced number of uterine CD4+Tregs is not associated with low concentrations of CCL17 and CCL22 in the uterine tissues. Further research is required to investigate whether LPS-induced CCL17 and CCL2 are indeed functional. Another possibility is that endotoxin administration leads to apoptosis of CD4+ Tregs at the maternal–fetal interface. This hypothesis was not tested in our study, but it is supported by two facts: endotoxin induces apoptosis of CD4+ T cells[66] and pre-eclampsia, a pregnancy complication during late pregnancy, is characterized by a deficit of apoptotic CD4+ Tregs.[67]

Effector T cells. Current hypotheses suggest that an imbalance between CD4+ Tregs and Th17 cells, T cells with opposing actions, underlies the pathophysiology of immune diseases,[68] inflammatory processes,[69] and pregnancy pathologies such as PTB.[70] Indeed, there is an increase of Th17 cells in the chorioamniotic membranes from women who underwent preterm labor with acute chorioamnionitis.[44] Our results did not support this hypothesis at the maternal–fetal interface since no changes were observed in uterine Th1, Th17, or Th9 cells prior to LPS-induced PTB. This disparity can be attributed to several factors, including the species under study (mouse vs. human) and the mechanisms of action of the causal agent (a microbial product (LPS) vs. live bacteria).

An imbalance between splenic Tregs and Th17 cells was observed 72 h prior to IL1β-induced PTB in rhesus monkeys.[70] Our results support this finding since we also observed that the administration of an inflammatory stimulus, such as LPS, leads to an expansion of Tregs and a reduction of Th17 cells in the spleen. However, the mechanisms whereby the Treg/Th17 imbalance in the spleen is implicated in the pathophysiology of PTB are yet to be determined.

Endotoxin administration to pregnant mice caused an expansion of IFNγ+CD8+ T cells in the spleen. CD8+ T cells,

also called cytotoxic T cells, exhibit effector functions by expressing IL2, IFNγ, TNFα, perforin, granzymes A/B/C/K, and many other effector factors.[71] An expansion of effector splenic CD8+ T cells occurs in acute inflammatory processes, e.g., influenza infection.[72] When CD8+ T cells produce IFNγ, this cytokine facilitates the expansion of the cell itself and other neighboring CD8+ T cells.[73] We, therefore, suggest that endotoxin administration to pregnant mice causes an expansion of effector CD8+ T cells, which participate in the maternal pro-inflammatory response that leads to PTB. The question of whether IFNγ also participates in the expansion of splenic CD8+ Tregs promoted by endotoxin administration needs further investigation.

An increase of decidual and uterine neutrophils occurs prior to endotoxin-induced PTB

A large body of evidence demonstrates that decidual and uterine neutrophils increase during infection-induced PTB in humans and mice.[22,25,28,29] Decidual/uterine neutrophils play a central role during the process of labor by releasing inflammatory cytokines and mediators, and secreting matrix metalloproteinases.[26,27,74,75] A recent study has demonstrated that neutrophils are not required for the onset of endotoxin-induced PTB as the depletion of these cells does not restore parturition on time.[23] However, it was also demonstrated that these cells contribute to the inflammatory response at the maternal–fetal interface that leads to endotoxin-induced PTB.[23] As expected, the administration of endotoxin to pregnant mice caused a large influx of neutrophils into the maternal–fetal interface, which validates our PTB animal model.

A reduction of MHC II+ neutrophils occurs in the uterine tissues prior to LPS-induced PTB

In addition to serving as sources of pro-inflammatory cytokines at the sites of infection and inflammation, neutrophils and macrophages have anti-inflammatory functions.[30–32] These cells can also act as APCs since they express MHC class II and co-stimulatory molecules to mediate effector T-cell responses.[33,34] Here, we show that although most of the uterine macrophages express MHC class II molecules, these cells were not altered, and a small proportion of uterine neutrophils expressing MHC class II molecules was reduced prior to LPS-induced PTB. These findings lead us to suggest that antigen presentation and CD4+ Treg function are processes implicated in microbial-induced preterm labor. To our knowledge, this is the first time that MHC class II+ neutrophils have been observed in the uterine tissues during late pregnancy, and their reduction is associated with endotoxin-induced PTB. Further research is needed to investigate these neutrophils as paternal antigen-specific cells and to determine their roles in maternal immune tolerance during pregnancy.

In summary, our findings demonstrate that prior to endotoxin-induced PTB the uterine microenvironment comprises scarce CD4+ Tregs, abundant neutrophils, elevated concentrations of CCL2, CCL3, CCL17, and CCL22, and reduced proportions of APC-like cells such as macrophages and MHC II+ neutrophils. These results suggest that a microbial product is able to disrupt the antigen presentation process at the maternal–fetal interface, which provides insight into the immunological mechanisms that lead to microbial-induced preterm labor.

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