Unique Roles of Infiltrating Myeloid Cells in the Murine Uterus during Early to Midpregnancy

Hui Zhao, Flora Kalish, Stephanie Schulz, Yang Yang, Ronald J. Wong and David K. Stevenson

J Immunol published online 16 March 2015
http://www.jimmunol.org/content/early/2015/03/14/jimmunol.1401930

Supplementary Material http://www.jimmunol.org/content/suppl/2015/03/14/jimmunol.1401930.DCSupplemental
Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription
Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Unique Roles of Infiltrating Myeloid Cells in the Murine Uterus during Early to Midpregnancy

Hui Zhao,* Flora Kalish,* Stephanie Schulz,* Yang Yang,† Ronald J. Wong,* and David K. Stevenson*

Leukocyte infiltration into the uterus is a characteristic feature in early to midpregnancy, but the composition and function of these leukocytes are not well understood. Using a pregnant murine model, we showed that myeloid cells and uterine NK (uNK) cells were the predominant populations in uteri during early to midgestation, whereas T and B cells were constrained. Uterine myeloid populations included cells that infiltrated from the circulation (myeloid-derived suppressor cells [MDSCs], monocyte-derived macrophages [M<Mps], and dendritic cells [DCs]) or proliferated from resident precursors (resident M<Mps [Re-M<Mps] and DCs). CD11b<sup>hi</sup> Ly6-C<sup>hi</sup> cells, representing neutrophils in both blood and uterine MDSCs, significantly increased from embryonic days 8.5 to 9.5.

To understand their putative functions, we used anti–Gr-1 Ab to deplete circulating neutrophils and uterine MDSCs. In the absence of MDSC suppression, uterine DCs, T cells, and regulatory T cells expanded. Conversely, uterine MDSCs responded to LPS-induced inflammation and transformed into CD14<sup>+</sup>-activated neutrophils, resulting in an upregulation of tolerogenic DCs. A high dose of LPS (2.5 μg/mouse) significantly increased the influx of neutrophils and production of proinflammatory cytokines, such as IL-1β and TNF-α, resulting in the reduction of Re-M<Mps and uNK cells, and led to placental hemorrhages and fetal deaths.

In summary, uterine MDSCs are important in early to midpregnancy by responding to the maternal immunologic milieu and shaping uterine leukocyte populations. This work was supported by the March of Dimes Prematurity Research Center at Stanford and the Stanford Child Health Research Institute (to H.Z., F.K., R.J.W., and D.K.S.), the Mary L. Johnson Research Fund (to H.Z., F.K., R.J.W., and D.K.S.), and the Christopher Hess Research Fund (to H.Z., F.K., R.J.W., and D.K.S.).

Received for publication July 29, 2014. Accepted for publication February 2, 2015.

*Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305; and †Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305

The PCR data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/query/acc.cgi?acc=GSE63246) under accession number GSE63246.

Address correspondence and reprint requests to Dr. Hui Zhao, Department of Pediatrics, Division of Neonatal and Developmental Medicine, Stanford University School of Medicine, 300 Pasteur Drive, Grant Building Room S230, Stanford, CA 94305-5208. E-mail address: huizhao2@stanford.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: Bmp-2, bone morphogenetic protein 2; DBA, Dolichos biflorus agglutinin; DC, dendritic cell; dM<Mps, decidual macrophages; E, embryonic day; M2, alternatively activated M<Mps; MDSC, myeloid-derived suppressor cell; Mo-M<Mps, monocyte-derived M<Mps; Re-DC, resident DC; Re-M<Mps, resident ψ; Treg, regulatory T cell (CD4<sup>+</sup>Foxp3<sup>+</sup>); uNK, uterine NK.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00

The other less abundant cells are dendritic cells (DCs) and T cells, which are hypothesized to be involved in immune tolerance (1). In the second trimester, other leukocyte subsets emerge, such as CD4<sup>+</sup> Th cells, regulatory T cells (Tregs), alternatively activated M<Mps (M2), and proangiogenic neutrophils (9–11). In late gestation, especially during the onset of labor, macrophages (M<Mps) and eosinophils, but not neutrophils, have been shown to infiltrate into human and murine deciduae, suggesting their critical roles in labor induction and postpartum uterine remodeling (12–15). Dysfunction and improper deposition of uterine leukocytes have been implicated in pregnancy disorders such as spontaneous miscarriages, pre-eclampsia, and infection-related preterm births (16, 17).

During pregnancy, the maternal immune system undergoes major adaptations to not only prevent its attack on the semi-allogeneic fetus (18), but also to preserve its ability to mount a defense in response to infection. Evidence from murine and human pregnancy studies points to a strong association between maternal Th2-type immunity and successful pregnancies, whereas Th1-type immune reactivity may be associated with pregnancy loss (18, 19). In addition, based on studies of women with autoimmune diseases, Th1 immunity appears to be suppressed during pregnancy. For example, rheumatoid arthritis is a chronic systemic inflammatory disorder that is mediated by CD4<sup>+</sup> T cells, and its symptoms improve during pregnancy and then deteriorate postpartum (20). In fact, the numbers of maternal suppressive CD4<sup>+</sup> Tregs are increased, whereas the proliferation of effector T cells is constrained during pregnancy. The recruitment of T and B cells to the fetomaternal interface is restricted, and DCs present in the decidua are in a tolerogenic state, preventing them from priming a fetal Ag-specific T cell response (1, 18, 21).

Although accumulating evidence has shown that uterine M<Mps and DCs are important in fetal and placental development, little is known about the role of neutrophils, another major component of
Myeloid cells. It may be due to the fact that neutrophils are rarely present in healthy peripheral tissues, but found only in sites of infection where they execute strong antibacterial actions. Recently, a new type of immature neutrophils, called myeloid-derived suppressor cells (MDSCs), was identified from tumor studies (22). These cells infiltrate into tumors or inflammatory sites to suppress local immune responses by inhibiting T cells, DCs, and NK cells (22). MDSCs can also accelerate angiogenesis, tumor progression, and metastasis. Interestingly, pregnancy is also associated with local placental immune suppression, with the development of the fetus and placenta sharing similar mechanisms that are exploited by tumors, such as invasiveness, vessel angiogenesis/vasculogenesis, and immune tolerance. Some tumors, such as breast cancer, cervical cancer, lymphomas, malignant melanoma, and leukemias, are indeed more aggressive during pregnancy and have greater metastatic activity, suggesting the presence of maternal MDSCs. Mauti et al. (23) studied tumor metastasis during pregnancy and found an increase in the MDSC population in the maternal mouse spleen. Fainaru et al. (24) showed that mouse placental MDSCs had striking similarities to MDSCs found in malignant tumors and could promote angiogenesis. In human pregnancies, CD33+ myeloid cells (MDSCs) isolated from maternal PBMCs are increased and have a stronger inhibitory capability to suppress T cell proliferation than those from nonpregnant women (25, 26). Recently, Amsalem et al. (10) have identified a novel human decidua-specific neutrophil population with proangiogenic properties. They expressed high levels of the angiogenesis-related proteins, such as vascular endothelial growth factor, arginine-1, and CCL2, and displayed similar properties to the MDSCs found in tumor cells.

MqCs and DCs are found in all tissues and are critical effectors and regulators of immune responses. The dogma is that they all originate from bone marrow–derived circulating myeloid cells (27, 28). However, after host irradiation, bone marrow transplantation does not lead to a full replacement of tissue MqCs. Therefore, two origins of tissue MqCs and DCs have been proposed: either arising from the infiltration of circulating cells derived from the bone marrow (“infiltration”) or from the proliferation of local progenitor cells (“residential”) (29, 30). Residential myeloid cells are actually derived from the yolk sac and colonize all embryonic tissues early in development. Tissue MqCs from the yolk sac are CD11b<sup>+</sup>F<sub>48/80</sub><sup>+</sup>Cx3CR1<sup>+</sup>, whereas those from the bone marrow are CD11b<sup>+</sup>F<sub>48/80</sub><sup>-</sup> (30–34).

Due to the nature of the adaptive immunity being suppressed in the fetomaternal interface (16), we hypothesized that, in early to midpregnancy, it is innate immunity that executes critical roles in immunotolerance, as well as immune reactivity against infection, with myeloid cells being the key players. In this study, we used multiparameter flow cytometry to identify and characterize the subpopulations of uterine myeloid cells based on their phenotypic markers during early to midpregnancy (embryonic days [E] 8.5–10.5), when spiral artery remodeling occurs and the vasculature in the labyrinth begins to develop. We then investigated the putative functions of CD11b<sup>+</sup>Ly6-G<sup>+</sup> cells, representing neutrophils in blood and MDSCs in the pregnant uterus, by administering either Gr-1 Ab (targeting both Ly6-G and Ly6-C) (35) to deplete neutrophils and MDSCs, or LPS to induce neutrophil influx due to uterine inflammation. The roles of infiltrating myeloid cells were also investigated and explored as mediators between the mother and the fetoplacental unit.

**Materials and Methods**

**Animals and tissue collection**

FVBn and BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Syngeneic matings of FVBn mice were set up at 6–10 wk of age. All animals were maintained and bred under strict adherence to Stanford University institutional guidelines. Gestational ages were determined by visualizing the presence of a vaginal plug (E0.5 = vaginal plug day). Uteri were collected at various gestational ages. Allogeneic matings of FVBn (females) crossed with BALB/c (males) were set up for studies to identify the origin (maternal or fetal) of uterine leukocytes.

**Preparation of single-cell suspensions from uteri**

Pregnant mice were euthanized at various gestational ages by CO<sub>2</sub> inhalation. Blood (0.8–1.0 ml) was then immediately collected by intracardiac puncture and placed into Microtainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). RBCs were removed from whole blood using a RBC Lysis buffer (eBioscience, San Diego, CA). For collection of uteri, after intracardiac puncture to remove up to 1 ml blood, uterine horns were harvested in toto, blood vessels and connection tissues were removed, and uteri and placentas were carefully dissected away from the fetuses. Uterine tissues were further separated from the labyrinth (fetal origin) and any remaining maternal blood was removed by blotting with paper towels. Blanched uteri were then rinsed in MACS buffer (Miltenyi Biotec, San Diego, CA). Uteri were then pooled from the same dam, finely minced into ~1-mm<sup>3</sup> pieces in a small volume of MACS buffer, and pushed through a 60-μm mesh screen (Sigma Aldrich, St. Louis, MO) using a syringe plunger (6). The resulting cell suspension was then passed through a 70-μm filter.

**Multiparameter flow-cytometric analyses**

Freshly isolated cells were washed in PBS, incubated with a fixable viability dye (eBioscience), and FcRs were blocked with purified anti-CD16/CD32 (clone 93). Fluorescent-conjugated mAbs, for example, CD54 (30–F11), CD45.1 (A20), CD45.2 (104), CD3e (500A2), CD4 (GK1.5), CD8a (53–6.7), CD25 (PC61.5), Fopx3 (FJK-16s), CD19 (6D5), CD122 (TM-b1), NKp46 (29A1.4), CD11b (M1/70), CD11c (N418), Ly6-G (RB-6-8C5), Ly6-C (HK1.4), MHCII (MS/114.15.2), CD80 (16-10A1), CD86 (GL1), CD205 (205), CD86 (Yts158), CD14 (46-7.2), CD68 (FA-11), F<sub>48/80</sub> (BM8), CD11c (C068/2C), and others were purchased from eBioscience, Biolegend (San Diego, CA), and BD Biosciences (San Jose, CA). Anti-Gr-1 Ab (RB6-8C5; Bio X Cell, West Lebanon, NH) was added to FACS buffer and incubated with cells on ice for 30 min. Intracellular staining (for unK Dolichos biflorus agglutinin [DBA], CD68, Foxp3) was performed after fixation and permeabilization protocols according to manufacturers’ instructions (eBioscience). Biotinylated DBA (Vector Laboratories, Burlingame, CA) and Qdot605 Streptavidin (Conjugate, Life Technologies, Carlsbad, CA) were used to stain UCNK cells. Compensation was performed using OneComp E beads (eBioscience). Flow cytometry was performed using a BD Biosciences LSRII at the shared FACS Facility at Stanford. Percentages of positive cells were calculated against the background set on a fluorescence-minus-one negative control. Analyses of all flow-cytometric data were performed using FlowJo (Tree Star, Ashland, OR) software. The total viable CD45<sup>+</sup> cells per dam were first determined using flow cytometry and then divided by number of implantation sites, to calculate the number of leukocytes per implantation site.

**PCR arrays**

After sacrifice, uterine tissues from pregnant mice were immediately dissected and stored in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Frederick, MD). cDNA was synthesized using a first-strand Kit (Qiagen). PCR array kits (Mammalian Inflammatory Cytokines & Receptors catalog no. PAMM-011A) and Cyto-kines & Chemokines (catalog no. PAMM-150Z) were used. RT<sup>2</sup> Profiler Array Real-time PCR was performed using RT<sup>2</sup> Real-Time SYBR Green/ROX PCR Master Mix (Qiagen) on a Stratagene MX3005P QPCR system (Agilent Technologies, Palo Alto, CA). Data analyses were performed using the ΔΔCt method and fold-changes of gene expression in uteri were calculated at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63246.

**Gr-1 Ab and LPS administration**

Anti-mouse Gr-1 Ab (RB6-8C5; Bio X Cell, West Lebanon, NH) was administered by i.p. injection to pregnant mice (100 μg/mouse) at various gestational ages. After 24 h, depletion efficiency was assessed through flow cytometry. LPS S-form from Salmonella abortus equi (TLRgrade) was purchased from Enzo Life Sciences (Farmingdale, NY). LPS 0.25 μg or 2.5 μg per mouse was administered i.p. to pregnant mice. Leukocyte profiles in the blood and uteri were analyzed using flow cytometry.
Statistical analyses

For comparisons of experimental groups, one-way ANOVAs were first performed for each set of experiments to determine significant differences when \( p < 0.05 \). To determine differences between individual experimental and control groups, we used Dunnett’s test, which adjusts for multiple comparisons using the same control group.

Results

Cytokines and receptors profiles in uteri are significantly different at E8.5 versus E10.5

There are dynamic alterations of the leukocyte population in the uterus in early and midgestation. To investigate the production cytokines/chemokines in this tissue, we used a mouse Inflammatory and Cytokine/Receptor RT² Profiler Array to compare the expression of 84 cytokine/chemokine genes and their receptors between E8.5 and E10.5. Of the 84 genes screened, 12 were significantly upregulated and 3 genes were downregulated at E10.5 compared with E8.5 (Table I). Most of the upregulated genes (Ccl6, Cc12, Cxcl4, Cx3cl1, Ccr1, Ccr2, and Il-15) are produced by myeloid cells such as neutrophils, Møs, monocytes, and DCs. The function of these upregulated genes is also primarily related to myeloid cell recruitment and stimulation (36–38), suggesting that uterine myeloid cells play a critical role in placental development by dynamically secreting cytokines and chemokines, and actively recruiting new myeloid cells into the placenta. Interestingly, the three downregulated genes (Ccl17, Cc19, and Cx3cl1l) all target and affect T and B cells (36), implying that T and B cell infiltration and function may be suppressed as gestation progresses (39).

Myeloid and uNK cells are the predominant leukocyte populations present in uteri in early to midpregnancy

To characterize the major uterine immune cell populations, we isolated uteri from syngeneic-bred pregnant FVBn mice at E8.5, E9.5, and E10.5. Single-cell suspensions were prepared and each isolated uteri from syngeneic-bred pregnant FVBn mice at E8.5, E9.5, and E10.5. In pregnant uteri, we found the presence of very small percentages of B (CD45⁺CD19⁺) and T cells (CD45⁺CD3e⁺; Fig. 1A). B cells accounted for \(<2\%\) of the total leukocytes, whereas T cells constituted 4–5% (Fig. 1A, 1B). NK cells in pregnant uteri were mostly DBA⁺ uNK cells (Fig. 1B); whereas, the cells with the similar markers as blood NK (CD11b⁺CD122⁺) were barely detectable (<1%; Fig. 2A). CD25⁺ Foxp3⁺ Tregs accounted for 6–7% of the total CD4⁺ population (data not shown), which was equivalent to \(<0.3\%\) of the total leukocytes.

Table 1. Comparison of uterine cytokines and receptor profiles between E8.5 and E10.5

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>E10.5/E8.5</th>
<th>( p )</th>
<th>Cell Source</th>
<th>Target and Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl6</td>
<td>6.8</td>
<td>(&lt;0.05)</td>
<td>Neutrophils, Møs</td>
<td>Mixed leukocyte recruitment</td>
</tr>
<tr>
<td>Cc18</td>
<td>6.4</td>
<td>(&lt;0.05)</td>
<td>Stromal cells</td>
<td>Mixed leukocyte recruitment</td>
</tr>
<tr>
<td>Cc12</td>
<td>7.8</td>
<td>(&lt;0.05)</td>
<td>Møs</td>
<td>Monocytes and lymphocytes</td>
</tr>
<tr>
<td>Cc17</td>
<td>(-4.4)</td>
<td>(&lt;0.05)</td>
<td>Monocytes</td>
<td>T cells</td>
</tr>
<tr>
<td>Cc19</td>
<td>(-5.6)</td>
<td>(&lt;0.05)</td>
<td>Møs</td>
<td>T and B cell trafficking, DCs</td>
</tr>
<tr>
<td>Cxcl4</td>
<td>6.9</td>
<td>(&lt;0.01)</td>
<td>Platelets</td>
<td>Neutrophils, monocytes, fibroblasts</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>10.9</td>
<td>(&lt;0.05)</td>
<td>Møs</td>
<td>T cells</td>
</tr>
<tr>
<td>Cxcl12</td>
<td>27.4</td>
<td>(&lt;0.05)</td>
<td>DCs and endothelial cells</td>
<td>MDSCs, Møs, T cells</td>
</tr>
<tr>
<td>Cx3cl1l</td>
<td>(-3.2)</td>
<td>(&lt;0.05)</td>
<td>Activated endothelial cells</td>
<td>T cells and monocytes</td>
</tr>
<tr>
<td>Ccr1</td>
<td>5.4</td>
<td>(&lt;0.05)</td>
<td>Myeloid cells</td>
<td>Myeloid cell infiltration</td>
</tr>
<tr>
<td>Ccr2</td>
<td>5.6</td>
<td>(&lt;0.05)</td>
<td>Monocytes</td>
<td>Monocyte recruitment</td>
</tr>
<tr>
<td>Il-1β</td>
<td>6.4</td>
<td>(&lt;0.01)</td>
<td>Epithelial cells</td>
<td>Proinflammatory</td>
</tr>
<tr>
<td>Il-16</td>
<td>4.3</td>
<td>(&lt;0.05)</td>
<td>Møs</td>
<td>NK cell proliferation</td>
</tr>
<tr>
<td>Il-17β</td>
<td>21.9</td>
<td>(&lt;0.01)</td>
<td>T cells</td>
<td>Monocyte stimulation of TNF and IL-1β</td>
</tr>
<tr>
<td>Other</td>
<td>8.0</td>
<td>(&lt;0.05)</td>
<td>Hepatocytes</td>
<td>Møs stimulation of phagocytosis</td>
</tr>
</tbody>
</table>

Bold text represents fold increases. Italicized text represents fold decreases.

CRP, C-reactive protein.
For comparison, in the maternal blood, B cells were ∼10–12%, T cells were 55–68%, and NK cells (CD11bmidCD122+) were ∼6% of the total leukocytes (Fig. 1B). Tregs (CD25+Foxp3+) were only 0.5% of the total CD4+ T cells (data not shown). In nonpregnant uteri, B cells accounted for only ∼2%, T cells for ∼17%, and NK cells for ∼17% of the total leukocytes, but no uNK cells were detected (Fig. 1B, 2C).

Myeloid cells of two different origins identified in uteri

When cells were stained with anti-CD45 and anti-CD11b Abs, two distinct populations were observed in pregnant uteri: CD45hiCD11bhi and CD45midCD11bmid (Fig. 2A). Because other studies have identified two origins of tissue M\(_{\text{w}}\)s (30, 31, 34, 40), we speculated that CD45hiCD11bhi cells represented the infiltrating population, whereas the CD45midCD11bmid cells were residential myeloid cells. For reference, we also stained the maternal blood (Fig. 2B) to compare the phenotypes of infiltrating myeloid cells. Each subtype of infiltrating (uterine) myeloid cells displayed the same cell-surface markers as their circulating counterparts (Fig. 2B): MDSCs (Ly6-GhiCD14+), monocyte-derived M\(_{\text{w}}\)s (Mo-M\(_{\text{w}}\); Ly6-ChiF4/80lo), and DCs (CD11C+Ly6-C-); whereas residential cells, which included F4/80hiCD11C+ M\(_{\text{w}}\)s and F4/80+CD11C+ DCs (Fig. 2A), showed no similarities to any circulating populations.

In nonpregnant uteri, we also identified two groups of myeloid cells: infiltrating CD45hiCD11bhi and residential CD45midCD11bmid. In addition, we observed infiltrating (CD11bmidCD122+) NK cells (Fig. 2C) (41, 42). Similar to cells in pregnant uteri, infiltrating cells in nonpregnant uteri were mainly neutrophils (and MDSCs), Mo-M\(_{\text{w}}\)s, and DCs. However, two major differences were found: (1) the majority of residential myeloid cells in nonpregnant uteri were F4/80loCD11C+ resident DCs (Re-DCs), and not F4/80hiCD11C+ resident M\(_{\text{w}}\)s (Re-M\(_{\text{w}}\); Fig. 2A, 2C), and (2) significantly more infiltrating T cells and NK cells were also present (Fig. 1B, 2C).

The phenotypes of each subpopulation were also characterized and are summarized in Table II. The blood neutrophil and uterine MDSCs were CD68lo, whereas all other myeloid cell subtypes were CD68hi. Among the Re-M\(_{\text{w}}\) population in the pregnant uterus, roughly half were CD206+, indicating that they were M2 M\(_{\text{w}}\)s. To determine whether infiltrating CD11c+ cells were either mature (immunogenic) or immature DCs (tolerogenic), we stained cells with MHCII, CD86, and CD80, and found they are MHCIIhi and CD80-/CD86-. This indicated that these cells were immature and in a tolerogenic state and, therefore, can induce T cell anergy or Treg expansion (Table II and Supplemental Table I).
Changes in myeloid cells are significant in the maternal blood and uteri between E8.5 and E9.5

To understand the dynamic changes in the myeloid cell populations of maternal blood during early to midgestation, we collected blood from pregnant mice and nonpregnant mice (controls) at E8.5 to E10.5. Cells were analyzed by flow cytometry and also verified through measurements of CBCs with automated differentials. A significant increase in the total myeloid cell populations (CD11b+) was observed at E9.5 (data not shown) and was found to be mainly due to a significant increase in blood neutrophils (Fig. 3A). Other subpopulations, such as monocytes, DCs, and NK cells, remained relatively unchanged from E8.5 to E10.5 (data not shown).

In pregnant uteri, infiltrating cells represented the majority of myeloid cells (Fig. 3B). These infiltrating cells significantly increased from E8.5 to E9.5 (6.2 × 10^3 to 8.9 × 10^3, respectively; p < 0.001; Fig. 3B). When each subpopulation was analyzed, we found that the most significant increases also occurred from E8.5 to E9.5. In particular, the MDSC population increased almost 10-fold (3.0 × 10^2 to 2.5 × 10^3; p < 0.001) from E8.5 to E9.5 (Fig. 3C).

Table II. Phenotypes of each leukocyte subpopulation in maternal blood and pregnant uteri

<table>
<thead>
<tr>
<th></th>
<th>CD45</th>
<th>CD11b</th>
<th>CD68</th>
<th>Ly6-G</th>
<th>Ly6-C</th>
<th>CD11C</th>
<th>MHCII</th>
<th>CD80/86</th>
<th>F4/80</th>
<th>CD206</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Monocytes</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DCs</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pregnant uteri</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDSCs</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mo-Møs</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
</tr>
<tr>
<td>DCs</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>++/+</td>
<td>+/+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Re-Møs</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>+/+</td>
<td>+/-</td>
<td>–</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

-, not detected; +/-, partially present; +, low staining intensity; ++, moderate staining intensity; ++++, high staining intensity.
Depletion of Gr-1+ cells increased DCs, T cells, and Tregs at E8.5

To see how a change in circulating myeloid cells can affect the influx of uterine leukocytes, we gave anti-mouse Gr-1 Ab (RB6-8C5) i.p. to pregnant mice to deplete Gr-1+ (both Ly6-G+ and Ly6-C+) cells at different gestational ages. Gr-1 Ab had no adverse effect on pregnancy when it was given at E5.5, E9.5, E10.5, and E12.5 (n = 3 for each gestational age). All dams proceeded to have normal deliveries, occurring at the expected time of E19.5 ± 0.5, with normal litter numbers and pup weights and appearance. However, we observed an 80% pregnancy failure rate after Gr-1 Ab administration at E8.5 (n = 5).

We then collected blood and uterine tissues 24 h post-administration of Gr-1 Ab at E8.5 and found that all circulating neutrophils and monocytes (Fig. 4A, 4B), as well as uterine MDSCs and Mo-Mφs (Fig. 4C, 4D), were completely depleted from the maternal blood. Interestingly, even without these two populations (uterine MDSCs and Mo-Mφs) present, the percentages of total myeloid cells, as well as uNK cells, did not significantly change in Gr-1 Ab-treated uteri compared with untreated controls. However, infiltrating DCs increased significantly from 17.2 ± 5.4 to 39.1 ± 2.2% (p < 0.005) and Re-Mφs also significantly increased from 2.8 ± 1.6 to 10.4 ± 4.4% (p < 0.01; Fig. 4D). Although Gr-1 Ab treatment did not change the tolerogenicity of infiltrating DCs (data not shown), upregulation of T cells/CD45 (3.5 ± 0.9 to 7 ± 1.8%; p < 0.005) and the Tregs/CD4 ratio (6.7 ± 1.5 to 12.2 ± 0.8%; p < 0.005; Fig. 4E, 4F) were observed.

The high dose of LPS induced a significant expansion of neutrophils in maternal blood (22.5 ± 2.5 to 46.0 ± 8.3%; p < 0.001) and their infiltration into uteri (10.8 ± 5.5 to 68.4 ± 11.3%; p < 0.001; Fig. 5B). The increases of uterine proinflammatory neutrophils were associated with significant decreases in other infiltrating populations, such as Mo-Mφs and DCs, as well as Re-Mφs and uNK cells (Fig. 5B).

PCR arrays for mouse cytokines and chemokines were performed on the pregnant uterine samples from untreated pregnant controls and LPS-treated pregnant dams 8 and 16 h post-administration. Large differences in cytokines and chemokines were detected between LPS-treated and control uteri. Similar profiles were found between 8 and 16 h post-LPS-treated samples (Table III). The majority of the upregulated genes encoded for chemotactic factors for neutrophils, monocytes, and T and B cells (36). In addition, G-CSF, which can stimulate the production and promote the release of neutrophils from the bone marrow, also increased >100-fold. Although both proinflammatory factors (IL-1β and TNF-α) and anti-inflammatory factors (IL-1rn, IL-22, IL-10) were induced (37, 38), IL-1β progressively increased 8- and 30-fold at 8 and 16 h post-LPS, respectively. The only downregulated gene was bone morphogenetic protein 2 (Bmp-2), which significantly decreased between 8 and 16 h post-LPS treatment (Table III).

Discussion

Leukocytes account for a substantial proportion of the cell population within the uteri during early to midpregnancy. Using multi-parameter flow cytometry to simultaneously stain up to 13 surface or intracellular cell markers, we were able to identify and characterize the subpopulations of leukocytes in the circulation and in uteri of nonpregnant and pregnant (E8.5–E10.5) mice. Different from blood, where T and B cells were predominant, pregnant uteri were dominated by myeloid and uNK cells; whereas, T and B cells were significantly constrained (Fig. 1B, 1C). This adaption reflects an immunotolerant mechanism that is necessary for the fetomaternal interface to support the development and survival of the semiallogeneic fetus and placenta.

Based on the phenotypic surface markers, as well as the depletion by anti-Gr-1 Ab, we identified two myeloid groups in pregnant uteri: infiltrating cells (such as MDSCs, Mo-Mφs, and DCs) that are recruited from bone marrow–derived cells; and activated neutrophils migrate to uteri after LPS administration.

To study how uterine leukocytes respond to systemic infection or inflammation, we administered LPS at a low (0.25 μg/mouse) or high (2.5 μg/mouse) dose i.p. to pregnant mice at E8.5–9.5. Sixteen to 24 h post-LPS administration, the high LPS dose induced fetal deaths (60% in n = 5 pregnant dams) with gross placental hemorrhages visible; whereas, the low dose caused only maternal weight loss, but no gross placental damage or fetal loss.

Compared with non–LPS-treated pregnant controls, mice given the low LPS dose did not have a significant increase in the neutrophil or MDSC populations in either the maternal blood or uteris, respectively (Fig. 5A, 5B); however, these cells were transformed from inactivated immature (CD14+CD80–) to activated CD14+CD80+ cells (Fig. 5C). Re-Mφs were also activated as CD14+ surface markers increased, but the extent was much less than that for neutrophils (Fig. 5C). Moreover, treatment with LPS at both doses significantly reduced the Mo-Mφs infiltration while increasing the uterine DC population (16.0 ± 5.1 to 30.9 ± 9.3%; p < 0.01; Fig. 5B). However, the immature and tolerogenic (MHCIICD80–CD86–) state of CD11c+ DCs was unchanged (Fig. 5D). In contrast, CD11c+ became CD80+ mature DCs in LPS-treated nonpregnant uteri (Fig. 5D).

Activated neutrophils migrate to uteri after LPS administration.

**FIGURE 3.** Dynamic changes in myeloid cell subpopulations in maternal blood and pregnant uteri. (A) The percentages of neutrophils in the total CD45+ leukocyte population in maternal blood are shown from E8.5 to E10.5. A significant increase was found between E8.5 and E9.5 (n = 10 for each time point. **p < 0.001). (B) Infiltrating myeloid cells accounted for the majority of the total number of myeloid cells in pregnant uteri and significantly increased from E8.5 to E9.5. In contrast, residual myeloid cells gradually increased from E8.5 to E10.5 (n = 10 for each time point). (C) The total cell numbers per implantation site of each myeloid subpopulation are shown from E8.5 to E10.5. The most significant increase was found in the number of MDSCs between E8.5 and E9.5 (n = 5 for each time point. *p < 0.05).
resident cells (such as Re-M$\text{ws}$ and Re-DCs) that proliferated from uterine myeloid precursors, which may be derived from the yolk sac (Fig. 2A). All these populations were also found in nonpregnant uteri, although total cell numbers were much less (Fig. 2C). Compared with pregnant uteri, nonpregnant uteri had significantly more infiltrating T and NK cells (percentage-wise), no DBA$^+$ uNK cells, and a predominance of Re-DCs, suggesting that the onset of pregnancy induced dramatic changes in the uterine immune microenvironment. These myeloid cell profiles we observed in the uteri of FVBn mice were not strain specific because we have also observed similar subpopulations in the C57BL/6 strain (see Supplemental Fig. 1 and Supplemental Table II).

Cytokines and their receptors are believed to play critical roles in leukocyte recruitment. Using PCR arrays, we showed that the majority of upregulated genes from uterine at E8.5 to E10.5 were primarily those involved in myeloid cell recruitment and stimulation (Table I), which is consistent with our flow-cytometric data, which showed a significant increase in total myeloid and uNK cells from E8.5 to E10.5 (Fig. 1C). It is still unclear as to what triggered these changes, but we speculate that the hormones of pregnancy (i.e., estrogens and progesterone) may have direct or indirect effects on leukocyte redistribution. Cytokines, such as Ccl6, Ccl8, Ccl12, and Cxcl12, bind to CCR1, CCR3, CCR2, and CSCR4, respectively, and recruit many myeloid cells, such as monocytes, neutrophils, mast cells, eosinophils, and basophils (36).

Tissue M$\text{ws}$ are a heterogeneous population that can arise from hematopoietic (infiltrating) versus self-renewing embryo-derived populations (residential) (30). In both pregnant and nonpregnant uteri, M$\text{ws}$ have been identified to derive from two origins: infiltrating Mo-M$\text{ws}$ and Re-M$\text{ws}$, both of which were CD68$^+$ (Fig. 2B, 2C, and Table II). The presence of two M$\text{w}$ populations in mouse and human uteri has also been reported by others (43–46). Therefore, it would be important to correlate human dM$\text{ws}$ with Mo-M$\text{ws}$ and Re-M$\text{ws}$ found in our mouse studies. The origin of uterine Mo-M$\text{ws}$ was determined to arise from the peripheral blood because Gr-1 Ab treatment resulted in their depletion (Fig. 4D). Tagliani et al. (21, 45) demonstrated that the recruitment of M$\text{w}$s in pregnant uteri were CSF-1– and CCR-2–dependent. CSF-1 can stimulate the recruitment of blood monocytes (CCR2$^+$) and promote the differentiation and proliferation of monocytes into M$\text{w}$s in uteri. In contrast, because uterine Re-M$\text{w}$s share similar phenotypic markers with other resident M$\text{w}$s, such as Kupffer cells, alveolar M$\text{w}$s, and skin Langerhans cells, we speculate that they proliferated from precursors derived from the yolk sac and/or fetal liver (30). M2 M$\text{w}$s (CD206$^+$) were only observed in Re-M$\text{w}$s, but not from Mo-M$\text{w}$s.

DCs in the uteri also have infiltrating and residential origins, in agreement with the subsets that have been reported in humans (CD11b$^+$CD11c$^-$/CD103$^-$ versus CD11b$^+$CD11c$^+$CD103$^+$, respectively) (47). Infiltrating DCs account for the majority DCs in pregnant uter i and can be recruited from blood via a CSF-1–dependent, but CCR-2–independent, pathway (45). Re-DCs, with minimal in pregnant uteri, were predominant among the resident cells in nonpregnant uteri (Fig. 2B, 2C). We speculate that Re-DCs in nonpregnant uter i and Re-M$\text{ws}$ in pregnant uteri may share the same progenitor cells. Upon the hormonal stimulation or changes in uterine microenvironment, they may set out to differentiate into M$\text{w}$s or DCs. DCs in pregnant uteri appeared to be tolerogenic (Table II), which is in agreement with other studies (47–50). When we administered a low LPS dose or Gr-1 Ab to pregnant mice, the DC population increased, but their tolerogenicity did not change (Fig. 5D).

**FIGURE 4.** Upregulation of DCs, T cells, and Tregs when MDSCs were depleted by Gr-1 Ab administration. Twenty-four hours after Gr-1 Ab administration at E8.5, neutrophils and monocytes were completely eliminated from maternal blood [(A) and (B) $n = 5$, $**p < 0.001$] and pregnant uteri [(C) and (D) $n = 5$, $*p < 0.005$, $**p < 0.001$] compared with untreated controls. In contrast, DCs (D), T cells (E), and Tregs (F) in uteri significantly increased ($n = 5$, $*p < 0.005$).
Uterine MDSCs share the same cell-surface markers (CD11b^hiLy6-G^+) as neutrophils in blood and nonpregnant uteri (Fig. 2). So far, mouse markers that can distinguish MDSCs from neutrophils using flow cytometer are as yet not available (51). Studies by other groups have clearly shown that the CD11b^hiLy6-G^+ population, isolated from maternal spleens and placentas, has T cell–suppressive functions in vitro (23, 24). MDSCs do not need to be present in large numbers to exert their suppressive functions as it has been previously shown that only 3% of MDSCs are required to completely block T cell proliferation in vitro (23, 52). Two subpopulations of MDSCs have been characterized based on their nuclear morphology: “monocytic” (immature monocytes) and “granulocytic” (immature granulocytes) (53, 54). Although we understand that most MDSCs found in placenta are reported to

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Significant changes of myeloid subpopulations in maternal blood and pregnant uteri after a low (0.25 µg/mouse) or high (2.5 µg/mouse) LPS dose at E8.5–9.5. (A) In maternal blood, most significant changes were found in neutrophils in mice treated with a high LPS dose (n = 5, **p < 0.001). (B) In pregnant uteri, a low LPS dose resulted in the expansion of DCs and Re-Mφs (n = 5, *p < 0.01). A high LPS dose induced a significant influx of neutrophils (n = 5, **p < 0.001), whereas Re-Mφs and uNK cells were significantly reduced (n = 6, *p < 0.01 and **p < 0.001, respectively). Both doses of LPS depleted Mo-Mφs infiltration. (C) Low LPS dose induced the differentiation of blood neutrophils and uterine MDSCs (CD14^+) to the activated neutrophils (CD14^+)(n = 5). LPS also increased the expression of CD14 in Re-Mφs (F4/80^+) (n = 5). (D) However, low LPS dose administration had no effect on the tolerogenicity of DCs (CD80^−) in maternal blood and uteri (right panel) compared with untreated pregnant controls (n = 5). As a positive control, LPS-treated DCs in nonpregnant uteri (n = 5) showed a CD80^+ phenotype.

Table III. Cytokine and chemokine profiles of uterine samples harvested at 8 and 16 h post-LPS compared with untreated pregnant controls

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>8 h Post-LPS</th>
<th>16 h Post-LPS</th>
<th>Putative Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cxcl1 (141), Cxcl3 (55), Cxcl5 (57)</td>
<td>Cxcl1 (78), Cxcl3 (77), Cxcl5 (84)</td>
<td>Neutrophil recruitment</td>
<td></td>
</tr>
<tr>
<td>Ccl2 (19), Ccl3 (11), Ccl4 (13), Ccl5 (29), Ccl7 (11), Ccl11 (8), Ccl20 (43), Ccl22 (10)</td>
<td>Ccl2 (17), Ccl3 (16), Ccl4 (8), Ccl5 (39), Ccl7 (13), Ccl22 (5)</td>
<td>Mixed leukocyte recruitment</td>
<td></td>
</tr>
<tr>
<td>Cxcl9 (89), Cxcl10 (44), Cxcl11 (96), Cxcl13 (55)</td>
<td>Cxcl9 (65), Cxcl10 (11), Cxcl11 (117), Cxcl13 (155)</td>
<td>T and B cell recruitment</td>
<td></td>
</tr>
<tr>
<td>G-CSF (101), GM-CSF (9), Lif (5)</td>
<td>G-CSF (25), GM-CSF (6)</td>
<td>Proliferation and differentiation</td>
<td></td>
</tr>
<tr>
<td>IL-1β (8.2), TNF-α (5)</td>
<td>IL-1β (30)</td>
<td>Proinflammatory</td>
<td></td>
</tr>
<tr>
<td>IL-1α (17), IL-22 (10.6)</td>
<td>IL-1α (12), IL-10 (4)</td>
<td>Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td>IL-27 (9), Bmp2 (−10)</td>
<td>C5 (8), Bmp2 (−34)</td>
<td>Others</td>
<td></td>
</tr>
</tbody>
</table>

*The putative function of each cytokine and chemokine is based on Abbas et al. (36–38). Numbers in parentheses represent the fold induction (positive) or reduction (negative) over untreated pregnant control levels; all values are significantly different compared with untreated pregnant controls, p < 0.005.
“granulocytic,” we chose to use RB6-8C5, a potent Gr-1 Ab, to deplete both “monocytic” and “granulocytic” MDSCs.

We have observed a significant increase of neutrophils (neutrophilia) in maternal blood, as well as uterine MDSCs, from E8.5 to E9.5 (equivalent to 11–13 wk in humans) (Fig. 3A, 3C); acute disruption of neutrophil infiltration during this period by Gr-1 Ab could induce fetal abortions (Fig. 4). Interestingly, neutrophilia has also been observed in human pregnancies (at 11–15 wk), as well as in pregnancies of other mouse strains (10, 55), which strongly implies a significant role of neutrophils in this period of pregnancy. In fact, neutropenic patients (low neutrophils) have higher spontaneous miscarriage rates (26%). When they were treated with G-CSF to increase neutrophils, miscarriage rates reduced to 5% (56).

The gestational period from E8.5 to E9.5 is a time when the uteroplacental circulation is beginning to establish and is critical for spiral artery angiogenesis and remodeling. It is also the time when we observed a dramatic proliferation and accumulation of uNK cells in pregnant uteri (Fig. 1C). As Amsalem et al. (10) had proposed, upregulation of neutrophils might be related to spiral artery remodeling via an interaction with uNK cells. Our study focusing on MDSC immunosuppression may offer additional insights that neutrophilia occurring in this period may also promote immunotolerance toward fetal cells in the uteri by suppressing DCs and T cell proliferation, as well as NK cell cytotoxicity (Fig. 4D).

After treating pregnant mice with Gr-1 Ab (to deplete MDSCs) or a low dose of LPS (to transform MDSCs into activated neutrophils), we observed significant increases in uterine DCs and T cells, suggesting that MDSCs may mediate the suppression of the adaptive immunity in the fetoplacental unit by limiting the proliferation and infiltration of DCs and T cells in vivo. Interestingly, we also found an increase of Tregs after Gr-1 Ab administration. It has been suggested that MDSCs can promote CD25+Foxp3+ Tregs; therefore, a depletion of MDSCs should reduce Tregs (57, 58). However, because tolerogenic DCs are capable of promoting the activation and expansion of Tregs (59), we speculate that MDSC depletion induces DC infiltration and differentiation into tolerogenic DCs, subsequently promoting Treg proliferation. Therefore, it appears that “MDSC-tolerogenic DC-Tregs” may provide a redundant or compensatory immunosuppressive mechanism to ensure normal fetal and placental development.

Infilitrating myeloid cells can be seen as a buffer between the mother and fetoplacental unit by sensing maternal stress and responding accordingly to protect the fetus and placenta. However, excess maternal immune responses to infection are believed to be harmful. In our model, after administration of a high dose of LPS, we found a dramatic influx of neutrophils (60–70% of the total leukocytes) and a limited recruitment of Mo-Mψs (Fig. 5B). LPS appeared to transform immature and inactivated MDSCs into activated neutrophils (Ly6-GCD14) and the expression level of CD14, a coreceptor of TLR4, in neutrophils was the highest among all leukocytes after LPS treatment (Fig. 5C). Other cases of excessive neutrophils or Mψ-induced pregnancy loss include anti-phospholipid syndrome (60), T cell–dependent fetal allograft rejection (61), and TLR-9, which can trigger adverse pregnancy induced by Cpg oligodeoxynucleotide (62, 63).

Significant cytokine and chemokine changes were observed in LPS-treated uteri compared with control (Table III). Upregulation was found in those genes that were associated with the recruitment of neutrophils (Cxc1, Cxc5, and Ccl20), as well as the promotion of neutrophil production and release from the bone marrow (G-CSF) (36, 38). This confirms our flow-cytometric findings (Fig. 5A, 5B), which demonstrated that neutrophils were the first responders to LPS-induced inflammation. Interestingly, chemotactic factors for other leukocytes, such as monocytes, Mψs, and T and B cells, were also significantly induced, but without significant induction of cell numbers in uteri (Fig. 5A, 5B). This may be due to their slow production in bone marrow and limited numbers in blood. Proinflammatory factors, IL-1β and TNF-α, were induced along with anti-inflammatory factors, IL-1rn and IL-10. These cytokines were also induced in LPS-treated human dMψs (43). Moreover, TNF-α has been shown to be a key player in LPS- or Cpg-oligodeoxynucleotide–induced fetal loss in IL-10−/− (null) mice or nonobese diabetic mice (62–64). In contrast, Murphy et al. (64) found that infiltrating NK1.1+ uNK cells were the critical mediators in a chronic LPS-induced inflammation model, suggesting that the fetomaternal unit might respond differently to an acute versus a chronic infection.

When studying uterine leukocytes, one of the technical challenges is the removal of peripheral blood from uterine vessels. Initially we tried to use standard perfusion technique but ended with no success. Later, we performed intracardiac puncture to collect peripheral blood (up to 1 ml) and found that this procedure greatly eliminated the majority of blood from the fetomaternal interface. However, the efficiency of blood removal using this technique may also be dependent on the gestational ages. Based on our experience, it eliminates almost all of the blood in the nonpregnant uterus and the uterus before E10.5. However, as gestation progresses and fetomaternal circulation advances, more blood cells were found in uterine single-cell suspension. Alternatively, Tagliani et al. (45) have reported to use two different fluorophore-conjugated anti-CD45 Abs to distinguish leukocytes arising from either the circulation or locally within uteri. They injected CD45 Ab (1 μg i.v. per mouse) into pregnant mice 5 min before sacrifice, which led to the labeling of only peripheral blood leukocytes. In addition, Amsalem et al. (10) used the ratio of decidual NK (CD56brightCD16−) to blood NK (CD56dimCD16+) to correct for the contamination of PBMCs in human decidua samples.

In conclusion, the fetomaternal interface becomes immunotolerant to support placental and fetal development. T cell suppression together with the failure of DCs to present Ags appears to play a key role in this process. In this study, we showed that infiltrating MDSCs could limit T cell and DC expansion in uteri, whereas neutrophils served the critical role of responding to systemic inflammation. In addition, our data demonstrated that the profile of subpopulations in circulating myeloid cells could directly alter the local uterine immune microenvironment via the infiltration of these circulating myeloid cells. These cells may, in turn, affect resident Mψs and uNK cells, leading to impaired spiral artery remodeling and abnormal trophoblast invasion, and result in abnormal placentation. Failure to orchestrate this complex interplay in the timing and coordination of immune cell changes in early to midpregnancy may cause placental malformation to result in pregnancy failure.

Acknowledgments

We thank Drs. David B. Lewis and Virginia D. Winn for critical reviews of this manuscript. We also thank the staff at the Stanford FACS facility for invaluable help in starting this project.

Disclosures

The authors have no financial conflicts of interest.

References


