Increased maternal T cell microchimerism in the allogeneic fetus during LPS-induced preterm labor in mice

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Fetal surgery is a promising strategy to treat fetuses with severe congenital abnormalities but its clinical applications are often limited by preterm labor. In normal pregnancy, multiple mechanisms protect the semi-allogeneic fetus from attack by maternal T cells. Maternal microchimerism (the presence of maternal cells in the fetus) has been suggested to be one mechanism of maternal-fetal tolerance in that it exposes the fetus to non-inherited maternal antigens and leads to the generation of fetal regulatory T cells that can suppress a maternal T cell response. Preterm labor may represent a breakdown of this robust tolerance network. We hypothesized that during inflammation-associated preterm labor, maternal leukocytes cross the maternal-fetal interface and enter the fetal circulation. Consistent with this hypothesis, we found that during preterm labor in mice, the percentage of maternal microchimerism in fetal blood increased and the frequency of fetuses with high levels of trafficking (greater than 0.5%) also increased. Finally, we showed that the maternal leukocytes trafficking into the fetus are primarily Gr-1⁺ cells in both syngeneic and allogeneic pregnancy, while T cell trafficking into the fetus specifically increases during allogeneic pregnancies. Our results demonstrate that trafficking of maternal leukocytes during pregnancy is altered during preterm labor. Such alterations may be clinically significant in affecting maternal-fetal tolerance.

Introduction

Preterm birth (defined as birth prior to 37 weeks) is the most important cause of neonatal mortality and childhood morbidity in the developed world.¹ Surviving babies face long-term health problems, including neurodevelopmental impairments such as cerebral palsy and other learning disabilities.² Preterm labor (PTL) is multifactorial, but the leading cause is inflammation and infection.³

Although multiple mechanisms protect the semi-allogeneic fetus from the maternal immune system during normal pregnancy,^{4–11} it is not known whether PTL entails recognition and rejection of the fetus by the maternal immune response. Maternal and fetal cells routinely traffic into each other's circulation^{12,13} and the resultant bidirectional microchimerism may promote maternal-fetal tolerance at baseline.¹⁴ Maternal cells in the fetus (Maternal microchimerism, MMc) reportedly expose the fetus to non-inherited maternal antigens and lead to the generation of fetal regulatory T cells (Tregs) that can suppress a maternal T cell response.¹⁴ In healthy adults, maternal leukocytes have been shown to persist as various immune cell types.¹⁵ Maternal cells have also been identified in children with Type I diabetes.^{16,17}

Although cellular trafficking may be a component of maternal-fetal tolerance, it has also been suggested that fetal-to-maternal trafficking is altered during pregnancy complications. For example, high levels of fetal DNA and cells have been found in maternal plasma in patients with spontaneous PTL¹⁸ and levels of trafficking into the fetus increase after fetal surgery.¹⁹

Our lab has investigated the trafficking of maternal leukocytes into the fetus after fetal intervention. We recently reported that maternal T cells traffic into the fetus after allogeneic in utero haematopoietic cell transplantation in mice and may prevent engraftment of transplanted cells.²⁰ Moreover, maternal T cells play a role in the selective demise of fetuses after fetal stem cell transplantation in mice.²¹ We have also observed that patients undergoing fetal surgery have increased levels of maternal cells in fetal blood.¹⁹ However, trafficking in a model of PTL that more closely resembles human inflammation-induced PTL has not been reported.

Here, we hypothesized that during inflammation-associated preterm labor, maternal leukocytes cross the maternal-fetal interface and enter the fetal circulation. To test this hypothesis, we examined maternal-fetal cellular trafficking using an established method of lipopolysaccharide (LPS)-induced PTL in mice.¹⁵ We

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demonstrate that LPS injection into the mouse uterus leads to increased MMc in the fetal blood. Moreover, there is a specific increase in the number of maternal T cells found in the fetus in allogeneic but not syngeneic pregnancies. These results suggest that maternal T cell awareness of fetal antigens is increased during PTL.

Results

LPS injection into the pregnant uterus results in preterm labor

We tracked maternal leukocytes in fetal blood in syngeneic and allogeneic pregnancies in a classic mouse model of PTL, intrauterine LPS injection.²² We first did a dose-response study for intrauterine LPS in syngeneic pregnancies, injecting doses ranging from 0.05 μ g to 1.00 μ g. Injection of 0.05 μ g resulted in delivery after 64 ± 21 (mean $\pm$ SEM) hours, whereas while higher concentrations (0.075, 0.100, 0.500, 1.00) resulted in an earlier delivery (36 ± 12 – 40 ± 16 hours) (Fig. 1). Time of delivery after treatment with 0.05 μ g of LPS did not differ between syngeneic and allogeneic pregnancies (64 ± 21 hours vs 66 ± 15 hours) (Fig. 1). Based on these results, for our next set of experiments, we injected 0.05 μ g of LPS to examine trafficking in fetal blood 12 hours after treatment to ensure viable pups for harvesting blood to analyze maternal cell trafficking.

Comparison of 2 methods to quantify maternal-fetal cellular trafficking during preterm labor

We next compared 2 methods for quantifying maternal cells in fetuses (MMc) (Fig. 2A and B). One was our previously published “leukocyte method,”²⁰ in which CD45.2 mothers are bred to CD45.1 fathers such that the fetuses are CD45.1⁺/CD45.2⁺ and maternal leukocytes are identified by lack of CD45.1. The second was the “GFP method,”²³ in which GFP^{+/−} mothers are

bred to GFP^{−/−} fathers such that maternal cells can be tracked in the GFP^{−/−} pups. To directly compare the 2 methods, we bred B6.CD45.2.GFP^{+/−} mothers to B6.CD45.1.GFP^{−/−} fathers and identified maternal cells as CD45.1[−] leukocytes in the pups (which are all CD45.1^{+/−}) or as GFP⁺ in GFP^{−/−} pups (half of the litter, Fig. 2A and B). We induced PTL by intrauterine LPS injection on E16.5 and examined MMc in the blood of the pups after 12 hours using both methods. We found that the levels of MMc were the same with each method ($P = 0.76$ by paired t-test), such that they could be used interchangeably in subsequent analyses. (Fig. 2C).

Enhanced maternal cellular trafficking during preterm labor at sites of highest blood flow

We next quantified MMc during intrauterine LPS-induced PTL in syngeneic and allogeneic pregnancies. As above, we induced PTL on E16.5 and quantified the percentage and lineage composition of maternal leukocytes in fetal blood after 12 hours. We found low but detectable levels of maternal leukocytes in uninjected fetuses ($0.1 \pm 0.1\%$), with no increase in trafficking after intrauterine PBS injection (syngeneic PBS, $0.3 \pm 0.1\%$; allogeneic PBS, $0.5 \pm 0.2\%$) (Fig. 3A). However, during LPS-induced PTL, the percentage of maternal leukocytes increased strikingly in the fetus whether the pregnancy was syngeneic or allogeneic (syngeneic LPS, $2 \pm 0.7\%$ vs uninjected, $P < 0.05$; allogeneic LPS, $2 \pm 0.6\%$ vs uninjected, $P < 0.05$ by Kruskal-Wallis test with Dunn’s post-hoc comparison) (Fig. 3A). When we specifically examined fetuses with high levels ($>0.5\%$) of MMc, we determined that the pups of allogeneic matings undergoing PTL were most likely to have high MMc compared to the other groups (allogeneic PTL, 64%; allogeneic PBS, 38%; syngeneic PTL, 47%; syngeneic PBS, 27%; uninjected, 5%) (Fig. 3B). In allogeneic matings, the percentage of pups with high MMc increased during PTL compared to PBS injection (64% vs 38%, $p = 0.03$ by chi square test) (Fig. 3B). In syngeneic matings, there was no difference in the percentage of pups with high MMc during PTL compared to PBS injection ($p = 0.09$ by chi square test). We also compared allogeneic and syngeneic pregnancies that were treated with LPS and found no difference either in the average number of trafficking cells or in the percent of pups with high trafficking ($p = 0.16$ by chi square test).

Figure 1. LPS injection into the pregnant uterus results in preterm delivery. (A) The time of delivery (in hours) after injecting various concentrations of lipopolysaccharide (LPS) into the uterus in syngeneic pregnancies (0.05 μ g, $n = 6$; 0.075 μ g, $n = 3$; 0.100 μ g, $n = 3$; 0.500 μ g, $n = 3$; 1.000 μ g, $n = 3$). (B) The percentage of dams pregnant at 24, 48, 72 and 96 hours after injection of 0.5 μ g of LPS into the uterus in syngeneic ($n = 6$) and allogeneic ($n = 4$) pregnancies.

We next analyzed the location of fetuses with high levels of trafficking and determined that in both syngeneic and allogeneic pregnancies treated with LPS, fetuses with the highest levels of MMc were at the tops of the uterine horns

Figure 2. Comparison of 2 methods to track maternal-fetal cellular trafficking. (A) Breeding scheme in which GFP^{+/−}CD45.2 mothers are bred to GFP^{−/−}CD45.1 fathers such that resulting fetuses are either GFP^{+/−} or GFP^{−/−} and all fetuses are CD45.1⁺/CD45.2⁺. (B) Gating strategy to quantify maternal cells in fetal blood. In the leukocyte model, fetal cells are CD45.1⁺/CD45.2⁺ (gate F) and maternal cells are CD45.2⁺ (gate M). In the GFP model, maternal cells are GFP⁺ and are identified only in GFP^{−/−} pups (gate F). The percentage of maternal cells obtained using each method is demonstrated for 2 fetuses undergoing PTL. (C) Comparison of maternal cell chimerism levels using the leukocyte or GFP models (n = 15 fetuses). P = 0.76 by paired t-test.

or near the cervix, and not necessarily near the site of LPS injection (Figs. 3C–D). These areas reportedly have the highest levels of blood flow,²⁴ suggesting a link between blood flow and trafficking.

Increase in maternal Gr-1 cells in fetal blood with a specific increase in T cells during preterm labor in allogeneic pregnancy

We further analyzed the lineage composition of the trafficking maternal leukocytes using markers for T cells (CD3), B cells (B220 or CD19) and granulocytes (Gr-1) (Fig. 4A). Most trafficking maternal leukocytes were Gr-1⁺. When we compared the lineage distribution of maternal Gr-1, T, and B cells found in fetal blood to that found in maternal blood, the concentration of Gr-1 cells was higher in fetal blood, suggesting specific recruitment of these cells into the fetus rather than a non-specific breakdown of the maternal-fetal interface (Fig. 4B). Interestingly, the percentage of maternal T cells found in fetal blood during PTL was significantly higher in allogeneic pregnancies than that in syngeneic pregnancies ($13 \pm 3\%$ vs $4.1 \pm 1.1\%$, $P = 0.01$ by t-test) (Fig. 4B). Thus, although there were no differences in the overall leukocyte MMc between syngeneic and allogeneic matings, the lineage analysis indicated that T cell MMc was specifically increased in allogeneic matings. These results suggest that trafficking is one mechanism by which maternal T cells may be exposed to fetal antigens and develop increased awareness of the allogeneic fetus.

Discussion

This study demonstrates that during inflammation-associated PTL, there is increased maternal microchimerism of granulocytes in fetal blood, with a specific increase in T cells during allogeneic matings. In our experiments, we showed that during PTL, the percentage of MMc in fetal blood increased and the frequency of fetuses with high levels of trafficking (greater than 0.5%) also increased. We also showed that the maternal leukocytes

trafficking into the fetus are primarily Gr-1⁺ cells in both syngeneic and allogeneic pregnancy, and that T cell trafficking into the fetus is specifically higher during allogeneic pregnancies. The significant increase of maternal leukocytes in fetal blood compared to the composition in maternal blood suggests a preferential recruitment of cells or enhanced proliferation after trafficking into the fetus, rather than an anatomical breakdown of the placental barrier.

The route of entry of maternal leukocytes into the fetus remains a fascinating unanswered question. The most direct route of maternal cell trafficking may be through the maternal-fetal interface in the placental labyrinth. We also see a correlation between trafficking and maternal blood flow, in that fetuses with higher levels of MMc are located in the uterine horns or near the cervix, and suspect that T cells and Gr1⁺ cells are specifically recruited to the inflamed uterus via the circulation and may become concentrated at the maternal-fetal interface. This interface is made up of 3 layers: the fetal mononuclear trophoblasts and a continuous double-layer of syncytiotrophoblasts.²⁵ In the vasculature, capture and adhesion of recruited cells occurs through the interaction between leukocytes and the endothelium, a process that is mediated by selectins. In the placenta, however, leukocytes do not come into contact with the endothelium, but rather with a mononuclear trophoblast layer and a continuous double-layer of syncytiotrophoblasts. In the murine maternal-fetal interface, there are increases in E-selectin expression on the trophoblasts that line maternal blood sinuses, and in VCAM-1 expression on maternal blood vessels during mid-gestation, and these changes correlate with neutrophil infiltration.²⁶ A study in humans has shown that L-selectin expression on trophoblasts could potentially enable leukocyte capture from the maternal circulation.²⁷ In addition, maternal T cells have been observed in fetal tissues of patients with villitis of unknown etiology,^{28,29} with coordinate changes in placental chemokines,³⁰ suggesting that maternal leukocytes can penetrate the maternal-fetal interface in the placenta.

Figure 3. Increased presence of maternal leukocytes in fetal blood during preterm labor at sites of highest blood flow. **(A)** The percentage of trafficked maternal leukocytes among fetal leukocytes from uninjected ($n = 19$), PBS-injected (syngeneic (Syn PBS), $n = 15$; allogeneic (Allo PBS), $n = 8$), and LPS-injected fetuses (syngeneic (Syn PTL), $n = 32$; allogeneic (Allo PTL), $n = 42$). $\S P < 0.05$ by Kruskal-Wallis with Dunn's post-hoc comparison. **(B)** Frequency of pups with high levels ($>0.5\%$) of maternal microchimerism among all pups in the experimental group. $*P < 0.05$ by Student's t -test. **(C)** Cartoon schematic defining specific locations within the mouse uterus. H = uterine horn; CTR = center; CRV = cervix. **(D)** The percentage of trafficked maternal leukocytes among fetal leukocytes from LPS-injected fetuses (syngeneic, $n = 32$; allogeneic, $n = 42$) at various locations in the uterus (uterine horn, center or cervix).

Another route whereby maternal leukocytes may be gaining entry into fetal circulation is at the interface of the decidua (maternal) and trophoblast giant cells in the placenta. In normal pregnancy, epigenetic silencing of chemokine genes inhibits T cell migration into the decidua.³¹ Alterations in chemokines during pregnancy complications are likely responsible for increased trafficking into the fetus. Previous studies in humans have shown that various chemokines produced by fetal membranes (CXCL8, CCL2, IP10, and MIP-1 α) are associated with monocyte and T cell recruitment to the pregnant uterus during the onset of labor.^{32,33} Selective recruitment of maternal leukocytes to the decidua has been described in mice²⁶ and humans³⁴ and it has been proposed that alterations in type of cells recruited to the decidua may result in pregnancy complications.³¹

One strength of this study is in our demonstration that 2 methods of quantifying MMC are equivalent. Our lineage analysis indicated that determination of lineage composition is a critical addition to quantifying overall levels of MMC. Thus, studies of human MMC should take into account the composition of trafficked maternal leukocytes in addition to the absolute levels of microchimerism. One weakness in our study is that we could not determine whether increased MMC is secondary to increased trafficking or secondary to increased proliferation of cells after entering the fetal circulation. Although we previously showed that adoptively transferred antigen-specific maternal T cells proliferate at the maternal-fetal interface,²¹ there is an overall low level of trafficking in the fetus that precludes more detailed analysis of these cells. Our study also does not address whether maternal T cells contribute to the onset of preterm labor or whether

they simply proliferate in the inflammatory environment induced by LPS.

In conclusion, our study demonstrates that during inflammation-associated PTL, there is increased maternal microchimerism of granulocytes and T cells in fetal blood. We speculate that cellular trafficking after fetal intervention may influence maternal-fetal tolerance by facilitating maternal T cell activation through trafficking of maternal T cells and APC into the fetus and, possibly, release of fetal antigen into the maternal circulation. These results are consistent with our recent report that maternal T cells mediate resorption in a fetal intervention model.²¹ Further studies are required to determine whether enhanced microchimerism has a functional role in the onset of LPS-induced preterm labor.

Materials and Methods

Reagents and antibodies

The following reagents were used: ACK Lysing Buffer (Lonza), dihydrochloride (DAPI, Invitrogen), Lipopolysaccharide from *Salmonella abortus equi* S-form (TLR-gradeTM) (LPS, Alexis Biochemicals). The following antibodies for flow cytometry were purchased from Becton Dickinson: CD3 (145–2C11), CD19 (1D3), CD45.1 (A20), CD45R/B220 (RA3–6B2); Ebioscience: CD45.1 (A20), CD45.2 (104); UCSF Hybridoma Core: Gr-1 (RB6–8C5), Fc receptor (2.4G2).

Mice

The inbred strains, BALB/c, C57BL/6.CD45.2 (B6), and C57BL/6.CD45.1 (CD45.1) were obtained from either NCI or Jackson Laboratories. B6.uGFP transgenic mice (strain 004353) were obtained from Jackson Laboratories. BALB/c.actin-GFP were a gift from Abul Abbas (University of CA, San Francisco, USA). All mice were bred and maintained in a specific pathogen-free facility at UCSF. All mouse experiments were performed according to the UCSF Institutional Animal Care and Use Committee approved protocol.

LPS-induced preterm labor

BALB/c and B6 mice were bred to create syngeneic and allogeneic pregnancies. On E16.5, a laparotomy was performed and 0.05 µg of LPS (or PBS) was injected into the uterus between the first and second fetus of the right horn to induce PTL.²²

Figure 4. Increase in maternal Gr-1 cells in fetal blood with a specific increase in T cells during preterm labor in allogeneic pregnancy. **(A)** Gating strategy to phenotype trafficked maternal cells. Maternal leukocytes were separated into Gr-1⁺ and Gr-1⁻ gates. Gr-1⁻ cells were divided into B220⁺ (B cells) or CD3⁺ (T cells). **(B)** Lineage analysis of trafficked maternal leukocytes during PTL shown as the percentage of all trafficked maternal leukocytes (e.g. trafficked Gr-1⁺ cells/total trafficked maternal leukocytes) and compared to the lineage composition found in maternal blood. Syn: syngeneic mating; Allo: allogeneic mating. **P* < 0.05, ****P* < 0.001 by Student's *t*-test.

Fetuses were harvested twelve hours later. The dose of LPS (0.05 µg, 0.075 µg, 0.100 µg, 0.500 µg, 1.000 µg) was titrated to ensure that it is a dose high enough to induce preterm delivery, but pups were harvested prior to delivery so that viable blood could be analyzed.

Tissue harvesting and flow cytometry

Fetuses were harvested 12 hours after maternal injection of LPS. Pups were washed twice in PBS prior to decapitation in heparinized HBSS to minimize contamination with maternal blood. Maternal blood was collected from the maxillary vein. The samples were stained for flow cytometry after first lysing the red blood cells in ACK lysis buffer as previously described.²⁰ At least 1,000,000 events were collected during the flow cytometry to ensure accurate analysis of microchimeric cells. “High traffickers” were defined as fetuses showing greater than 0.5% maternal microchimerism in the peripheral blood as previously described.²⁰

Statistics

Comparisons involving 2 groups were evaluated using either the Chi-square test (for changes in frequency) or Student's *t* test. Comparisons between 2 or more groups were evaluated using ANOVA with a Tukey's multiple comparison test for pairwise comparisons, or with a Kruskal-Wallis test with Dunn's post-hoc comparison (the latter when the data were not normally distributed). A *p* value of less than 0.05 was considered to be significant. Data represent means ± SEM (or medians, for non-normally distributed data).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

M.W., Q.T., and T.C.M. designed the research and analyzed the data. M.W. and T.L. performed the experiments. M.W. and T.C.M. wrote the paper.

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