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Alloreactive fetal T cells promote uterine contractility in preterm labor via IFN-γ and TNF-α

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Author contributions

M.F., Q.T., and T.C.M. conceived and designed the experiments. M.F., L.C., T.-H.L., S.K., and G.C. performed the experiments. M.F., L.C., R.W., C.J., D.E.M., S.F.-D., T.-H.L., S.K., G.C., R.R., Q.T., and T.C.M. analyzed the data. M.P.B., P.J.N., L.F.B., N.G.-L., and R.R. contributed the reagents/materials/analysis tools. M.F. and T.C.M. wrote the manuscript, with input from all authors.

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Abstract

Healthy pregnancy is the most successful form of graft tolerance, whereas preterm labor (PTL) may represent a breakdown in maternal-fetal tolerance. Although maternal immune responses have been implicated in pregnancy complications, fetal immune responses against maternal antigens are often not considered. To examine the fetal immune system in the relevant clinical setting, we analyzed maternal and cord blood in patients with PTL and healthy term controls. We report here that the cord blood of preterm infants has higher amounts of inflammatory cytokines and a greater activation of dendritic cells. Moreover, preterm cord blood is characterized by the presence of a population of central memory cells with a type 1 T helper phenotype, which is absent in term infants, and an increase in maternal microchimerism. T cells from preterm infants mount a robust proliferative, proinflammatory response to maternal antigens compared to term infants yet fail to respond to third-party antigens. Furthermore, we show that T cells from preterm infants stimulate uterine myometrial contractility through interferon-γ and tumor necrosis factor–α. In parallel, we found that adoptive transfer of activated T cells directly into mouse fetuses resulted in pregnancy loss. Our findings indicate that fetal inflammation and rejection of maternal antigens can contribute to the signaling cascade that promotes uterine contractility and that aberrant fetal immune responses should be considered in the pathogenesis of PTL.

One Sentence Summary:

Activated fetal T cells promote preterm labor through the induction of maternal uterine contractions.

INTRODUCTION

Preterm birth (defined as delivery before 37 weeks of gestation) is the leading cause of neonatal morbidity and mortality in the developed world, accounting for 35% of infant deaths in the first year of life (1). Although preterm birth has multiple etiologies (2), infection and inflammation are the most common causes of spontaneous preterm labor (PTL) (3). Research on the immunological causes of PTL has mainly focused on activation of the innate immune system (3), with a relative lack of information regarding the possible role of the adaptive immune system. Healthy pregnancy is the most robust form of tolerance, in which the semiallogeneic mother and fetus tolerate each other: PTL, often associated with maternal infections, could potentially arise from a breakdown in maternal-fetal tolerance. For example, infections can activate the adaptive immune system and trigger T cell–mediated allograft rejection (4, 5). Thus, it is important to understand whether maternal or fetal T cell activation plays a role in the pathogenesis of PTL. In healthy pregnancies, multiple overlapping mechanisms maintain tolerance at the maternal-fetal interface (6). On the maternal side, reactive T cells are prevented from crossing the placenta (7), and their activation is kept under control by the expansion of regulatory T cells (T_{regs}) (8–10). In addition, uterine dendritic cells (DCs) are unable to migrate into uterine-draining lymph nodes and prime maternal T cells (11), and decidual B cells further counteract inflammatory responses during PTL (12). Immaturity of fetal antigen-presenting cells (APCs) (13) is another mechanism of tolerance reported in mouse models. Although maternal T cells that recognize fetal antigens presented by fetal APCs (using the “direct” pathway of antigen

presentation) comprise most of the alloreactive repertoire (14), the relative immaturity of fetal APCs and their low numbers in the maternal circulation mean that there is inefficient antigen presentation using this pathway, which effectively prevents maternal T cells from becoming activated in a healthy murine pregnancy (15). However, most of these mechanisms responsible for dampening T cell responses have been only described for maternal, not fetal, T cells. The possible contribution of fetal T cells has not been well examined, perhaps secondary to the predominant use of murine models, in which fetal T cells mature later than in humans (16).

One important mechanism for maintaining maternal-fetal tolerance arises on the fetal side because of formation of fetal T_{regs}. It has been shown that there is a baseline level of trafficking of cells between the mother and the fetus, leading to microchimerism of maternal cells in the fetus ("maternal microchimerism") (17, 18). These maternal cells induce the generation of fetal T_{regs} against noninherited maternal antigens in healthy pregnancies (19). Conversely, there is some evidence that alterations in microchimerism occur during pregnancy complications in murine models (9, 20–22), and it is possible that changes in microchimerism may lead to aberrant fetal T cell activation and perturb the balance between tolerance and rejection.

Although activation of the human fetal innate immune system during inflammation has been described (23–25), additional effects on fetal T cell activation remain unknown. Despite earlier evidence that fetal T cells were hyporesponsive (26, 27), recently, there has been a growing awareness of the ability of human fetal T cells to respond in lymphoid organs (19), cord blood (28), and intestine (29). We therefore asked whether the inflammatory environment in patients with PTL leads to increased activation of the innate and adaptive fetal immune cells. Here, we report that preterm fetuses show maturation of their DCs and priming of their T cells in response to maternal antigens. We demonstrate that the inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α) produced by fetal T cells cause contractility of human myometrial cells, a biological hallmark of parturition. In a murine model of fetal adoptive transfer of activated T cells, we also find that activated T cells mediate fetal resorption, a commonly seen outcome of aberrant immune activation at the maternal-fetal interface (21, 30–33). Thus, the fetal immune system is a previously unrecognized critical player in the breakdown of maternal-fetal tolerance and the onset of uterine contractions during PTL.

RESULTS

There is evidence of *in utero* inflammation and DC activation in preterm cord blood

We prospectively enrolled 89 patients with healthy pregnancies and 70 patients with spontaneous PTL secondary to preterm premature rupture of membranes (PPROM), which has been associated with subclinical infection (34), and/or chorioamnionitis (table S1). Overall, histological chorioamnionitis was seen in 28 (40%) patients with PTL (table S2).

The link between infection or inflammation and PTL led us to first analyze plasma cytokines in maternal and cord blood. For simplicity, we will refer throughout the text to fetal cells as cord blood cells because cord blood cells are considered to be fetal in origin (35). We found

that preterm infants have significantly elevated amounts of multiple proinflammatory cytokines and chemokines in the cord blood compared to infants born at term ($P < 0.05$) (table S3). In contrast, maternal cytokines did not differ between patients with PTL and controls (table S4).

Because some of these cytokines, such as interleukin-1α (IL-1α), TNF-α, IL-6, and macrophage inflammatory protein–1β (MIP-1β) (Fig. 1A), are produced by activated DCs (36–38), we next asked whether fetal DCs were activated during PTL. We analyzed the expression of the activation markers CD80 and CD86 on myeloid DCs (mDCs; defined as $Lin^{-}CD11c^{+}HLA\text{-}DR^{+}$) and plasmacytoid DCs (pDCs; defined as $Lin^{-}CD123^{+}HLA\text{-}DR^{+}$) (39) in cord and maternal blood. Both mDCs and pDCs in the cord blood of infants born preterm showed significant increases in expression of CD80 and CD86 compared to term controls (Fig. 1, B to D). When we analyzed the effect of chorioamnionitis on these findings, we found that these changes were seen in preterm patients both with and without chorioamnionitis, but CD80 expression on fetal pDCs was significantly higher in preterm patients with chorioamnionis ($P = 0.043$). CD86 expression was also increased in maternal pDCs during PTL, but otherwise, DCs were equivalent between the two groups (Fig. 1D). Collectively, these findings support the presence of an inflammatory microenvironment, manifested predominantly on the fetal side, in patients with PTL.

Central memory $CD4^{+}$ T cells are increased in the cord blood of preterm infants

Cell surface up-regulation of CD80 and CD86 by APCs is crucial for delivering the costimulatory signals necessary for T cell priming. We reasoned that the observed increase in systemic inflammation and DC activation in the cord blood of infants during PTL might lead to early activation of fetal T cells. We identified $CD4^{+}$ and $CD8^{+}$ T cells in maternal and cord blood peripheral blood mononuclear cells (PBMCs) and detected naïve, effector memory (EM), and central memory (CM) T cell subsets on the basis of CCR7 and CD45RA expression (40). We found that the cord blood of preterm infants is characterized by an increased proportion of CM $CD4^{+}$ T cells with a concomitant reduction in the proportions of naïve CD4+ T cells compared to term infants (Fig. 2, A and B). These changes were seen globally in our PTL cohort, and there were no differences in the percentages of CM T cells among preterm patients with or without chorioamnionitis ($P = 0.3704$). No changes in T cell phenotype in maternal blood were observed during PTL (Fig. 2B).

Because T_{regs} have a critical function in the suppression of T cell responses (41), we wondered whether CM $CD4^{+}$ T cell expansion in preterm infants might be due to reduced T_{reg} numbers and stained maternal and cord blood obtained during term and PTL deliveries. The percentages of T_{regs} did not differ between patients with PTL and controls (fig. S1).

Activated T cells can be subdivided into distinct T cell subsets based on their secreted cytokines, homing capacity, and effector functions. Thus, we investigated key surface markers and inflammatory mediators produced by the CM T cells of preterm infants. We detected an increased expression of the chemokine receptor CXCR3 in the CM T cells of preterm compared to term infants (Fig. 2, C and D). CXCR3 has been described as a surrogate for the type 1 T helper (T_H1) phenotype in adult T helper cells with homing capacity to inflamed tissues (42, 43). Accordingly, we demonstrated that $CD4^{+}CD45RA$

$^{-}$CCR7^{+}CXCR3^{+} fetal T cells from preterm infants can produce IFN-γ after stimulation *in vitro* (Fig. 2E), whereas those from term patients do not. Together, these findings support the existence of a distinct population of T_H1-like CM CD4^{+} T cells in the cord blood of preterm infants that is absent in the cord blood of term infants.

Maternal microchimerism is increased in preterm infants

In healthy pregnancies, maternal cells can be present in the fetal circulation at low frequencies and promote the generation of fetal T_{regs} against noninherited maternal antigens (19). Therefore, in this context, maternal microchimerism is a critical element in promoting maternal-fetal tolerance at baseline. There is evidence that the amount of naturally occurring maternal microchimerism can increase during pregnancy complications (22, 44). We therefore examined whether there are changes in maternal microchimerism in the fetal circulation during PTL by identifying nonshared alleles [human leukocyte antigen–DR (HLA-DR) and In-Del] between the mother and the fetus and using quantitative reverse transcription polymerase chain reaction (RT-PCR) to amplify nonshared maternal alleles in fetal blood or nonshared fetal alleles in maternal blood (45, 46). Maternal microchimerism was significantly increased in preterm infants compared to term controls (Fig. 3A), whereas fetal microchimerism in maternal blood did not change in mothers experiencing PTL compared to those who delivered at term (Fig. 3A). Maternal cells express HLA molecules that are not shared by fetal cells and, therefore, can be recognized as nonself by the fetal immune system. Accordingly, there was a strong association between maternal microchimerism and the presence of CM T cells in the cord blood of preterm infants ($\rho = 0.93$, $P = 0.007$) (Fig. 3B), suggesting that maternal cells might be a source of antigen that drives fetal T cell activation. Thus, in the inflammatory context of PTL, the combination of early activation of DCs and increase in maternal microchimerism could result in fetal T cell priming against maternal antigens.

Fetal T cells are activated specifically against maternal antigens in PTL

Because the cord blood of preterm infants shows increased maternal microchimerism, activated fetal DCs, and an expanded memory T cell population, we next tested whether fetal T cells are activated specifically against maternal antigens by using mixed lymphocyte reactions (MLRs). We reasoned that fetal T cells are more likely to be activated using the indirect pathway of antigen presentation (14), in which fetal APCs present maternal antigens to fetal T cells. This mechanism has been shown to be relevant for maternal T cell activation against fetal antigens (15) and is likely also relevant in considering the mechanism of fetal T cell activation; although there are some maternal cells present in the fetus, they may not be professional APCs (11, 20), and their numbers are likely not abundant enough to effectively present antigen directly to fetal T cells.

To test this hypothesis, we first established a novel MLR of indirect reactivity, in which T cells and APCs are of the same origin, either maternal or fetal (Fig. 4A). We cultured maternal or fetal T cells with *in vitro*-derived autologous APCs; maternal or fetal PBMC lysates were used as the source of antigen. In addition, to test the maternal or fetal T cell response to a fully nonrelated HLA type, we used lysate of PBMCs from adult women of reproductive age as a source of antigen and named “third-party control.”

When we examined the proliferation of maternal and fetal T cells against each other and against third-party controls, we found that fetal T cells from preterm infants proliferated and readily up-regulated CD25 in response to stimulation [$CD4^+$ T cells (Fig. 4B) and $CD8^+$ T cells (Fig. 4C)]. As expected, there was low proliferation of fetal T cells from term patients against their maternal antigens and against third-party controls. However, there was a significant increase in the proliferation of both fetal $CD4^+$ and $CD8^+$ T cells against maternal alloantigens in pregnancies complicated by PTL compared to term pregnancies [$CD4^+$ T cells (Fig. 4D) and $CD8^+$ T cells (Fig. 4E)]. This proliferation was specific for maternal antigens because it wasnot seen when preterm fetal T cells were stimulated with antigens from third-party controls. The observed increases in fetal T cell proliferation were seen broadly among preterm patients with no differences in the proliferation of $CD4^+$ or $CD8^+$ T cells from patients with and without chorioamnionitis. Thus, the frequency of alloreactive fetal $CD4^+$ and $CD8^+$ T cells that can recognize noninherited maternal antigens is significantly increased in patients with PTL.

In contrast to the observed fetal T cell activation, maternal T cell proliferation against fetal alloantigens was consistently low (Fig. 4, D and E). However, maternal T cell proliferation in response to anti- CD3 and anti-CD28 stimulation was preserved (fig. S2, A and B). The lack of maternal cell proliferation in this assay is consistent with the known low precursor frequency of indirectly reactive T cells (14), as well as with a previous study in mice that showed that there is little maternal T cell activation against fetal antigens with the physiologically relevant indirect pathway of antigen presentation (15).

The degree of HLA mismatch between a mother-fetus pair can affect the amount of proliferation and data interpretation. To rule out the possibility that the proliferation of fetal T cells from patients with PTL in our MLR assay was primarily due to increased HLA mismatch in the PTL group, we genotyped the HLA loci for each mother-fetus pair. We found no difference in histocompatibility between cases and controls at any of the classical HLA class I or class II loci investigated (table S5). Thus, the finding of increased frequency of maternal-reactive fetal T cells in patients with PTL is not simply secondary to increased HLA mismatch between these particular maternal-fetal pairs in our study.

We next asked whether proliferating fetal T cells produce effector cytokines that might augment inflammation in the fetus. We detected an increase in IFN-γ production by proliferating maternal-responsive $CD4^+$ and $CD8^+$ fetal T cells from preterm infants compared to term controls (Fig. 4F). Proliferating fetal $CD4^+$ and $CD8^+$ T cells from preterm infants also produced increased amounts of TNF-α (Fig. 4G). Thus, alloreactive fetal T cells in preterm infants maintain a stable T_H1 phenotype upon antigen-specific stimulation.

T cells from preterm infants stimulate myometrial contractility

Spontaneous PTL is defined by the onset of uterine contractions before term, but the molecular mechanisms connecting immune activation to the onset of labor are not well understood. Given the finding of fetal T cell activation in PTL, we next asked whether fetal immune activation could directly promote uterine contractility. We used an established assay of uterine contractility in which an immortalized human myometrial cell line (PHM1–41) is

plated in collagen gels and the relative ability of various agents to cause myometrial cell contractions can be read out as a decrease in the area of the collagen gels (Fig. 5A) (47). Because we had demonstrated that TNF-α was increased in the plasma of preterm infants (Fig. 1A) and that fetal alloreactive T cells produced TNF-α and IFN-γ (Fig. 4, F and G), we first tested the ability of these cytokines to promote contractility. We found that purified TNF-α resulted in myometrial cell contractility at 48 hours (Fig. 5B), whereas purified IFN-γ did not. To determine whether fetal T cells can directly stimulate contractility, we next cocultured a human myometrial cell line with sorted cord blood T cells. When we plated sorted, unstimulated $CD4^+$ or $CD8^+$ T cells from preterm infants on collagen-embedded myometrial cells, we observed a consistent increase in contractility at all time points tested, whereas T cells from term infants did not enhance contractility (Fig. 5, C and D). Blockade of TNF-α with a neutralizing antibody almost completely abolished the contraction of the myometrial cells (Fig. 5, C and D), in accordance with the direct effect of TNF-α in the contractility assay. Surprisingly, and in contrast with the inability of IFN-γ to directly exert an effect on the myometrial cells, anti–IFN-γ treatment also blocked T cell-induced contractility (Fig. 5, C and D). The simultaneous neutralization of TNF-α and IFN-γ did not further reduce the contractility of the myometrial cells (Fig. 5, C and D, and fig. S3, A and B), suggesting that these inflammatory cytokines may have a sequential, rather than a synergistic, effect on human myometrial cell contractility.

To determine the mechanism of this sequential effect, we performed an enzyme-linked immunosorbent assay (ELISA) for IFN-γ and TNF-α on the supernatant of these contractility assays. We observed that both $CD4^+$ and $CD8^+$ T cells from preterm infants secrete more IFN-γ and TNF-α compared to their term counterparts (Fig. 5E). The amount of TNF-α produced by T cells from preterm infants was in the range that was sufficient to cause contraction when purified TNF-α was added to these cultures (fig. S3 C). Moreover, the blockade of IFN-γ with a neutralizing antibody prevents the detection of TNF-α by ELISA (Fig. 5E, right), whereas the blockade of TNF-α does not affect the secretion of IFN-γ (Fig. 5E, left). Because IFN-γ alone has no effect on human myometrial cell contractility, we conclude that IFN-γ produced by alloreactive T cells acts in an autocrine and/or paracrine loop to enhance the production of TNF-α, as previously reported (48), which, in turn, promotes the contraction of the myometrial cells. Overall, these results suggest that T cells from preterm fetuses are already primed *in utero* to secrete effector cytokines that can stimulate contractions.

Adoptive transfer of activated T cells into fetal mice induces pregnancy loss

The relatively late development of fetal T cells in the mouse poses a challenge for examining the role of the fetal adaptive immune system *in vivo*. To overcome this limitation, we took advantage of our mouse model of *in utero* injection, in which cytokines or cells can be directly injected into mouse fetuses during a survival surgery (Fig. 6A) (20, 49). We and others have shown that immune activation at the maternal-fetal interface in mice results in resorption of the fetuses (21, 30–33). We first tested the effect of TNF-α and IFN-γ, the relevant cytokines produced by activated human fetal T cells in our MLR assays, on fetal survival. We found that TNF-α and IFN-γ induced a high and comparable rate of resorption when injected in embryonic day 14.5 (E14.5) fetuses (Fig. 6B). Because murine fetal T cells

are virtually absent from the periphery at this gestational age, we next developed an adoptive transfer model in which we injected *in vitro*-activated syngeneic T cells that produce both TNF-α and IFN-γ. We sorted adult C57BL/6 $CD4^+$ and $CD8^+$ splenic T cells, activated them with anti-CD3 and anti-CD28 antibodies, and then transferred them into E14.5 fetuses. An equal number of nonactivated $CD4^+$ and $CD8^+$ T cells were transplanted into separate litters as a control. *In vitro*-activated $CD4^+$ and $CD8^+$ T cells up-regulated CD44 and produced more IFN-γ and TNF-α compared to nonactivated T cells (fig. S4). We found that *in utero* adoptive transfer of activated T cells resulted in increased fetal resorption compared to transplantation of nonactivated T cells (Fig. 6C). This phenomenon was dependent on the production of TNF-α or IFN-γ because adoptive transfer of T cells from mice deficient in either cytokine failed to cause fetal resorption (Fig. 6C). These results demonstrate that TNF-α and IFN-γ produced by activated fetal T cells are central in breaking down maternal-fetal tolerance.

DISCUSSION

Here, we demonstrate that the fetal environment in PTL is characterized by inflammation, resulting in a cascade that involves early activation of DCs and priming of fetal T cells against maternal antigens. We show that aberrant fetal immune activation and the resulting inflammatory cytokines can then lead to the initiation of the labor pathway by stimulating uterine contractility. We further demonstrate a key role for TNF-α and IFN-γ produced by activated T cells in an *in vivo* fetal adoptive transfer model. Thus, our results indicate that activation of the fetal adaptive immune system, previously thought to be immature, may contribute to the breakdown of maternal-fetal tolerance in patients with PTL.

Our finding that the fetal immune system becomes activated in the context of PTL is compatible with a model in which inflammation at the maternal-fetal interface is the initial signal that triggers the maturation of fetal DCs and early activation of the fetal adaptive immune system (fig. S5). In this setting, fetal T cells can be easily activated to secrete T_H1 cytokines that can, in turn, stimulate uterine contractions. This model is consistent with the notion that, at least in mice, the fetus can secrete compounds that signal normal labor, such as surfactant protein A (50, 51); in the context of prenatal inflammation, it is logical that the fetus might produce other mediators that initiate parturition before term. The concept of a “fetal inflammatory response syndrome,” in which fetal inflammation allows the fetus to exit a hostile uterine environment, has been proposed in the past (23, 24). Our results provide further mechanistic details for this idea and implicate the sequential production of IFN-γ and TNF-α by fetal T cells as crucial mediators in this process. Activation of fetal T cells could be either the primary event in this cascade or a tipping point that enhances the cascade through the production of inflammatory cytokines.

In healthy pregnancies, fetal T cells that recognize noninherited maternal antigens are present but have a propensity to become T_{regs} upon encountering microchimeric maternal cells (19). Thus, maternal microchimerism is a critical element in promoting maternal-fetal tolerance at baseline. However, there is evidence in mouse models that microchimerism may serve to tolerize or sensitize, depending on the context (52, 53). Our study adds a new dimension to this idea: In the inflammatory context of PTL, alterations in maternal

microchimerism, coupled with the early activation of DCs, can instead result in the priming of maternal-reactive T cells and their differentiation into effector T cells, ultimately resulting in the breakdown of maternal-fetal tolerance.

There are likely other antigens, in addition to noninherited maternal antigens, that contribute to fetal T cell activation. For example, some patients with spontaneous PTL also have subclinical infections, as suggested by other studies of patients with PPROM (54–56). Although we did not observe significant differences in T cell activation between patients with proven chorioamnionitis compared to those without clinical or histological chorioamnionitis, it is possible that many of our patients have subclinical infections or even dysbiosis that could trigger the inflammation observed in these patients. For example, the presence of bacteria and pathogen-associated molecular patterns can induce proinflammatory cytokines that enhance fetal DC activation and alloreactive T cell differentiation (4). In addition to maternal-reactive T cells, pathogen-reactive fetal T cells might also be present and amplify the immunopathology of PTL. Sterile inflammation may also contribute to aberrant fetal T cell activation (30, 57–59).

Our new assay of indirect reactivity lends important insights into mechanisms of fetal T cell activation during pregnancy. Given the physical (albeit porous) separation of maternal and fetal leukocytes, indirect reactivity is a more physiologically relevant readout of the increase in allospecific T cells in infants during PTL, mirroring what has been reported for maternal T cell activation in mouse pregnancy (15). The low alloreactivity observed in healthy controls suggests that the low precursor frequency of indirectly alloreactive T cells, on both the maternal and fetal sides, contributes to maintain maternal-fetal tolerance in a healthy pregnancy. During inflammation, a well-orchestrated set of events results in maturation of DCs, resulting in the activation of alloreactive fetal T cells. These results are consistent with our previous findings that inflammation induced by allogeneic fetal hematopoietic stem cell transplantation activates fetal T cells and drives fetal loss in a T cell receptor (TCR) transgenic mouse model (60). Although we did not detect significant changes in maternal T cell activation in our patients, we only have access to circulating maternal blood and, therefore, cannot rule out the participation of the maternal immune cells at the maternal-fetal interface in this process (61).

Our mouse model of direct fetal adoptive transfer of activated T cells could be a useful tool for dissecting fetal immune disturbances in pregnancy by allowing the introduction of immune cells that are relevant in human fetal development but not present in mouse fetuses. The ability to manipulate the fetal immune system in a tractable mouse model, whether by cellular transfer, genetic knockouts, or direct fetal administration of reagents, can bring much-needed insights into the field of maternal-fetal immunology. In addition, this model can also be used to test the administration of specific agents that block immune activation by targeting fetal or maternal cells. Although immune activation at the maternal-fetal interface in mouse leads to fetal resorption, rather than preterm delivery (9, 10, 30), this model nevertheless provides mechanistic insights such as the contribution of IFN-γ and TNF-α in pregnancy loss.

Our study has several limitations. First, we did not compare spontaneous PTL with other categories of preterm birth (for example, induced preterm delivery for maternal or fetal indications) but only to term deliveries. We focused on spontaneous PTL because these patients are most likely to have underlying inflammation, allowing us to discern the effects of inflammation on the fetal immune system. Although we were careful to select a particular etiology for PTL, the observed variability between patients in each parameter that we measured suggests that PTL is likely to be a heterogeneous disease, and the current clinical definitions may include additional subgroups, as revealed here. It is possible that such variability is also reflected in the heterogeneous response to therapy in patients with PTL, and a better understanding of the causes of PTL may help tailor therapeutic modalities and inform novel interventions. Second, given the limited amount of cord blood available from each patient, we were not able to perform all the different experiments on all the patients. Finally, our mouse experiments led to resorption instead of preterm delivery, as is commonly seen in other experiments, resulting in immune responses during pregnancy (21, 30–33). Thus, although the mouse model allows detailed mechanistic experiments, the differences between human and mouse pregnancy preclude an exact parallel in the experimental outcomes.

Our study adds to the growing literature on the functional capacities of the fetal immune system (28, 62) and, in particular, the capacity of fetal T cells to assume an effector role. In addition to contributing to the initiation of preterm uterine contractions, altered immune activation during gestation may have downstream consequences for the health of the neonate and the adult. For example, numerous reports have correlated neonatal T cell activation with diseases such as cerebral palsy (63), necrotizing enterocolitis (64), and bronchopulmonary dysplasia (65). We show that immune dysregulation begins *in utero*, and its prevention therefore carries important clinical implications. Manipulating the complex cascade that links inflammation to labor may result in development of new treatment strategies for PTL.

MATERIALS AND METHODS

Study design

Matched maternal and cord blood samples were obtained from 96 patients with healthy term deliveries and 74 patients who had preterm birth secondary to spontaneous PTL or PPROM. Adult third-party controls used in the MLR assay were anonymous healthy subjects (15 nonpregnant females of reproductive age). Mice were bred and maintained in a specific pathogen-free facility at the University of California, San Francisco (UCSF). All mouse experiments were performed according to the UCSF Institutional Animal Care and Use Committee–approved protocol. C57BL/6J mice were bred to each other in house from Jackson stock (The Jackson Laboratory). TNF-$\alpha^{-/-}$ mice were obtained from the Ma Laboratory at UCSF and IFN-$\gamma^{-/-}$ mice from the Kim Laboratory at UCSF. Primary data of experiments with $n < 20$ are reported in table S6.

Study approval

Approval for this nonrandomized observational study was obtained from both UCSF and Wayne State University's Institutional Review Boards. After giving written informed

consent, patients were prospectively enrolled at UCSF Medical Center and Hutzel Women's Hospital between the years 2010 and 2016.

Sample processing

Samples collected at Wayne State University were shipped to UCSF by overnight mail. All maternal and cord blood samples were processed within 24 hours from delivery. Whole blood and plasma were frozen and stored for batch analysis of microchimerism or cytokines, respectively. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation and stored in liquid nitrogen. Phenotyping by flow cytometry was performed on freshly isolated PBMCs.

ELISA and cytokine assay

Cytokines in culture supernatants were measured by ELISA according to a standard protocol (R&S Systems). Absorbances at 450 nm were measured on a tunable microplate reader (VersaMax, Molecular Devices). Cytokine titers were calculated by extrapolating absorbance values from standard curves where known concentrations were plotted against absorbance using SoftMax Pro 5 software.

Cytokine profiles in the maternal and cord blood plasma samples were assayed using the standard-sensitivity MILLIPLEX Map kit (Millipore), as previously reported (46). Samples were acquired and analyzed on a LABScan 100 analyzer (Luminex) using Bio-Plex Manager 6.0 software (Bio-Rad).

Microchimerism measurement

Microchimerism percentages were assessed using a previously described real-time quantitative PCR (45) to amplify nonshared maternal alleles in fetal blood and nonshared fetal alleles in maternal blood. Briefly, paired maternal and cord blood samples were first genotyped for 12 HLA-DR and 12 In-Del alleles to determine nonshared ("informative") alleles between the mother and the fetus after genomic DNA was extracted. The presence of microchimeric cells was then determined by amplifying for the nonshared maternal alleles in cord blood or the nonshared fetal alleles in maternal blood. By amplifying the informative/nonshared allele, we quantified the event of minor population for each sample based on the serial dilutions of DNA from the major population. We also amplified HLA-DQ to quantify the total amount of genomic DNA in each sample. After characterizing the event of minor population and total quantity of genomic DNA, we divided the event of minor population (numerator) by the total amount of genomic DNA (denominator) to obtain the percent of microchimerism for each sample. The lower limit of detection of this assay has been established to be between 0.001 and 0.0001%, depending on the DNA input and primer pair. We excluded one outlier full-term patient from our study whose maternal microchimerism was 16%.

Antibodies used for flow cytometry and cell sorting

Single-cell suspensions were prepared in fluorescence-activated cell sorting (FACS) buffer [PBS plus 2.5% (v/v) fetal calf serum and 2 mM EDTA]. Initially, cells were incubated on ice with Fixable Viability Dye eFluor780 (eBioscience) to allow live/dead discrimination,

and cells were then surface-stained on ice with the following antibodies: fluorescein isothiocyanate (FITC)–labeled anti-CD3/CD19/CD20 (UCHT1, HIB19, and FB1, respectively), phycoerythrin (PE)–Cy7–labeled anti-CD4 (SK3), AmCyan-labeled anti-CD8 (SK1), AmCyan-labeled anti–HLA-DR (G46–6), PE-Cy7–labeled anti-CD11c (B-ly6), PerCP-Cy5.5 anti-CD25 (M-A251), Alexa Fluor 647–labeled anti- CD16 (3G8), PE-labeled anti-CXCR3 (1C6/CXCR3) (all from BD Biosciences), Alexa Fluor 700–labeled anti-CD80 (L307.4), PE-labeled anti-CD86 (IT2.2) (both from BD Biosciences), PerC-Cy5.5–labeled anti-CD123 (7G3) (from BD Biosciences), Alexa Fluor 647–labeled CCR6 (11A9) (from BD Biosciences), FITC-labeled anti-CD45RA (ALB11) (from Beckman Coulter), and purified anti-CCR7 (150503) (from R&D Systems), followed by anti-mouse immunoglobulin G2a-PE (from SouthernBiotech) and V450 Streptavidin (from BD Biosciences). Flow cytometry data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar Inc.).

For T cell sorting, $CD4^+$ and $CD8^+$ T cells were enriched by negative selection with a T cell isolation kit (Miltenyi Biotec) and were stained with the indicated antibodies. Cells were sorted as $CD19^-CD20^-CD14^-CD16^-CD8^-CD4^+$ or $CD19^-CD20^-CD14^-CD16^-CD4^-CD8^+$ to a purity of ≥99% with a FACSAriaII (BD Biosciences). Data were analyzed using FlowJo software (TreeStar Inc.).

T cell stimulation

The cytokine-producing capacity of lymphocytes was assayed after stimulation for 5 hours with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (2 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich). Cells were fixed and made permeable with the FOXP3 Transcription Factor Staining Buffer Set according to the manufacturer's instruction (eBioscience). Cells were incubated with V450-labeled anti–IFN-γ (B27) from BD Biosciences and PE-labeled anti–TNF-α (MAb11) from BD Biosciences, and then, cells were washed and were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar Inc.).

Tissue culture

We generated stimulated B cells (sBcs) to use as APCs in our MLRs by plating PBMCs on an irradiated feeder layer expressing CD40L and added cyclosporine A (100 μg/μl; Teva Pharmaceuticals) and IL-4 (16 ng/ml; PeproTech) to the culture on day 0, day 1, and every 3 days to deplete T cells. Cells were cultured in Iscove's modified Dulbecco's medium (Gibco) supplemented with 0.1% (v/v) ciprofloxacin (Sicor), plasmocin (5 μg/ml; InvivoGen), gentamicin (50 mg/ml; Gibco), human insulin (10 mg/ml; Sigma-Aldrich), and transferrin (30 mg/ml; Gibco) and containing 10% (v/v) human AB serum (Omega Scientific). At the time of harvest and at other time points, we checked for the purity of the culture for B cells (97 to 99%) and the expression of the activation markers by staining with FITC-labeled anti-CD80 (L307.4; BD Biosciences), PE-labeled anti-CD86 (IT2.2; BD Biosciences), and V450-labeled anti-HLA-DR (BD Biosciences). The expression of costimulatory molecules was equivalent in maternal and fetal sBcs (fig. S6). PHM1–41 cells used in the contractility assays were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 1% (v/v) GlutaMAX (Gibco), penicillin (50 U/ml) and

streptomycin (50 mg/ml), and G418 (0.1 mg/ml; Gibco) and containing 10% (v/v) fetal calf serum (Gibco).

Mixed lymphocyte reactions

Responder PBMCs were first labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (Invitrogen) in PBS at 37°C for 8 min, followed by two washes in RPMI 1640 + 10% fetal bovine serum. Labeled cells were added to 96-well U-bottom plates at a concentration of 150,000 cells per well in 200 μl of RPMI 1640 culture medium supplemented with 2 mM glutamine, 1% (v/v) nonessential amino acids, 1% (v/v) sodium pyruvate, penicillin (50 U/ml), and streptomycin (50 μg/ml) and containing 10% (v/v) heat inactivated Human AB Serum (Omega Scientific). For allogeneic/autologous MLRs, unlabeled sBcs were irradiated with 40 Gray, and 300,000 cells were added to wells for a final responder/sBc ratio of 1:2. The cell lysate was created by sonicating 500,000 PBMCs using the Fisher Model 100 Sonic Dismembrator (Fisher Scientific) with 20 1-s pulses at level 10 and added to wells. Control reactions included culturing with self-lysate only, culturing with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml), or third-party control. In some cases, low blood volume precluded performing all control reactions on a given pair. The cultures were harvested after 4.5 days and stained with the indicated antibodies. The cytokine-producing capacity of lymphocytes was assayed after incubation for 6 hours with brefeldin A (10 μg/ml; Sigma-Aldrich).

Uterine contractility assays

Collagen gels for contraction assays were prepared in 24-well plates at a concentration of 15 × 10^4 cells per well, following the manufactured instructions from the Cell Biolabs Inc. kit. The area of collagen gels embedded with PHM1–41 cells [a human myometrial cell line obtained from American Type Culture Collection (CRL-3046), as referenced in (47)] was assessed every 24 hours after treatment with medium, recombinant human TNF-α (10 ng/ml; PeproTech), recombinant human IFN-γ (10 ng/ml; PeproTech), and 15 × 10^4 sorted $CD4^+$ or $CD8^+$ T cells in the presence or absence of a blocking antibody for TNF-α (10 μg/ml; MAB610, R&D Systems) or IFN-γ (10 μg/ml; MAB285, R&D Systems). Gel treatments were performed in duplicates. Gels were visualized and photographed using an Olympus MVX10 microscope. The gel areas were calculated blindly using ImageJ software.

In utero injections

C57BL/6 fetal mice were injected with PBS, recombinant murine TNF-α (10 ng per fetus; PeproTech), recombinant murine IFN-γ (50,000 U per fetus; PeproTech), or 50 × 10^4 T cells. These T cells were sorted from the spleens of C57BL/6 or TNF-$\alpha^{-/-}$ or IFN-$\gamma^{-/-}$ female mice and were either nonactivated or activated [using anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) stimulation] for 4 days. The injections were made directly into the fetal liver (5 μl per fetus) using pulled glass micropipettes on E14.5, as previously described (20, 49). Briefly, mothers were anesthetized, and a laparotomy was performed to expose the uterus. The fetuses were injected individually through the translucent intact uterus. The laparotomy was closed in layers, and the survival of the pups was evaluated by monitoring daily for birth. Negative control mice were injected with PBS (5 μl per fetus).

Statistical analysis

Statistical analyses and graphs were carried out using Prism 5 software (GraphPad Software) or Stata version 13 (StataCorp LP). Nonparametric data were compared using the Mann-Whitney test (for paired samples) or a nonparametric analysis of variance (for groups of samples) after a normality test was performed. For multiple categories, one-way ANOVA was used with Bonferroni correction. Spearman's rank correlation test was used to assess the relationships between T cell subsets and maternal microchimerism. $P < 0.05$ was considered statistically significant. For the HLA analysis, we used Fisher's exact test to test for differences in histocompatibility using both approaches between PTL cases and controls. $P < 0.05$ was considered significant. To account for the possibility of confounding by ancestry, we conducted multidimensional scaling (MDS) analysis using markers across the genome. As a sensitivity analysis, we used logistic regression models to adjust for the first three components obtained from our MDS analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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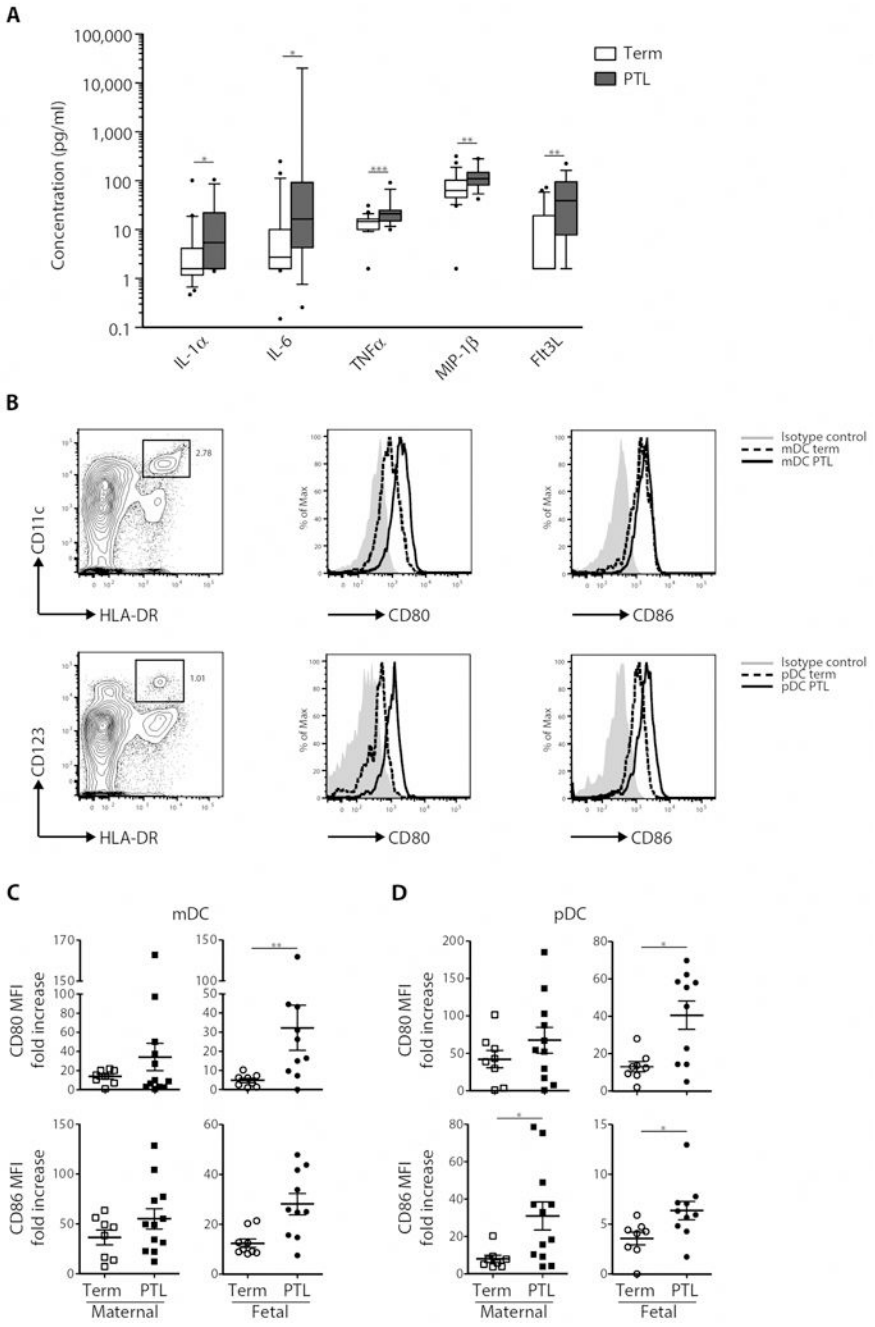

Figure 1.

Elevated levels of inflammatory markers in the fetal plasma and increased expression of activation markers on fetal DCs during PTL. (A) Luminex analysis of cytokines present in the cord blood plasma of infants born at term (term; n = 28) or preterm (PTL; n = 18). The horizontal line represents the median for each group. *P < 0.05, **P < 0.01, and ***P < 0.001 by Mann-Whitney test. Flt3L, Flt3 ligand. (B) Representative flow cytometric analysis of purified cord blood mononuclear cells from preterm infants stained to detect mDCs ($CD11c^+HLA\text{-}DR^+$) and pDCs ($CD123^+HLA\text{-}DR^+$) after gating out $CD3^+$ (T cells), $CD19^+$, and $CD20^+$ (B cells). On the right, representative histograms of CD80 and CD86 expression

on mDC and pDC in the cord blood of term (term) or preterm (PTL) infants. (C) Pooled data of the mean fluorescence intensity (MFI) of the activation markers CD80 and CD86 on maternal and cord blood mDC. **P < 0.01 by Mann-Whitney test. (D) Pooled data of the MFI of the activation markers CD80 and CD86 on maternal and cord blood pDC. The relative MFI was calculated over the intensity of an isotype control antibody. Each symbol represents a single patient; small horizontal bars indicate the mean ± SEM. Healthy term maternal samples (term), n = 8; healthy term fetal samples (term), n = 13; preterm maternal samples (PTL), n = 12; preterm fetal samples (PTL), n = 16. *P < 0.05 by Mann-Whitney test.

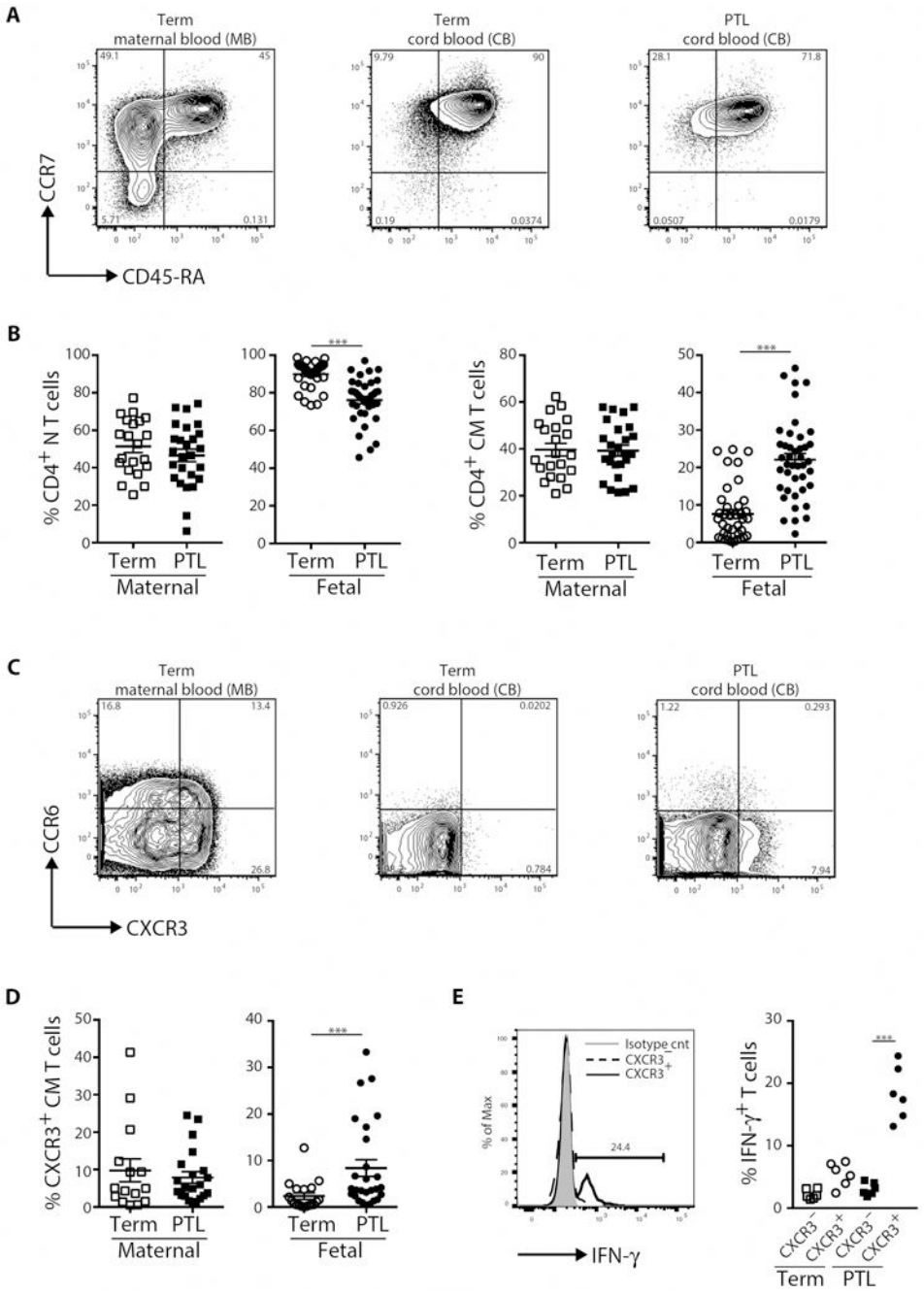

Figure 2.

Increased percentages of CM T cells with a TH1 phenotype in cord blood during PTL. (A) Representative flow cytometric analysis of purified maternal and cord blood PBMCs from healthy term controls (term) and patients with PTL (PTL) stained to detect $CD4^+$ naïve (N; $CCR7^+CD45RA^+$), $CD4^+$ EM ($CCR7^-CD45RA^-$), and $CD4^+$ CM ($CCR7^+CD45RA^-$) T cells. Numbers in quadrants indicate the percentage of cells in each. (B) Pooled data of the percentage of $CD4^+$ N cells and $CD4^+$ CM cells among the total $CD4^+$ T cells in maternal or cord blood. Each symbol represents a single patient; small horizontal bars indicate the mean ± SEM. Term, n = 35; PTL, n = 37. ***P < 0.001 by Mann-Whitney test. (C) Representative

flow cytometric analysis of purified maternal and cord blood PBMCs from healthy term controls (term) and patients with PTL (PTL) stained to detect T_H1 cells ($CXCR3^+CCR6^-$) and T_H17 cells ($CXCR3^-CCR6^+$). Numbers in quadrants indicate the percentage of cells in each. (D) Pooled data of the percentage of T_H1 cells among CM cells in maternal or cord blood. Each symbol represents a single patient; small horizontal bars indicate the mean ± SEM. Term, n = 19; PTL, n = 27. ***P < 0.001 by Mann-Whitney test. (E) Left: Representative histogram of intracellular cytokine staining for IFN-γ in $CD4^+$ CM T cells isolated from the cord blood of a preterm infant by cell sorting according to CXCR3 expression and then stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 hours. Right: Pooled data of the percentage of IFN-γ-secreting cells among $CXCR3^-$ and $CXCR3^+$ sorted cells from the cord blood of term and PTL patients. Each symbol represents a single patient; small horizontal bars indicate the mean ± SEM. Term, n = 6; PTL, n = 6. ***P < 0.001 by Mann-Whitney test.

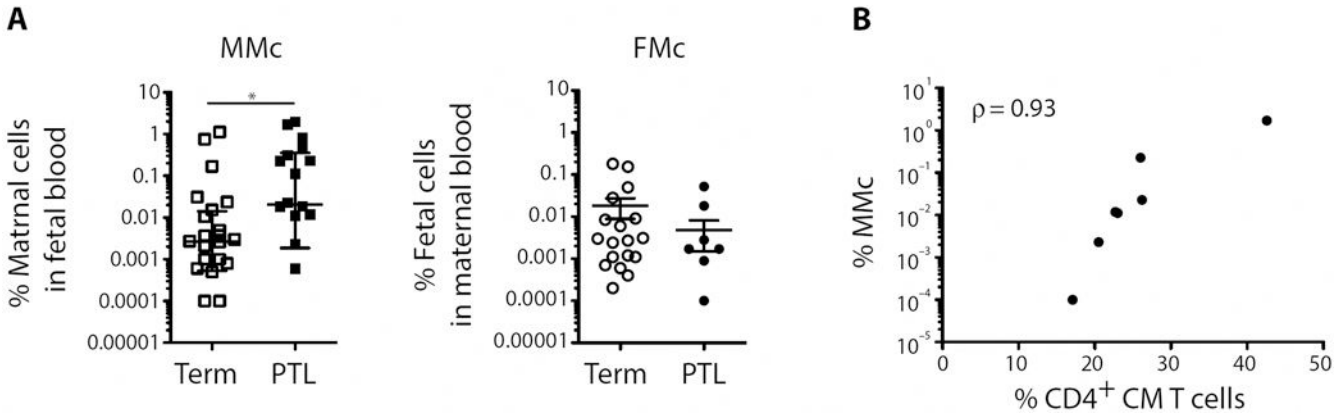

Figure 3.

Increased maternal microchimerism in preterm infants and correlation with CM cells. (A) Maternal microchimerism (MMc) in cord blood of preterm infants (PTL; n = 18) and healthy term controls (term; n = 24), and fetal microchimerism (FMc) in the peripheral blood of women with PTL (PTL; n = 16) and healthy term controls (term; n = 25). Each symbol represents a single patient; small horizontal bars indicate the mean ± SEM. *P < 0.05 by Mann-Whitney test. (B) Correlation between the levels of MMc and CM $CD4^+$ T cells in the cord blood of preterm infants ($\rho = 0.93$, P = 0.007 by Spearman's rank correlation test) (n = 7 samples with both MMc and CM data).

A

MLR components

	Responder T cells	APC	Antigen
Fetal T cells vs. maternal antigens	F	F	M
Fetal T cells vs. 3rd-party antigens	F	F	3rd
Maternal T cells vs. fetal antigens	M	M	F
Maternall T cells vs. 3rd-party antigens	M	M	3rd

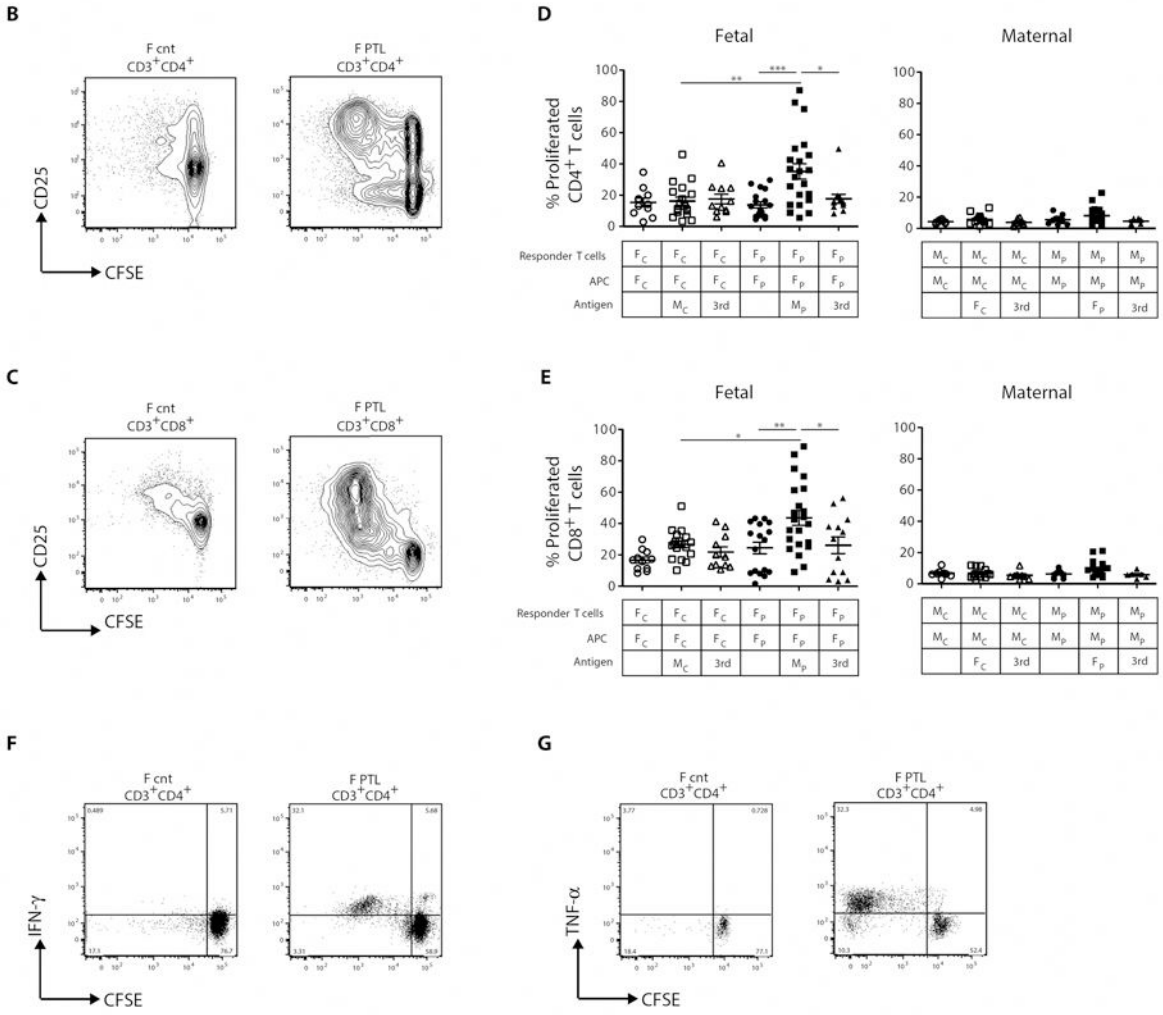

Figure 4.

Increased proliferation of fetal CD4$^+$ and CD8$^+$ T cells with indirect reactivity against maternal antigens during PTL. (A) Schematic representation of the components of the MLR indirect pathway assay. (B and C) Representative flow cytometric analysis of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4$^+$ T cells (B) and CD8$^+$ T cells (C) from the cord blood of control (term) or preterm (PTL) infants, cultured with autologous APC in the presence of maternal lysate. (D and E) Pooled data of the percentages of proliferated maternal and fetal CD4$^+$ (D) and CD8$^+$ (E) T cells cultured with autologous APC in the presence or absence of maternal or fetal lysate, respectively, and a third-party

lysate derived from adult women of reproductive age, as indicated under each graph. Open symbols represent term samples; filled symbols represent PTL samples. Each symbol represents a single patient; small horizontal bars indicate the mean ± SEM. M, maternal; F, fetal. $n \geq 4$ in ≥11 representative experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 by Mann-Whitney test. (F and G) Left: CFSE dilution profile and IFN-γ (F) and TNF-α (G) production in fetal $CD4^+$ and $CD8^+$ T cells in a representative term and a preterm infant. Right: Pooled data are also shown. $n \geq 4$ in ≥3 representative experiments. *P < 0.05 and **P < 0.01 by Mann-Whitney test.

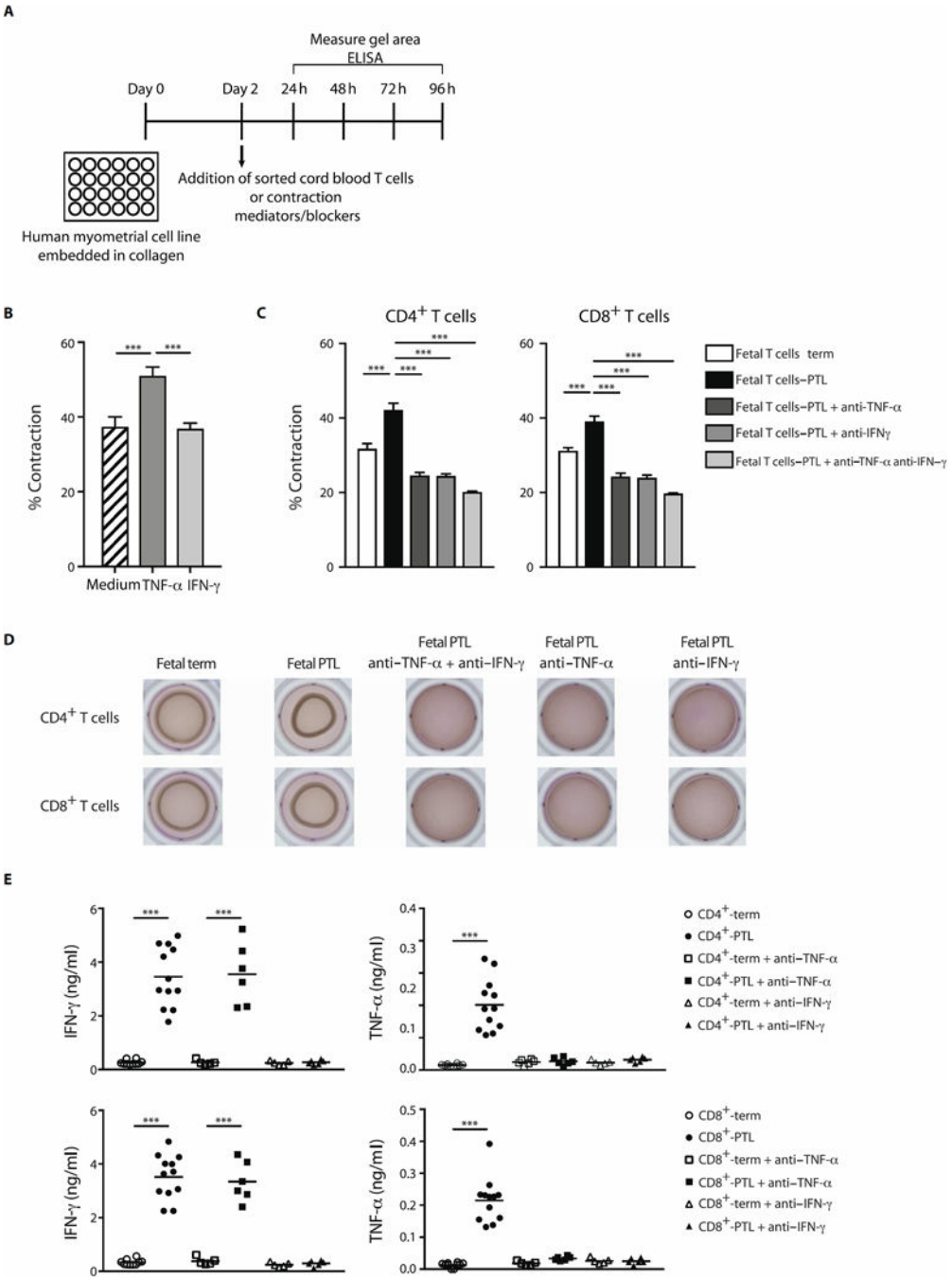

Figure 5.

Increased myometrial cell contractility in response to fetal T cells from preterm infants. (A) Experimental design. Uterine myometrial cells (PHM1–41) were embedded in collagen in a 24-well plate and incubated for 2 days at 37°C. Contraction mediators/blockers or sorted fetal $CD4^+$ and $CD8^+$ T cells from the cord blood of healthy term controls (term) or preterm (PTL) infants were added to the cultures before releasing the stressed matrix. The gel size was measured at the indicated time points. (B) Representative graph of the percentage contraction of PHM1–41 cells embedded in collagen gel matrices treated with medium,

TNF-α, or IFN-γ at 48 hours (n = 8). (C) Representative graph of the percentage contraction of PHM1–41 cells embedded in collagen gel matrices cocultured with fetal $CD4^+$ or $CD8^+$ T cells isolated from the cord blood of healthy term controls (term) or preterm (PTL) infants, either nontreated or treated with neutralizing antibodies for TNF-α, IFN-γ, or both, at 48 hours (n = 6). Small horizontal bars indicate the mean ± SEM. (D) Representative images of gels at 48 hours for different tested conditions. (E) IFN-γ (left) and TNF-α (right) secretion by $CD4^+$ and $CD8^+$ T cells from term or preterm infants either nontreated or treated with neutralizing antibodies for TNF-α or IFN-γ after 48 hours in coculture with PHM1–41 cells (n = 6). ***$P < 0.001$ by one-way analysis of variance (ANOVA) with Bonferroni correction.

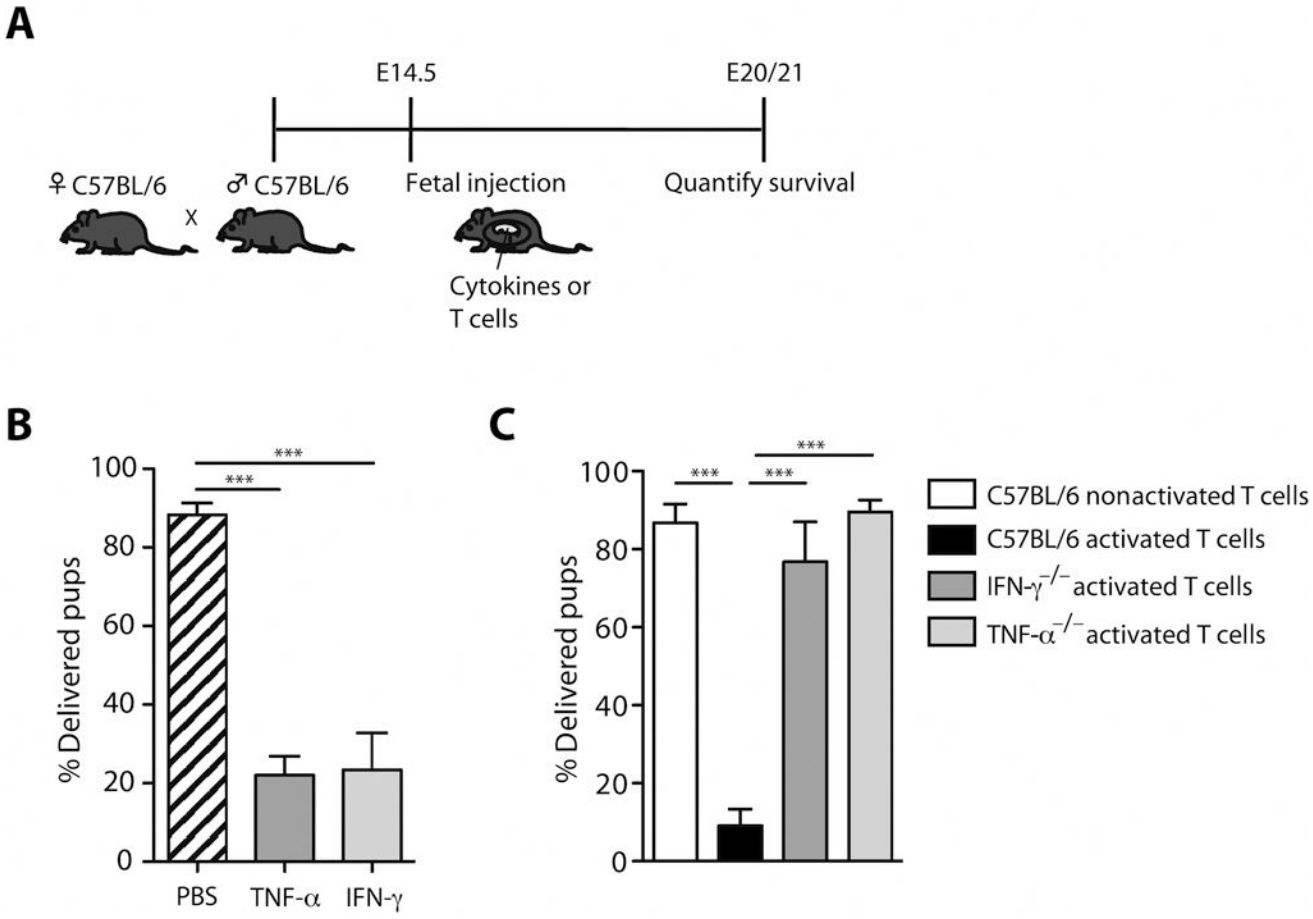

Figure 6.

In utero injection of inflammatory cytokines or adoptive transfer of activated T cells leads to pregnancy loss. (A) Experimental design. Mouse fetuses at E14.5 were injected *in utero* with cytokines or T cells, and the survival rate was quantified at birth. (B) Representative graphs of the percentage of delivered pups after fetal injection with TNF-α or IFN-γ. (C) Representative graphs of the percentage of delivered pups after fetal adoptive transfer of T cells. $CD4^+$ and $CD8^+$ T cells from C57BL/6, IFN-$\gamma^{-/-}$ or TNF-$\alpha^{-/-}$ mice were activated *in vitro* for 3 days with anti-CD3 and anti-CD28 antibodies before fetal injection. Nonactivated T cells from C57BL/6 mice were used as control ($n \geq 4$ experiments, with $n \geq 3$ dams per group). Small horizontal bars indicate the mean ± SEM. ***$P < 0.001$ by one-way ANOVA with Bonferroni correction.