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Maternal Immune Recognition of the Semi-Allogeneic Fetus During Fetal Intervention in Mice

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Maternal Immune Recognition of the Semi-Allogeneic Fetus During Fetal Intervention in Mice

by

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DISSERTATION

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in the

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of the

To the scientists whose mentorship, guidance and scientific brilliance has left a footprint on every page of this dissertation

&

To my parents, Maria and Bogdan, whose love and support is the glue holding these pages together

Thank you

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The postdocs in the lab served many roles throughout my training- mentors, role models and close friends. In particular, Amar Nijagal and Michela Frascoli embodied all of these roles. I emulated Amar's

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While pursuing my graduate education, I explored careers outside of academia and discovered a passion for communicating science to the public. I wanted to thank my committee for being open and supportive of my interests. I also wanted to thank Alex Schnoes and the GSICE program for the mentorship that helped me identify my excitement for science communication. It was the support and mentorship of all of these individuals that led me to create the blog, *StorkPhD*. I am so grateful for all of the support I have received from the UCSF community that allowed me to spend three months working on this blog during a part time internship. I also wanted to thanks Amar Nijagal who introduced me to Seth Bokser with whom I have been collaborating on this blog. Seth is not only a co-founder of *StorkPhD,* but an incredible mentor with admirable goals who always manages to inspire me during each meeting.

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CONTRIBUTIONS

Chapter 1 was written after helpful discussion with Adrian Erlebacher who in particular helped me define "conceptus-derived antigens." Figure 1.1 is a summary of that discussion and was created with the help of Michela Frascoli, a postdoc in the lab.

Chapter 2 is adapted from the publication Wegorzewska et al., 2014, which was published in *The Journal of Immunology* on February 15, 2014 (JI. 2014, 192(4):1938-45). Amar Nijagal was second author and was responsible for using the mouse model of in utero hematopoietic stem cell transplantation (IUHCTx) to describe the role of maternal T cell in the selective loss of the semiallogeneic fetus. Reese Wong assisted in characterizing cell types in the uterus and performing intracellular cytokine staining to study T cell function in the uterus. Tom Le and Ninnia Lescano maintained the animals required to conduct the experiments. Qizhi Tang provided advice and guidance. Tippi MacKenzie supervised this work.

Chapter 3 is the result of the publication Nijagal et al., 2011, led by primary author Amar Nijagal and published in *The Journal of Clinical Investigation* on January 18, 2011 (J Clin Invest. 2011, 121(2): 582–592). Although many experiments were not mentioned here, they contributed to shaping the experiments I contributed to the publication as described in this dissertation. The people who contributed are Amar Nijagal, Erin Jarvis, Tom Le, Qizhi Tang and Tippi MacKenzie. Amar Nijagal and Tippi MacKenzie supervised my contribution.

Chapter 4 is work being prepared for publication. Catherine Tsai made significant contribution to the data discussed in this chapter. She performed many of the fetal injections, diphtheria toxin injections, serum bleeds and alloantibody assays.

ABSTRACT

Maternal Immune Recognition of the Semi-Allogeneic Fetus During Fetal Intervention in Mice

Marta Wegorzewska

The semi-allogeneic fetus derives half of its genetic maternal from the mother. The other half, inherited from the father, leads to the expression of proteins that are foreign to the mother. In danger of potential immune recognition and rejection, the fetus is dependent on maternal immune regulation. Multiple mechanisms are in place to ensure the mother and fetus live in harmony during pregnancy, as outlined in **Chapter one**. This dissertation discusses what happens to some of these mechanisms during fetal intervention in mice.

Chapter two focuses on T cell specific mechanisms that prevent maternal T cell activation during pregnancy. These mechanisms include constraint in antigen presentation to maternal T cells, deletion of maternal T cells aware of fetal antigens and the lack of recruitment of maternal T cells to the uterine environment. Fetal intervention in mice results in enhanced antigen presentation with a reduction in apoptosis of activated cells and a more prominent presence of maternal T cells in the uterus. Maternal T cells also play a role in fetal demise (preterm labor) during fetal intervention.

Chapter three discusses changes in trafficking maternal cells during fetal intervention. Trafficking of maternal cells into fetal blood during normal pregnancy encourages generation of fetal Tregs that can suppress an anti-maternal T cell response. During fetal intervention, the presence of maternal T cells, maternal microchimerism (MMc), increases and may play a role in limiting engraftment of cells transplanted in utero. Because fetal intervention results in preterm labor, changes in maternal

microchimerism during fetal intervention may also play a role in mediating fetal rejection during preterm labor. Using a mouse model of preterm labor, we saw enhanced maternal microchimerism in fetal blood. The contribution of maternal cells in fetal blood to the pathogenesis of preterm labor is an open field for further investigation.

Chapter four deals with the role of maternal Tregs in regulating maternal immune responses during fetal intervention. This chapter more specifically examines maternal antibody production against "conceptus-derived" antigens in the absence of Tregs during normal pregnancy and fetal intervention. Tregs prevent production of maternal antibodies during pregnancy. During fetal intervention, however, maternal antibody production does not increase, even in the absence of Tregs. The presence of "conceptus-derived" antigens in maternal circulation may play a role in preventing detection of maternal antibodies during fetal intervention. Because maternal antibodies do not have an effect on the success of pregnancy, alternative mechanisms may play a role in protecting pregnancy.

This dissertation discusses how the regulatory mechanisms protecting the semi-allogeneic fetus during pregnancy are disrupted during fetal intervention and may play a role in preterm labor. The results discussed in this dissertation provide support for a novel role of the maternal adaptive immune system in the rejection of the fetus during pregnancy complications. Finally, **Chapter five** discusses the future directions that have come about because of this thesis work.

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CHAPTER 1

MATERNAL-FETAL TOLERANCE DURING PREGNANCY

Defining Fetal Antigens

Fertilization of the female egg by the male sperm begins the development of the conceptus. Made up of the fetus and the placenta, the conceptus derives it genetic material from both parents. During pregnancy, the maternal immune system is exposed to "conceptus-derived antigens" [1, 2]. These antigens, although poorly described in the literature, can refer to three classes: 1) placental-specific antigen (**Figure 1.1, red**) 2) minor or major histocompatibility antigens (MHC) inherited from the father and expressed on placental and/or fetal cells (**Figure 1.1, blue/purple**) or 3) fetal-specific antigen (**Figure 1.1, blue**). Currently, studies in the field have shown maternal T cells encounter "conceptus-derived antigens" that gain access to maternal circulation [2]. By mating female mice to Act m-OVA transgenic males, membrane-bound ovalbumin is ubiquitously expressed in the placenta and fetus by an active β-actin promoter [2]. In this study, ovalbumin could be detected in the maternal lymphatic system presumably through shedding of placental antigens from the maternal-fetal interface (discussed in the following section) [2]. Because ovalbumin is a surrogate for both fetal and placental antigens in this system, it is also possible that trafficking fetal cells could also be a source of ovalbumin detected in maternal tissues [3]. No study to date has determined the identity of the antigens, placental and/or fetal, driving maternal T cell awareness during pregnancy. However, it is feasible that in the eyes of the maternal immune system, all three classes of antigen are foreign and have the potential to trigger an immune response. An activated maternal immune response against any of these antigens may make the placenta and/or fetus a target for rejection.

Throughout this thesis, I will refer to the "conceptus-derived antigens" as fetal antigens for simplicity. However, those fetal antigens may be derived from the placenta and/or the fetus.

The Maternal-Fetal Interface

Maternal immune exposure to fetal antigens occurs when the mother makes contact with the conceptus at what is called the maternal-fetal interface [4]. This interface can occur in two places. The first interface is found between the uterus and the placenta. A maternal stromal structure derived from the uterine endometrium provides support for placental development. This structure, the decidua, comes into direct contact for the first time with the trophoblast giant cells (TGC) in the placenta (**Figure 1.2**) [5, 6] providing any maternal immune cells in the decidua access to placental antigens. The second interface is found within the placenta. Mononuclear trophoblasts and a continuous double-layer of syncytiotrophoblasts within the placental labyrinth come into direct contact with the maternal blood cells in sinuses at the maternal-fetal interface (**Figure 1.2**) [5, 6] allowing placental antigen direct access to the maternal circulation.

The maternal-fetal interface exposes the mother to placental antigens that may or may not be similar to the antigens expressed by the fetus (**Figure 1.1**). Access of fetal antigens to maternal circulation is dependent on trafficking of fetal cells into maternal blood, which has been previously described [3]. Although it is still unclear how fetal cells are able to cross into maternal blood, one possibility could involve fetal cells crossing the double-layer of syncytiotrophoblasts in the placental labyrinth that create the separation between the maternal and fetal circulatory systems (**Figure 1.2**)

The Immunology of the Maternal-Fetal Interface

The Uterine-Placental Interface

The uterine-placental interface is established upon implantation of the embryo to the uterine wall. The uterus undergoes decidualization to help support the development of the placenta [7, 8]. Trophoblasts differentiation and invasion of spiral arteries in the decidua occurs to provide maternal blood access to the placenta [4]. This process exposes maternal immune leukocytes in the decidua to semi-allogeneic placental trophoblast cells. In both mice and humans, the predominate populations of leukoyctes represented include NK cells, with restricted access of dendritic cells (DCs) and T cells [9-11].

The decidual natural killer (dNK) cells are defined as $CD56^{bright}$, $CD16^{-}$ NK cells in human and $B220^{+}CD11c^{+}$ NK cells in mouse decidua [9, 10, 12]. Transcriptionally different from peripheral NK cells living in the blood [13], dNK cells appear to be a highly specialized pregnancy-specific cell type important for maintaining pregnancy. Their primary role is to aid in the spiral artery remodeling that allows for a maternal blood source to the placenta (reviewed in [14]). In mice, Madeja et al. showed evidence for the interaction between dNK cells and tropoblast cells to influence decidual vessel remodeling [15].

DCs and T cells have limited access to the decidua during pregnancy. Collins et al. had shown a loss of DC presence upon decidualization in the mouse uterus with DC densities remaining constant in the surrounding myometrium [16]. Similar to DCs, T cell presence in the decidua is also limited. CD4+ and CD8+ T cells each represent about 3% of the leukocyte population in an E8.5 mouse decidua [17]. Their absence is attributed to epigenetic silencing of genes expressing Th1 chemokines including Cxcl9, Cxcl10 and Ccl5 [17]. Given the function of DCs as antigen presenting cells, limiting the presence of DCs and T cells in the decidua minimizes the chances DCs will encounter an antigen and activate a T cell at the maternal- fetal interface.

The Placental Labyrinth Interface

The microenvironment of the placental labyrinth is immunologically complex. During normal pregnancy, specialized maternal leukocytes reside in the placenta suggesting an important role for immune cells in maintaining pregnancy. Leukocyte recruitment to the maternal-fetal interface is dependent on the adhesion molecules and their ligands. In mid-gestation placentas, maternal blood vessels have increased ICAM-1 and VCAM-1 expression, recruiting neutrophils (40-60%), monocytes (25-30%), and some T (15-20%) and B cells (5-8%) to the maternal blood sinuses [18]. Infiltration of leukocytes to the placenta during pregnancy leads to the establishment of a cytokine milieu at the maternal-fetal interface. Chemokines are produced by leukocytes and endothelial cells and are able to induce cellular chemotaxis. Chemokines CXCL8 (IL-8) and monocyte chemoattractant protein-1 (CCL2/MCP-1) are upregulated during pregnancy and have been implicated in recruiting neutrophils and monocytes to the uterus in human pregnancy [19-21].

Mechanisms of T cell tolerance towards the Semi-Allogeneic Fetus

The potential for maternal immune cells to sample fetal antigens at the maternal-fetal interface emphasizes the need for regulation of these cells to prevent or inhibit immune responses against the fetus. The interaction between DCs and T cells has the potential to reject the fetus. A reduced presence of DCs and T cell in the local uterine environment during pregnancy suggests mechanisms are in place to limit their interaction [16, 17].

During pregnancy, it is possible that antigen-presenting cells (APCs) can pick up fetal antigens and activate maternal T cells. The migration of activated T cells to the fetus may result in fetal demise. This scenario is typical of graft rejection after organ transplantation when DCs activated by the presence of antigen in a graft migrate to the lymphatic system to present antigen to T cells. Activated T cells can then migrate out of the lymph nodes and to the graft where effector T cell function is responsible for graft rejection. Scientists have found, however, T cell specific mechanisms limit T cell activation during

pregnancy (reviewed in [1]). Hence, pregnancy is often described as the most successful model of allograft tolerance.

Constraints in Antigen-Presentation

The activation of T cells in the lymph nodes can occur via two pathways, the indirect and direct pathways of antigen presentation (reviewed in [22]). In the context of organ transplantation, the indirect pathway of antigen presentation involves presentation of graft antigens to recipient T cells by recipient APCs. The direct pathway of antigen presentation involves activation of recipient T cells by donor APCs that pick up antigens from the graft. Directly reactive T cells are ten times as frequent as indirectly reactive T cells and pose a greater threat for graft rejection during organ transplantation [22, 23]. Using the Act-mOVA system where expression of transmembrane form of ovalbumin (mOVA) is driven by the β-actin promoter [24], Erlebacher et al. mated females to Act-mOVA males such that the fetuses expressed ovalbumin to show CD4+ and CD8+ T cells proliferate significantly during pregnancy [2]. This T cell activation is restricted to the indirect pathway of antigen presentation (**Figure 1.3, #1**) [2]. Fetal APCs do not activate maternal T cells [2], which would require migration of fetal cells into maternal circulation. Although fetal microchimerism has been reported during pregnancy [3], it is possible that the low frequency of fetal APCs in the maternal circulation is not sufficient to activate maternal T cells. Alternatively, fetal APCs may not be mature enough to present antigen to maternal T cells. The inability of fetal APCs to activate the direct pathway eliminates a major immunological threat to the fetus.

Dendritic Cell Entrapment in the Decidua

Despite constraints in T cell activation during pregnancy, indirectly reactive maternal T cells can become aware of the fetus [2]. Additional mechanisms are required to limit activation of indirectly T cells. One of these mechanisms involves limiting maternal T cell and APC interaction. Activation of indirectly reactive

maternal T cells is dependent on maternal APCs that gain access to and present fetal antigens to T cells in the lymphoid organs. A major source of fetal antigens is found at the uterine-placental interface where the decidua and placenta come into contact. Collins et al. found during pregnancy the density of DCs significantly decreases in the decidua [16]. Reducing the presence of maternal DCs at the interface, reduces the chances DCs will encounter fetal antigens (**Figure 1.3, #2**). If DCs do gain access to the interface, they are trapped in the decidua and prevented from migrating out into the lymphoid organs where they would encounter T cells and induce T cell activation (**Figure 1.3, #2**) [16].

Deletion of Indirectly Reactive T cells

Although the maternal-fetal interface is a major source of fetal antigens, fetal cells can also cross into the maternal circulation [3]. Patrolling DCs that encounter fetal antigens will present the antigen to maternal T cells in the lymphoid organs. Evidence shows robust proliferation of indirectly reactive T cells during pregnancy [2]. However, these proliferating cells are poorly priming. Impaired cytokine and CD62L and CD25 expression results in reduced accumulation in the secondary lymphoid tissues [2]. To prevent activation, maternal T cells exposed to fetal antigen via the indirect pathway of antigen presentation get deleted (**Figure 1.3, #3**).

Expansion of Regulatory T cells with Fetal Specificity

An alternative mechanism involved in inhibit T cells priming includes immunosuppression by regulatory T cells. Studies in mice have revealed a systemic increase in Tregs associated with pregnancy [25]. Using two different systems, Rowe et al. and Samstein et al. found Tregs generated during pregnancy are antigen-specific [26, 27]. Depletion of Tregs during pregnancy resulted in an increase in fetal loss with higher rates seen in allogeneic compared to syngeneic pregnancy suggesting that Tregs may play a role in protecting the fetus in an antigen-specific manner (**Figure 1.3, #4**) [25-29].

Chemokine Gene Silencing to Prevent T cell Migration to the Decidua

Numerous mechanisms exist to prevent activation of maternal T cells during pregnancy. On the chance that T cells escape regulation and get activated, mechanisms are required to prevent effector T cells from attacking the fetus. Erlebacher et al. found artificial priming of anti-OVA+ CD8 T cells during pregnancy by anti-CD40 antibodies and poly(I:C) did not affect the survival of Act-mOVA+ fetuses suggesting the fetus is protected from activated maternal T cells [2]. Indeed, local mechanisms protect the maternal-fetal interface from maternal T cell attack. Nancy et al. showed the mouse decidua limits maternal T cell access [17]. The maternal decidua in the mouse does not express the genes encoding for key T cell-attracting chemokines such as CXCL9, CXCL10 or CCL5 [17]. The lack of transcription expression of the genes is to due to the epigenetic silencing of the genes which limits T cell access to the maternal-fetal interface in the decidua (**Figure 1.3, #5**) [17].

Disruption of Maternal-Fetal Tolerance During Pregnancy

Preterm Labor

Preterm labor (PTL) is the biggest cause of neonatal mortality and childhood morbidity [30]. The rate of PTL (birth prior to 37 weeks gestation) is 12-13% in the US, rising from 9.5% in 1981. Surviving babies face long-term health problems, including neurodevelopmental impairments such as cerebral palsy and language and learning disabilities [31]. The leading cause of PTL is inflammation and infection: 85% of cases are associated with chorioamnionitis [32] [33].

The Role of the Innate Immune System During Preterm Labor

Inflammation-induced PTL occurs when microorganisms entering through the vagina or maternal circulation gain entry into the sterile uterine environment by moving across the placenta or fetal membranes (chorion and amnion) [34]. The innate immune system plays an important role in the pathogenesis of PTL (reviewed in [35]). It can identify the presence of non-self pathogens through

pattern recognition receptors (PRR), inducing an inflammatory response. Inflammation-associated PTL in women results in increased mRNA levels of the PRR, Toll-like receptor 4 (TLR-4), in the chrioamniotic membranes [36]. TLR-4 recognizes lipopolysaccharide (LPS), a component of Gram-negative bacterial cell wall, to induce PTL in mice [37]. LPS signaling through TLR-4 activates the pro-inflammatory NF-kB signaling pathway in the maternal uterus and increases pro-inflammatory cytokines [38]. The inflammatory response helps to deliver cells that release antimicrobial peptides and cytokines, inhibits spreading of the infection, and aids in repairing damaged tissue. However, if exaggerated, the inflammatory response and its cellular and molecular mediators can result in tissue damage and diseases [39]. Despite evidence implicating the innate immune response during preterm labor, a role of the adaptive immune response in the pathogenesis of preterm labor has not been established. This thesis work is based on the questions, is there an adaptive immune response component to preterm labor in which maternal immune system rejects the fetus?

Trafficking of Maternal Cells During Pregnancy

Maternal microchimerism (MMc) or the presence of maternal cells in fetal blood has been detected in fetuses during normal pregnancy [40]. Billigham et al. defined the concept of acquired tolerance after in utero exposure of fetuses to foreign antigens [41] suggesting that MMc should induce fetal tolerance to maternal antigens. Mold et al. showed that a correlation exists between trafficking maternal cells and the development of fetal Tregs, suggesting the importance of acquired tolerance for maintaining pregnancy [42]. In healthy adults, maternal cells has been shown to persist as various immune cell types [43]. Maternal cells have also been identified in children with Type I diabetes [44, 45]. Although MMc has been suggested to play a role in maintaining tolerance during pregnancy, the role of trafficking maternal cells in disease states is unclear.

Fetal Surgery Results in Preterm Labor in Humans

Preterm labor is a major complication of fetal surgery. Fetal surgery started at the University of California, San Francisco (UCSF) to treat fatal or severe fetal congenital anatomic anomalies which could be corrected surgically [48]. A clinical trial performed at three maternal-fetal surgery centers including UCSF compared outcomes in prenatal versus postnatal surgery in children with Mylomeningocele (MMC, known as spinal bifida) [49]. MMC is a neural tube defect which results in diminished lower extremity motor function, bowel and bladder control, and cerebral fluid buildup in the brain that requires shunt placement to drain the cerebral fluid. The rationale for fetal repair is to protect the spinal cord from trauma during the fetal period and improve motor and neurologic outcomes. The clinical trial showed prenatal surgery improved neurological functions and decreased a need for shunt placement compared to postnatal surgery [49]. However, the clinical trial reported a major limitation of fetal surgery to be PTL [49]. Although patients undergoing fetal surgery received tocolytic therapy to delay PTL the average gestational age in the prenatal group receiving fetal surgery was reported to be 34.1 weeks and 13% of the fetuses delivered before 30 weeks of gestation [49]. Open fetal surgery is a valid model of PTL in which to study maternal-fetal tolerance.

Developing a Mouse Model of Fetal Surgery

We have developed a model of fetal surgery in the mouse to study the adaptive immune system during PTL (**Figure 1.4**). Figure 1.3 shows a picture of a fetal surgery performed on an anesthetized mouse. A laparotomy is performed to expose the uterus temporarily. The mouse uterus, unlike the human uterus, carries numerous fetuses ranging anywhere from 5-13 fetuses. A pulling machine is used to make a fine needle that is then attached to a microinjector allowing 5 ul of liquid to be injected directly into the fetal liver. We inject the fetal liver on E13.5-E14.5, a point in gestation where the uterus is transparent and the fetal liver is clearly visible. Using this system, we have injected hematopoietic cells, PBS or LPS to study tolerance induction, surgical trauma and surgical trauma in the context of inflammation, respectively. Fetal surgery in mice results in the resorption of the fetuses. Depending on type of

injection (cells, PBS or LPS), the rates of fetal loss vary and will be discussed in more detail in Chapter 2. However, it is important to note that fetal surgery in mice often results in resorption, which is different from what is seen in humans. This difference emphasizes the need to study the role of the maternal immune response on human samples from women with fetal surgery induced preterm labor. To study preterm labor in mice, LPS is injected into the uterus between the first and second fetus of the right horn in an previously established model [50] and will be discussed in detail in Chapter 3.

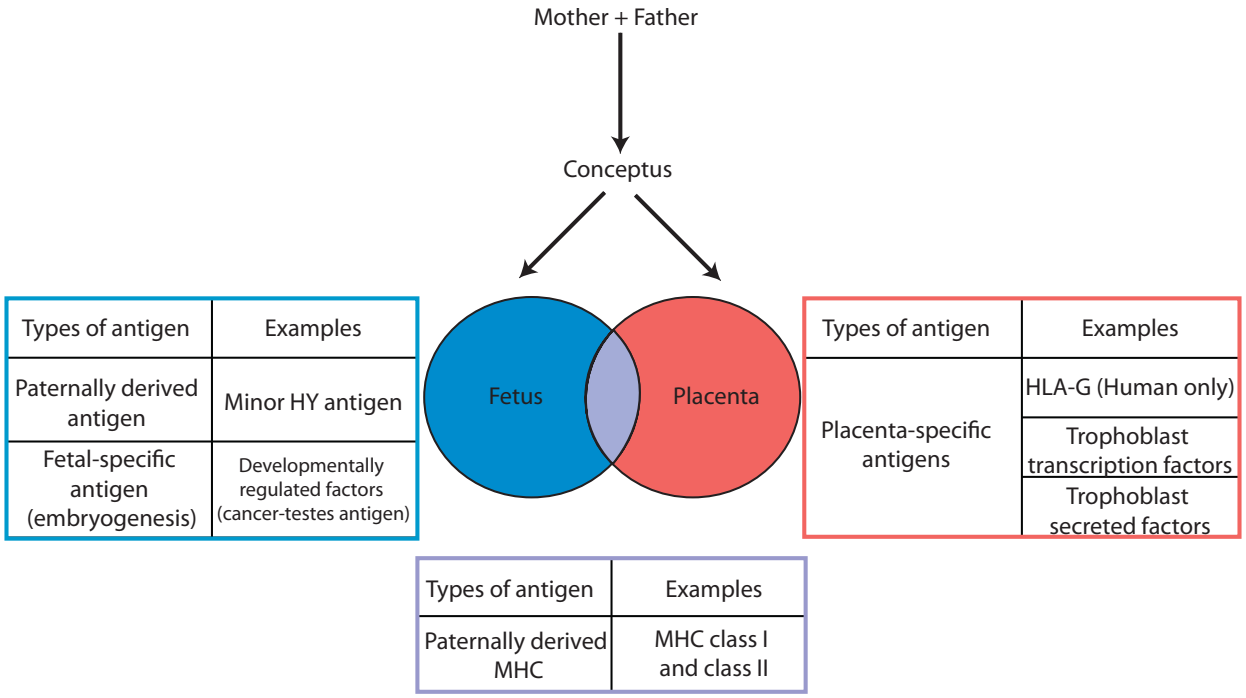

Figure 1.1. Potential placental and fetal antigens expressed during pregnancy. During pregnancy, the conceptus is made up of the fetus (blue) and placenta (red). The genetic material driving the expression of antigens expressed on the conceptus is derived from the mother and the father. These antigens can include 1) placental-specific antigens (red table) that are only expressed on the trophoblast cells of the placenta 2) paternal minor histocompatibility antigens (blue table) such as HY antigens or fetal-specific antigens (blue table) that can be expressed throughout embryogenesis or 3) Paternal minor and major histocompatibility antigens (purple table) expressed on both the fetus and placenta. *This figure was created with Michela Frascoli thanks to helpful discussion with Adrian Erlebacher.*

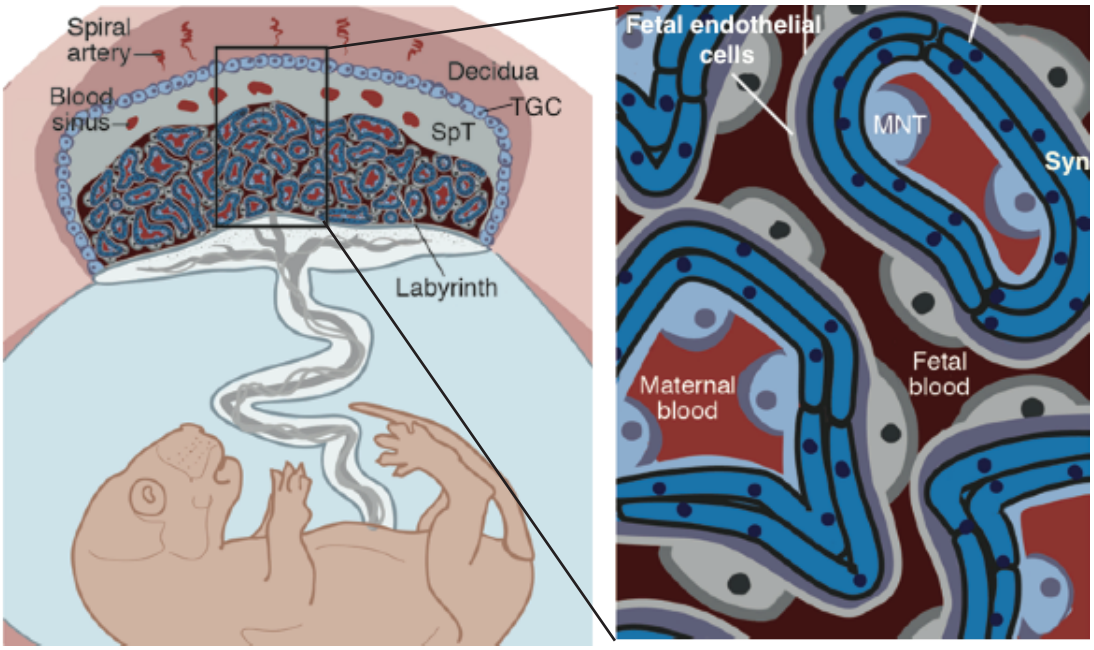

Figure 1.2. The Maternal-fetal interface in the mouse placenta. A schematized cross-section of a pregnant uterus (left) and a close up of the placental labyrinth (right) are shown. The mother and fetus come into direct contact through two surfaces. The first is located at the interface between the decidua, a maternal stromal structure derived from the uterine endometrium, and trophoblast giant cells (TGC) of fetal origin. The second is found in the placental spongiotrophoblast layer where fetal mononuclear trophoblasts (MNT) are found in direct contact with the maternal blood. Adapted from Maltepe et al., 2010.

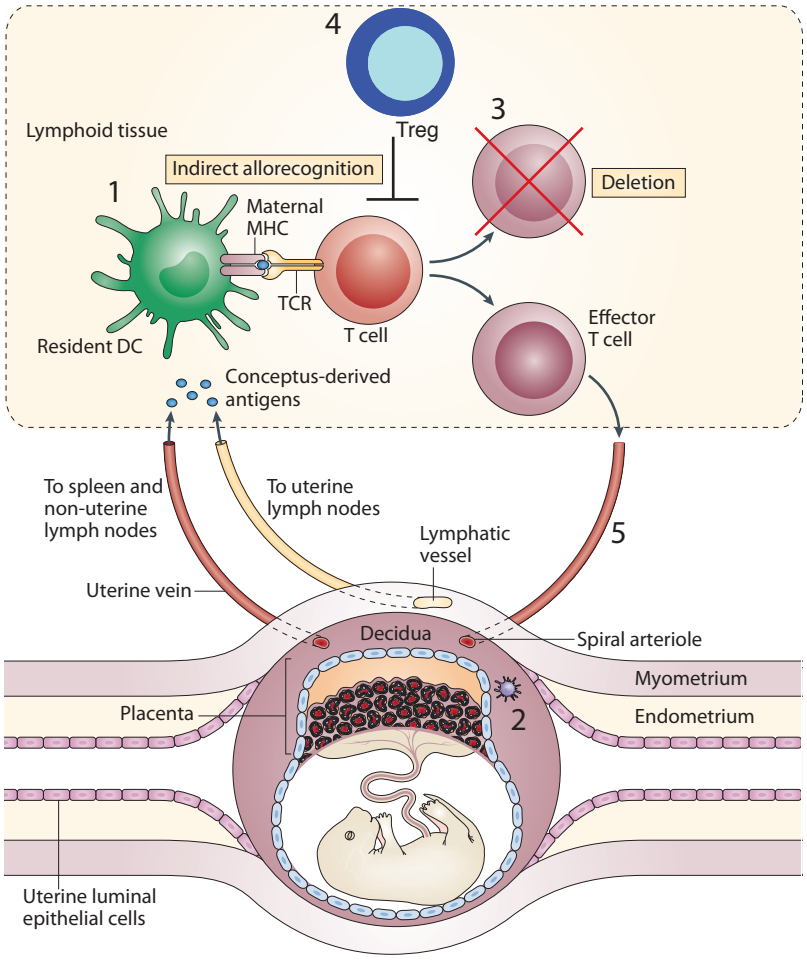

Figure 1.3. Mechanisms of T cell tolerance towards the semi-allogeneic fetus. Numerous mechanisms protect the fetus from T cell mediated rejection. 1) Maternal T cell activation is limited to the indirect pathway of antigen presentation where maternal APCs activate maternal T cells. 2) Entrapment of DCs in the decidua prevents migration of DCs to the lymphoid nodes. 3) T cells activated via the indirect pathway undergo deletion. 4) Maternal regulatory T cells specific for the fetus expand and are important for maintaining a successful pregnancy. 5) T cell recruitment to the maternal-fetal interface in the decidua is limited by the epigenetic silencing of genes expressing Th1 specific chemokines. Adapted from Erlebacher et al., 2013.

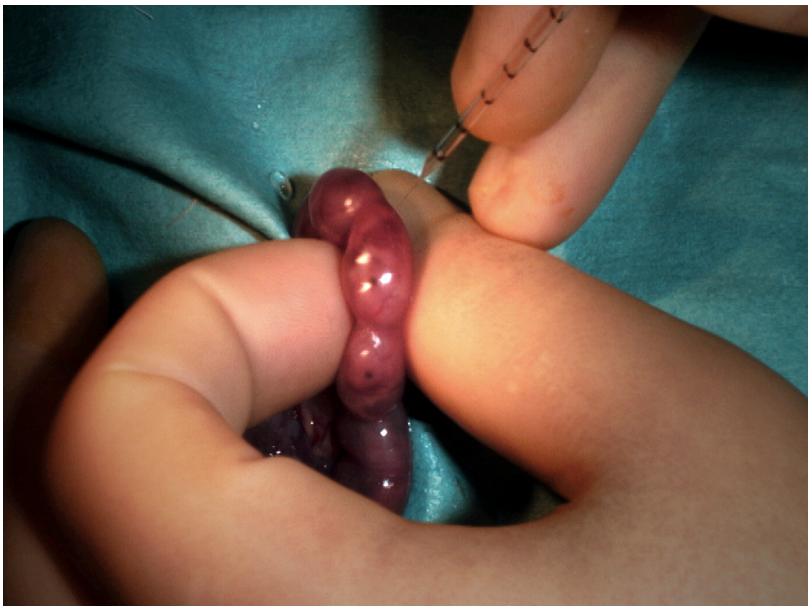

Figure 1.4. Mouse model of fetal surgery. A photo taken while performing fetal surgery on an anesthetized mouse. A laparotomy was performed on the pregnant dam to temporarily remove the uterus carrying numerous fetuses. Fetal mice were injected with PBS, LPS, or hematopoietic cells (5 ul/fetus) directly into the fetal liver using pulled glass micropipettes on embryonic day (E)13.5-14.5.

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CHAPTER 2

MATERNAL T CELLS EXACERBATE FETAL DEMISE AFTER FETAL INTERVENTION

ABSTRACT

Fetal interventions to diagnose and treat congenital anomalies are growing in popularity but often lead to preterm labor. The possible contribution of the maternal adaptive immune system to post-surgical pregnancy complications has not been explored. Fetal intervention in mice increases maternal T cell trafficking into the fetus. We hypothesized that this process may also lead to increased maternal T cell recognition of the foreign conceptus and subsequent breakdown in maternal-fetal tolerance. We show fetal intervention in mice results in accumulation of maternal T cells in the uterus and these activated cells can produce effector cytokines. In adoptive transfer experiments, maternal T cells specific for a fetal alloantigen proliferate after fetal intervention, escape apoptosis, and become enriched compared to endogenous T cells in the uterus and uterine-draining lymph nodes. Finally, we demonstrate such activation and accumulation can have a functional consequence: in utero transplantation of hematopoietic cells carrying the fetal alloantigen leads to enhanced demise of semiallogeneic fetuses within a litter. We further show maternal T cells are necessary for this phenomenon. These results suggest fetal intervention enhances maternal T cell recognition of the fetus and that T cell activation may be a culprit in post-surgical pregnancy complications. Our results have clinical implications for understanding and preventing complications associated with fetal surgery such as preterm labor.

INTRODUCTION

Fetal surgery is a promising strategy to treat fetuses with severe or fatal congenital anatomic anomalies such as diaphragmatic hernias or spina bifida (1). Beyond these conditions, fetal stem cell transplantation has the potential to cure congenital immunodeficiencies and hematopoietic stem cell disorders (2). However, fetal intervention is often associated with preterm labor (PTL), a complication that severely limits the widespread use of this approach (3). Clinical trials of fetal surgery have consistently demonstrated that frequent and severe PTL dampens much of the therapeutic benefit of the fetal intervention (4, 5). While PTL is a complication that curtails our ability to offer fetal treatments for congenital anomalies, the precise mechanisms that lead to PTL after surgery are poorly understood.

Pregnancy is the most robust form of allograft tolerance and multiple mechanisms protect the semi-allogeneic fetus from the maternal immune system (reviewed in (6-8)). The fetus is specifically protected from maternal effector T cells (Teff) by a unique combination of biological mechanisms that impede Teff function (reviewed in (9)). For example, it has been demonstrated that maternal T cells recognize the fetal allograft primarily using the relatively inefficient "indirect" pathway of antigen presentation (in which fetal antigen is presented by maternal antigen presenting cells, APCs) and that these indirectly-reactive T cells undergo clonal deletion after activation (10). Directly-reactive T cells (which recognize antigen presented by fetal APCs) represent a higher percentage of alloreactive T cells (11) but are not activated in normal pregnancy. Pregnancy is also associated with an increase in maternal regulatory T cells (Tregs) (12-19) whose loss leads to elimination of the semi-allogeneic fetus (13-15, 17-19). However, it is not known whether these maternal-fetal tolerance mechanisms are thwarted during after fetal intervention, leading to recognition and rejection of the fetus by maternal T cells. Since fetal surgery can trigger PTL without overt infection, it is possible that inflammation from surgical trauma can activate maternal T cells.

While patients experience preterm labor after fetal surgery, murine fetal intervention instead results in resorption of some of the fetuses in the litter. Resorption has also been observed during T cell-mediated rejection early in pregnancy in mice (15, 20, 21) but the possible contribution of maternal T cells to resorption after fetal intervention has not been examined. We have previously reported that fetal stem cell transplantation increases maternal T cell trafficking into the fetus and that these T cells limit the engraftment of transplanted cells in mice (22). Given that fetal injection also causes resorption, we hypothesized that maternal T cell activation after fetal intervention could perturb maternal-fetal tolerance, leading to enhanced maternal T cell recognition of the semi-allogeneic fetus and, possibly, increased fetal loss. We therefore tested whether various complementary methods of fetal intervention result in maternal T cell activation and used transgenic tools to study the antigen-specificity of such activation. We demonstrate that after fetal intervention, maternal T cells become activated and accumulate in the uterus, where they assume an effector phenotype. Furthermore, maternal T cells can exacerbate selective demise of allogeneic fetuses when triggered by an additional dose of paternal antigen. These results suggest that medical interventions to inhibit maternal T cells could be beneficial in treating pregnancy complications after fetal intervention.

RESULTS

Fetal PBS injection leads to increased resorption in allogeneic matings compared with syngeneic

We used our established method of fetal intervention (injection into the fetal liver through an intact uterus (22)) to study maternal T cell activation. We bred B6 females to B6 (syngeneic) or BALB/c (allogeneic) males and injected the fetuses with phosphate-buffered saline (PBS) to study the effect of surgical trauma alone, or with lipopolysaccharide (LPS), to study the effect of trauma along with a strong inflammatory stimulus on fetal survival **(Table 2.1)**. Baseline resorption in this allogeneic strain combination is low and we observed increased fetal loss with PBS injection in syngeneic matings compared to no intervention, indicating there is some fetal loss secondary to the trauma of the intervention. However, there was a significantly higher rate of resorption in allogeneic matings compared to syngeneic ($c^2 = 0.04$) with PBS injection, suggesting the contribution of an adaptive immune response to this process. With LPS injection, which provides a stronger innate inflammatory stimulus, there was near-total resorption in most experiments, which precluded discerning a difference between syngeneic and allogeneic matings ($c^2 = 0.16$). We therefore proceeded to define whether T cells become activated in the PBS injection model and to devise other experimental breeding schemes to read out a possible functional effect of such activation.

Maternal Teff accumulate in the uterus after fetal intervention

To determine whether fetal intervention results in expansion and proliferation of maternal lymphocytes at the maternal-fetal interface, we bred B6.CD45.2 females to BALB/c.CD45.1 males, injected fetuses with PBS on E13.5, and phenotyped the maternal lymphocytes in the uterus on E18.5 using flow cytometry (**Figure 2.1A)**. Since some fetuses are resorbed after injection while littermates are not, we analyzed the uterus surrounding resorbed fetuses (“resorbed uterus”) separately from the uterus surrounding live fetuses (“live uterus”) (**Figure 2.1A)**. To further define the maternal T cell population,

we used congenic alleles of CD45 to distinguish maternal and fetal cells when harvesting tissues at the maternal-fetal interface by flow cytometry as previously described (22) (**Figure 2.1A,B**).

We first analyzed the uterine T cell composition to detect changes in effector and regulatory T cell subsets (**Figure 2.1B**). The numbers of conventional Foxp3$^-$ CD4 T cells (Tconv) and CD8 cells increased after fetal intervention, with significant increases in resorbed uteri compared to uninjected (**Figure 2.1C).** We also detected an increase in the number of Foxp3$^+$ CD4 T cells (Tregs) in the resorbed uterus, as has been reported in other models of inflammation (25, 26). In addition, CD25 expression increased on all of these T cells subsets after fetal intervention (**Figure 2.1D,E**). CD25 expression on CD4 T cells further increased in resorbed compared to live uteri, suggesting increased activation of effector cells in this setting (**Figure 2.1E**). When we enumerated CD25$^+$ effector and regulatory CD4 cells in the uterus, we found increases in the number of CD25$^+$ T cells (Teff) in resorbed uteri compared to live uteri, such that the overall Teff/Treg ratio was significantly increased in resorbed uteri (**Figure 2.1F**). Given these increases in cell numbers, we next asked whether proliferation of certain T cell subsets increased after fetal intervention using Ki67 staining. We noted an increase in the proliferation of both Teff and CD25$^+$ Tregs, with a higher proportion of cycling Teff to CD25$^+$ Tregs in resorbed uteri **(Figure 2.1G)**. Collectively, these results indicate that fetal intervention leads to inflammation in the uterus, with an increase in local T cell and Treg activation and proliferation. In resorbed uterine segments, the net effect is a shift in the effector to regulatory T cell balance.

We also analyzed other leukocyte populations in the uterus and found increases in the percentage of Gr-1$^+$ myeloid cells (both Gr1^{low} (monocytes) and Gr1^{high} (neutrophils)) after fetal intervention (**Figure 2.S1**). There were no differences in the percentages of NK cells, B cells, or dendritic cells between groups (**Figure 2.S1**).

Increased IFN-g production by uterine T cells after fetal intervention

To determine whether the increased CD4 T cells in the uterus have functional significance, we next asked whether they produce effector cytokines. We harvested lymphocytes from the maternal uterus and uterine draining lymph nodes (udLNs) after fetal PBS injection, stimulated them with PMA and ionomycin, and stained for the intracellular cytokines IFN-g, TNF-a, and IL-17 (**Figure 2.2A**, IFN-g and TNF-a in a representative experiment shown). We found a significant increase in the percentage of IFN-g producing CD4 T cells and a trend for increased percentage of TNF-a producing cells in the uterus after fetal intervention (**Figure 2.2B**). Interestingly, for both cytokines, the percentage of cytokine-producing cells was significantly higher in the uterus than in the udLNs even in normal pregnancies (**Figure 2.2B**). There were no changes in IL-17 production with fetal intervention (**Figure 2.S2**). Thus, maternal T cells resident in the uterus can assume an effector phenotype after fetal intervention.

We also examined cytokine production (IFN-g, TNF-a, and IL-17) by Tregs to determine whether they also assume an effector phenotype in the context of inflammation. We detected no changes in cytokine production by Tregs for any of the cytokines examined (**Figure 2.S2**).

Fetal intervention results in activation, proliferation, and accumulation of Ag-specific maternal T cells

It is possible that the global T cell infiltration and activation we observed after fetal intervention is secondary to inflammation and that these T cells are not specific for fetal or placental antigens. To determine whether maternal T cell activation after fetal intervention is antigen-specific, we adoptively transferred fetal antigen-specific T cells from T cell receptor transgenic mice and analyzed their proliferation and accumulation in maternal tissues after fetal intervention. Maternal T cells may recognize fetal antigen presented by maternal APCs (indirect pathway) or by fetal APCs (direct pathway). Previous reports showed the predominance of the indirect pathway in normal pregnancy (10), using a model in which the β-actin promoter drives the expression of the fetal antigen (10,17). To quantify

maternal T cell activation to a fetal alloantigen that is endogenously expressed, we used TCR 75 mice (27), which have CD4 T cells that recognize fetal BALB/c class I antigen (H-2K^d) presented by B6 (maternal) APC. We mated B6 females to BALB/c males (or to B6 fathers, as syngeneic controls), adoptively transferred CFSE-labeled TCR75 cells into the dams on E12.5, injected the fetuses with PBS or LPS on E13.5, harvested maternal lymphoid organs on E18.5, and analyzed the proliferation of the transferred TCR75 cells using flow cytometry (**Figure 2.3A**). We first confirmed that the antigen recognized by TCR75 T cells is expressed in BALB/c fetal liver and placenta at E13.5-E14.5 (**Figure 2.3B**), consistent with the detailed analysis of H-2K and H-2D reported previously at E8.5 (28). In normal allogeneic pregnancies without fetal intervention, T cell proliferation was low but detectable in the udLNs and spleens, and even lower in the non-draining lymph node (ndLNs) (**Figure 2.3C**). Interestingly, in normal allogeneic pregnancies, the levels of T cell proliferation varied between litters, with some showing no proliferation and others showing detectable but abortive proliferation; this variation was not dependent on litter size (**Figure 2.S3)** but may instead represent occasional spontaneous resorption.

We next asked whether antigen-specific maternal T cells become activated during fetal intervention. Fetal injection of PBS or LPS consistently led to a significant increase in the percentage of proliferated TCR75 cells (**Figure 2.3D**). Proliferation was antigen-specific, since there was no proliferation in syngeneic pregnancies even in the presence of LPS (**Figure 2.3D,E)**. This TCR model was very sensitive in detecting any fetal antigen release after fetal intervention: TCR75 proliferation was robust with both PBS and LPS injection (**Figure 2.3E and Figure 3.S4**) and these cells proliferated even when no fetuses resorbed (data not shown), suggesting that the injection procedure alone can expose maternal T cells to fetal and placental antigens. Unlike the proliferation pattern observed in the udLNs, TCR75 cells in spleens and ndLNs were either not proliferated or highly proliferated (**Figure 2.3D**), suggesting migration of TCR75 cells that were initially activated and proliferated in the udLNs.

In normal pregnancy, fetal antigen-specific T cells proliferate but fail to become activated or to accumulate, suggesting clonal deletion (10). Given the robust proliferation and expansion we observed, we asked whether apoptosis of TCR75 cells decreases after fetal intervention. We stained TCR75 cells for Annexin V and DAPI at the time of harvest. We found that 37% of the proliferated and 24% of the non-proliferated TCR75 cells in the udLNs of mothers with normal pregnancy were apoptotic (Annexin V^+, $DAPI^-$) but this percentage decreased significantly during fetal intervention (**Figure 2.3F**). Apoptosis decreased only in TCR75 cells in the udLNs, but not in other lymphoid organs (**Figure 2.3F**).

We next asked whether the adoptively transferred antigen-specific T cells accumulate at the maternal-fetal interface after fetal intervention. In normal pregnancy, we detected few TCR75 cells in the uterus. However, both the absolute numbers and the percentage of TCR75 cells among CD4 cells in the uterus increased significantly after fetal PBS injection, indicating preferential recruitment or expansion of these antigen-specific cells over endogenous CD4 cells (**Figure 2.3G**). Similar increases in the proportion of TCR75 cells were seen in the udLNs, but not in placentas, ndLNs or spleens (**Figure 2.3G**). We also detected some TCR75 cells in the decidua, which is normally protected from maternal T cell infiltration (29) (**Figure 2.3G**). Thus, fetal intervention leads to an enrichment of antigen-specific cells in the uterus and udLNs. Collectively, our results suggest that fetal intervention leads to changes in normal tolerance mechanisms that usually prevent proliferation, migration, and accumulation of antigen-specific T cells.

Fetal intervention does not lead to activation of directly reactive maternal T cells

Maternal T cells can also recognize alloantigen presented on donor (fetal) APCs using the "direct" pathway. Although such directly alloreactive T cells constitute a significantly higher percentage of alloantigen-specific T cells compared to indirectly reactive T cells (11), they are not activated in normal pregnancy and the fetus is therefore protected from the majority of maternal antigen-specific T cells (10). We asked whether fetal intervention can increase antigen presentation by fetal APC and thus

enhance maternal recognition of the fetus by directly-reactive T cells, using adoptive transfer of T cells from 4C (30) and 2C (31) mice. 4C mice have CD4 T cells that recognize the BALB/c class II antigen I-A^d while 2C mice have CD8 T cells that recognize BALB/c class I antigen H-$2L^d$, both presented "directly" by fetal BALB/c APC. We adoptively transferred T cells from 4C and 2C mice into B6 mothers bred to BALB/c fathers on E12.5, performed fetal injections on E13.5, harvested lymphoid organs on E18.5, and analyzed the proliferation of antigen-specific T cells using flow cytometry. To maximize the possibility of detecting fetal antigen exposure, we used the fetal LPS injection model, which leads to higher rates of resorption (Table 1). We first analyzed the expression of the antigens recognized by these transgenic T cells in fetal tissues on E13.5-E14.5 and determined that I-A^d (recognized by 4C) is present in the placenta but not in the fetal liver (**Figure 2.4A**), while H-$2L^d$ (recognized by 2C) is present both in the placenta and in the fetal liver (**Figure 2.4D**). We found no increase in the proliferation of 4C (**Figure 2.4B,C**) or 2C (**Figure 2.4E,F**) T cells with fetal intervention over baseline in allogeneic matings in any lymphoid organ. Thus, fetal intervention does not result in direct antigen presentation, either because immature murine fetal APCs lack sufficient MHC expression (32, 33), or because maternal exposure to these antigens is not high enough to trigger activation in this TCR model, even when the majority of the litter is resorbed.

Maternal T cells exacerbate selective loss of semiallogeneic pups after in utero transplantation of additional paternal Ag

Given our findings that maternal T cells are activated and proliferate in the uterus after fetal intervention, we questioned whether these activated maternal T cells could be functional and cause resorption of the fetus in allogeneic matings. To address this question, we used our model of fetal hematopoietic cell transplantation, since we have previously shown that this intervention generates a maternal immune response to transplanted allogeneic cells (22). We reasoned that selective maternal rejection of the fetus could best be triggered by in utero transplantation of the same alloantigen carried

by the fetus, which exposes the mother to a higher dose of the antigen in the context of surgical inflammation. To minimize experimental variability secondary to differences between individual litters, we designed an F1 breeding scheme in which a maternal anti-fetal immune response could be read out as decreased survival of semi-allogeneic pups compared to syngeneic littermates. We bred BALB/c females to B6 x BALB/c (F1) males such that half of the resulting fetuses expressed the foreign paternal antigen H2-K^b and were semi-allogeneic to the mother, while half were syngeneic to the mother (F1 and BALB/c pups, respectively) (**Figure 2.5A**). We recorded both the overall survival to birth as well as the genotype distribution (BALB/c or F1) of surviving pups at baseline and after in utero transplantation of hematopoietic cells testing various experimental conditions (**Figure 2.5B**). Uninjected litters had the expected equivalent survival of BALB/c and F1 pups, with a slight but consistent preference for survival of syngeneic over semi-allogeneic pups (1.2/1 ratio). Transplantation of allogeneic cells from a third party donor (C3H) resulted in some resorption but a conserved 1.2/1 ratio of syngeneic and semi-allogeneic pups. However, after in utero transplantation with hematopoietic cells from B6 mice, which carry the paternal antigen, we observed a striking decrease in the percentage of surviving semi-allogeneic fetuses, resulting in a 2:1 ratio of syngeneic to semi-allogeneic pups. As another control, we performed breedings in which the mother was B6 x BALB/c F1, such that neither the fetuses nor not the transplanted B6 cells are allogeneic to the mother. Consistent with our hypothesis, resorption rates were significantly lower in this group compared to the experimental (paternal antigen transplantation) group, and there was no skewing in the genotype of the litter. These results indicate that there is enhanced resorption of pups expressing the foreign paternal antigen only when the same paternal antigen is transplanted in utero, suggesting that in utero transplantation triggers a maternal adaptive immune response that ultimately results in fetal demise.

We next asked whether maternal T cells, which we have determined to be activated after fetal intervention, were mediating the selective loss of the semi-allogeneic pups after fetal intervention. We

bred BALB/c.TCRa$^{-/-}$ females (which lack T cells) to B6 x BALB/c F1 males and performed in utero transplantation with B6 hematopoietic cells. We found that the survival of semi-allogeneic fetuses was not affected by in utero transplantation when the mother lacks T cells (1.3/1 ratio, **Figure 2.5B).** We have noted high overall rates of surgical complications in immunodeficient dams (including RagKO and B cell deficient mice, data not shown) and therefore do not expect the overall rate of resorption in this maternal strain to be comparable to the other experimental groups. These results are consistent with our observations of increased T cell activation and accumulation in the uterus with fetal surgery and suggest a functional role for maternal T cells in enhancing the loss of pups carrying the foreign transplanted antigen.

MATERIALS AND METHODS

Reagents and antibodies

The following reagents were used: Invitrogen Vybrant® CFDA SE Cell Tracer Kit (CFSE, Invitrogen), Ficoll-Paque Plus (GE Healthcare), Annexin V (BD Pharmingen), Qdot 605 Strepavidin conjugate (Invitrogen), Foxp3 staining buffer set (eBiosciences), 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen), Lipopolysaccharide from Salmonella abortus equi S-form (TLR-grade™) (LPS, Alexis Biochemicals), DAPI vector shield (Vector Laboratories), Paraformaldehyde Aqueous Solution (Electron Microscopy Science), LIVE/DEAD Cell Viability Dye (Invitrogen), DNase I (Roche), Collagenase D (Roche), Alexa Fluor 488 Goat Anti-Rat IgG Antibody (Life Technologies), Triton-x 100 (Sigma), Bovine Serum Albumin (Fisher Scientific), Goat serum (Jackson ImmunoResearch Laboratories, Inc), Sucrose (Fisher Scientific), Tissue-Tek OCT Compound (VWR), Phorbol 12-myristate 13-acetate (PMA) (Sigma), Ionomycin (Sigma), and Brefeldin A (Sigma). The following antibodies for flow cytometry were purchased from Becton Dickinson: CD3 (145-2C11), CD8 (53-6.7), CD19 (1D3), CD45 (30-F11), CD45.1 (A20), CD45R/B220 (RA3-6B2), H-2K^{b} (AF6-88.5), H-2K^{d} (SF1-1.1), H-2K^{k} (36-7-5), I-A^{d} (AMS-32.1), Thy1.1 (HIS51), Vβ8 (F23.1), Vβ13 (MR12-3), Ki67 (B56), NK1.1 (PK136); EBiosciences: CD4 (RM4-5), CD8 (53-6.7), CD45.1 (A20), CD45.2 (104), CD25 (PC61.5), Foxp3 (FJK-16s), IL-17A (eBio17B7), TNF- a (MP6-XT22), IFN-y (XMG1.2), CD11c (N418), IgG2a,k isotype control (eBM2a); Southern Biotech: CD8 (53-6.7); UCSF Hybridoma Core: Gr-1 (RB6-8C5), Fc receptor (2.4G2); Biolegend: CD25 (3C7), H2-L^{d} (28-14-8), IgG2a,k isotype control (MOPC-173); Abcam: CD3 (RM0027-3B19).

Mice

The inbred strains, BALB/c, C3H-HeJCr, C57BL/6.CD45.2 (B6), and the F1 hybrid strain, B6 x BALB/c (F1), were obtained from either NCI or Jackson Laboratories. BALB/c.CD45.1 were obtained from Dr. Michelle Hermiston (UCSF) and B6.Thy1.1.2C (2C), B6.Thy1.1.4C (4C), and B6.Thy1.1.TCR75 (TCR75) mice were

obtained from Dr. Sang-Mo Kang (UCSF). 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. B6 females used were all nulliparous.

In utero injections

Fetal mice were injected with PBS, LPS, or hematopoietic cells (5 ul/fetus in all experiments) directly into the fetal liver using pulled glass micropipettes as previously described on embryonic day (E)13.5-14.5 [24, 35]. The pregnant dam was anesthetized, a laparotomy was performed, and the fetuses were injected through the intact uterus. For LPS injections, a dose of 0.5ug was diluted among the total fetuses in the litter. We based our LPS concentration on a reported model of intrauterine injection of LPS [36] and performed titration experiments to achieve a dose that resulted in the loss of the litter within 40 hours without maternal death (0.5 ug, data not shown). For allogeneic stem cell transplantation experiments, hematopoietic cells were harvested from fetal livers of E13.5-E14.5 donor fetuses as previously described [24] and $2.5x10^6$ cells were injected into each fetus. For experiments involving survival of the semi-allogeneic and syngeneic fetuses, peripheral blood leukocytes were stained for H-2^b and H-2^d to genotype the surviving BALB/c (H-2^d) and F1 (H-2^b/H-2^d) pups at the time of weaning.

Tissue harvesting and processing

Tissues at the maternal-fetal interface were harvested by separating the uterus from the fetus and placenta; deciduas could be further separated from the placentas in live fetuses. Fetal livers were harvested from E13.5–E14.5 fetuses in PBS. Single-cell suspensions were made by gently pipetting the fetal livers and filtering through a 70-µm Nitex filter. Tissues surrounding each fetus were processed separately with DNase I (5 ug/ml) and Collagenase D (400 U/ml) to make a single cell suspension. Cells

were also harvested from spleens, uterine draining lymph nodes (udLNs; para-aortic), and non-draining lymph nodes (ndLNs; inguinal, axillary, brachial and mesenteric). For surface staining experiments, tissues surrounding individual fetuses were each analyzed separately whereas for intracellular cytokine staining and adoptive transfer experiments, all resorbed or all non-resorbed uterine segments within a dam were pooled to obtain adequate cell numbers. After staining with the indicated antibodies, samples were analyzed on a LSRII flow cytometer using FACS Diva or FlowJo software.

Intracellular cytokine stain

Maternal lymphocytes were stimulated with PMA (70 ng/ml) and Ionomycin (70 ng/ml) for three hours, treated with Brefeldin A (200 mg/ml) for two hours, then stained for flow cytometry.

Proliferation of adoptively transferred fetal antigen-specific lymphocytes

Whole lymphocytes were harvested from the spleen and lymph nodes of TCR75, 2C or 4C mice and labeled with CFSE. 1-5x10^6 T cells were adoptively transferred intravenously into pregnant females at E12.5 followed by injection of PBS or LPS into the fetuses one day later. Five days after in utero injection, the dams were sacrificed and the adoptively transferred T cells were identified in the maternal and fetal tissues by their congenic marker, Thy1.1. Positive controls for 2C and 4C proliferation were B6 females sensitized with 5x10^6 BALB/c splenocytes intravenously prior to adoptive transfer for 2C or 4C cells.

Statistics

Differences between two groups were evaluated using either a Chi-square test (c^2, for changes in survival) or a Student's *t* test (or Mann-Whitney test, for non-normally distributed data) and those among more than two groups were evaluated using ANOVA with Tukey's multiple comparison test (or

Kruskal-Wallis with Dunn's post-test, for non-normally distributed data) using Graphpad Prism. P values of less than 0.05 were considered to be significant. Data are summarized as means ± SEM.

DISCUSSION

In this study, we tested the hypothesis that fetal intervention perturbs maternal-fetal tolerance, leading to activation of fetal antigen-specific maternal T cells. We first showed that fetal PBS injection leads to higher rate of fetal resorption in allogeneic matings compared to syngeneic. We then demonstrated that this intervention results in expansion and proliferation of maternal T cells in the uterus, with an increase in local production of IFN-g. Using an adoptive transfer model, we demonstrated that fetal intervention results in activation and proliferation of antigen-specific maternal T cells, which escape apoptosis and accumulate in the uterus. We also showed that maternal T cells can exacerbate demise of semi-allogeneic fetuses after in utero transplantation of additional paternal antigen. The finding that maternal T cells become activated after fetal intervention and can contribute to an adverse outcome suggests that treatments aimed at blocking the maternal adaptive immune response may be useful to treat complications of fetal surgery.

In normal pregnancy, multiple overlapping mechanisms keep maternal T cells in check such as lack of direct antigen presentation (10), physical entrapment of dendritic cells in the uterus (34), chemokine gene silencing (29), and dominant suppression by regulatory T cells (12-19). Fetal intervention may increase antigen presentation to maternal T cells through the release of fetal antigen into the maternal circulation. We have also observed increased trafficking of maternal cells into the fetus after fetal surgery in both this mouse model (22) and in patients (35) and such trafficking may facilitate maternal T cell activation. In addition, bleeding in the uterus after surgical trauma might release the physical entrapment of maternal dendritic cells (34) and upregulate Class II expression on these cells, in addition to recruiting other inflammatory cells such as macrophages. Finally, inflammatory signals may hinder Treg function, as has been demonstrated during Listeria infection (36) or render effector T cells less sensitive to Treg suppression (37).

In many models of pregnancy complications, it is difficult to distinguish the effects of non-specific inflammation from a true antigen-specific immune activation. Resorption in our fetal intervention model is multifactorial and includes a component of non-specific inflammation since there is some baseline resorption in syngeneic matings after PBS injection. We have used several experimental settings to show that fetal alloantigens are critical to the maternal T cell response. First, we noted a significantly higher rate of fetal loss in the allogeneic setting compared to syngeneic in our fetal PBS injections. Second, in our adoptive transfer experiments, we detected TCR75 proliferation only when fetal alloantigen is present, and not in the syngeneic setting. Finally, with the stem cell transplantation experiments, we detected fetal loss only when the fetus carries the same alloantigen that is transplanted into the fetus. These latter experiments were designed to also tease out the possible functional contribution of maternal T cells in enhancing fetal loss after the intervention. Although the degree of inflammation induced by surgery may vary between animals, comparing the survival of syngeneic and semi-allogeneic pups within the same litter allows a more accurate quantification of an allospecific immune response. It is interesting that in spite of the maternal T cell activation and enhanced loss of some of the pups in the litter, the entire litter is not lost, highlighting the importance of local tolerance mechanisms in this complex biological system.

We used several TCR transgenic mice to study maternal T cell recognition of the fetus in the context of fetal intervention. Our results are complementary to the studies of maternal T cell activation against fetal ovalbumin observed at baseline (10) and after infection (36), and provide further information regarding the response to an MHC antigen expressed physiologically. We have also performed a detailed analysis of immune cells in the uterus and udLNs to show that there is antigen-specific T cell infiltration locally after fetal intervention, supporting the concept of maternal rejection of the foreign conceptus. During fetal intervention, maternal T cells can be exposed to fetal antigens that are released from the fetal liver at the time of injection as well as those present in resorbed fetal and placental tissues. We

devised the LPS injection model to mimic a more severe inflammatory insult, such as that seen with a microbial infection after fetal intervention. Chorioamnionitis has been reported in after fetal surgery (4) and its true incidence is likely higher than the reported rate since preterm premature rupture of membranes, seen commonly after fetal intervention, can represent a subclinical infection (38). Although we did not detect activation of directly-reactive T cells, the question of whether this pathway is relevant after clinical fetal surgery remains open. While it has been suggested that the indirect pathway of antigen presentation may be predominant for human pregnancies (39), the gestation period after fetal surgery is longer in patients than in mice and it is possible that human fetal APCs may have enough time to mature and stimulate directly-reactive maternal T cells after surgery.

One limitation of our study is that we could not examine the activation of indirectly-reactive CD8 T cells, which may play a role in fetal rejection, since there is no BALB/c allospecific TCR transgenic model for these cells. CD8 T cells expressing markers of differentiated effector memory cells have been observed in human deciduas and may be controlled locally (40). Our analysis of uterine T cells showed an increase in CD8 cells and both CD4 and CD8 cells are likely involved in a maternal immune response.

Our experiments indicate that surgical inflammation can perturb maternal-fetal tolerance by shifting the Teff/Treg balance, similar to what has been observed after infection-induced immune activation (36). Although the mouse feto-placental unit is significantly different from the human, it is likely that clinical fetal surgery would also expose maternal T cells to fetal and placental antigens and enhance maternal T cell recognition of the fetus. Furthermore, maternal T cells have been observed in fetal tissues of patients with villitis of unknown etiology (41, 42), with coordinate changes in placental chemokines (43), suggesting that T cell activation may be a culprit in other human pregnancy complications.

Fetal intervention is becoming ever more frequent— in addition to surgeries for fatal anatomic abnormalities, it is now common to perform diagnostic and therapeutic interventions for a variety of

reasons. Therefore, determining whether maternal T cells are activated in patients who undergo uterine manipulation has vital clinical significance. Our results suggest that inhibiting maternal T cells may be a therapeutic target for complications of fetal surgery such as preterm labor.

TABLE

Table 2.1 Complementary models of fetal intervention in mice

Models of Fetal Intervention	Rationale	% Resorption	# of litters (L)/# of fetuses (F)
Uninjected	Baseline	Syngeneic - 3.2±2 Allogeneic - 1.5± 1	7L/58F 12L/114F
PBS injection	Sterile inflammation	Syngeneic - 34±7 Allogeneic - 44±6*	20L/155F 43L/355F
LPS injection	Strong inflammatory stimulus	Syngeneic - 90±6 Allogeneic - 79±4	10L/74F 32L/252F

* c^2 = 0.04 between Syngeneic and Allogeneic matings undergoing fetal PBS injection; c^2 = 0.16 between Syngeneic and Allogeneic matings undergoing fetal LPS injection; c^2 = 0.33 between uninjected Syngeneic and Allogeneic matings. n≥4 independent experiments in each group.

FIGURES

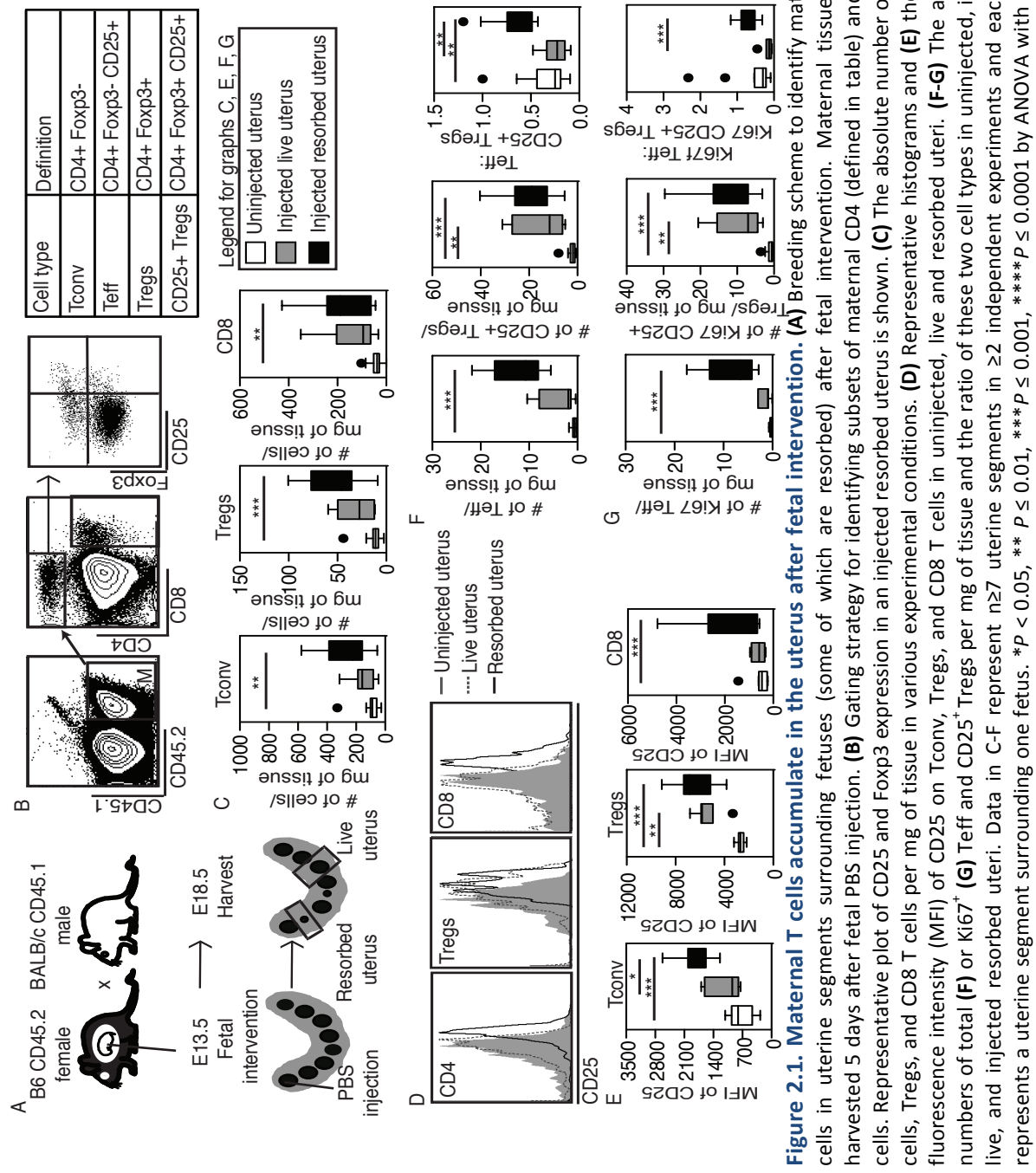


Figure 2.1. Maternal T cells accumulate in the uterus after fetal intervention. **(A)** Breeding scheme to identify maternal T cells in uterine segments surrounding fetuses (some of which are resorbed) after fetal intervention. Maternal tissues were harvested 5 days after fetal PBS injection. **(B)** Gating strategy for identifying subsets of maternal CD4 (defined in table) and CD8 T cells. Representative plot of CD25 and Foxp3 expression in an injected resorbed uterus is shown. **(C)** The absolute number of Tconv cells, Tregs, and CD8 T cells per mg of tissue in various experimental conditions. **(D)** Representative histograms and **(E)** the mean fluorescence intensity (MFI) of CD25 on Tconv, Tregs, and CD8 T cells in uninjected, live and resorbed uteri. **(F-G)** The absolute numbers of total **(F)** or Ki67^{+} **(G)** Teff and CD25^{+} Tregs per mg of tissue and the ratio of these two cell types in uninjected, injected live, and injected resorbed uteri. Data in C-F represent n≥7 uterine segments in ≥2 independent experiments and each point represents a uterine segment surrounding one fetus. *P < 0.05, ** P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by ANOVA with Tukey's multiple comparison test.

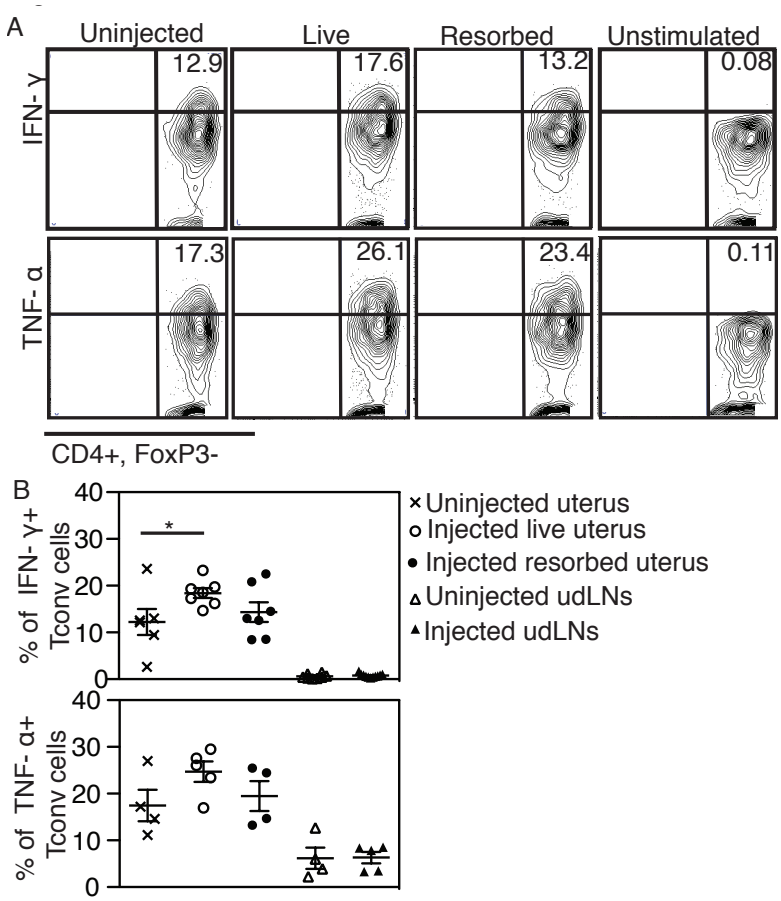


Figure 2.2. Increased IFN-g production by uterine T cells after fetal intervention. **(A)** Representative flow cytometry plots of uterine CD4 Tconv cells after stimulation with PMA and ionomycin to detect the production of IFN-g (top) and TNF-a (bottom). Maternal tissues were harvested 5 days after fetal PBS injection in an allogeneic mating. Percentages obtained in one representative experiment shown. **(B**) The percentage of Tconv cells producing a given cytokine in uterine segments and uterine draining lymph nodes (udLNs) harvested from pregnant dams with or without fetal injection of PBS. Each point represents tissues from one dam. N≥4 dams per group in n≥4 independent experiments. *$P < 0.05$ by ANOVA with Tukey's multiple comparison test.

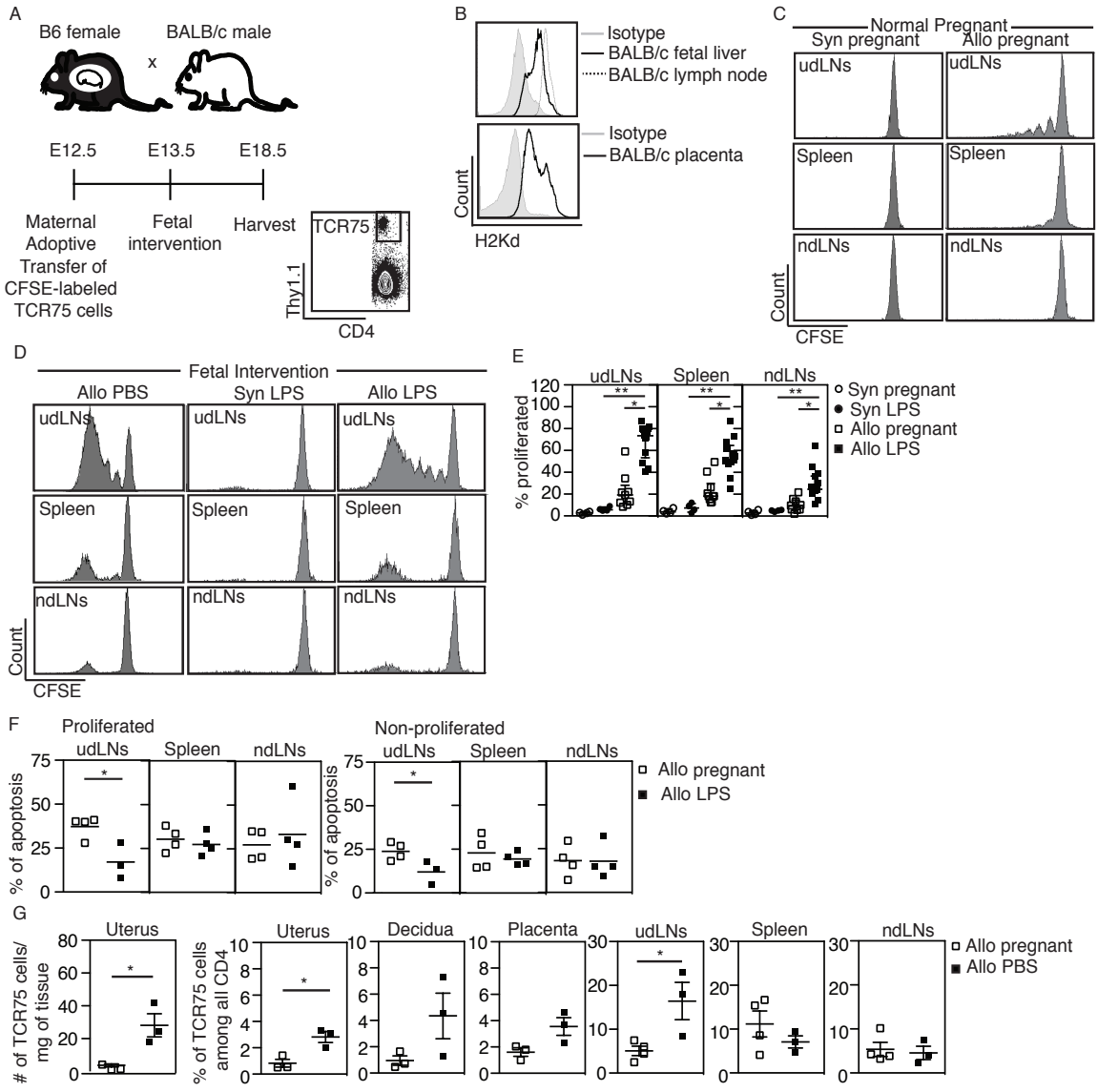


Figure 2.3. Fetal intervention results in activation, proliferation, and accumulation of antigen-specific maternal T cells. (A) Experimental design and gating strategy to track proliferation of adoptively transferred fetal antigen-specific TCR75 ($CD4^+$ $Thy1.1^+$) cells after fetal intervention. TCR75 proliferation is detected as dilution of CFSE. **(B)** Expression of MHC class I antigen H-$2K^d$ in BALB/c fetal liver (top) and placenta (bottom) on E13.5-E14.5. Adult BALB/c lymph nodes (top) and an isotype antibody (top and bottom) were used as controls. N ≥ 3 in each group in 3 separate experiments. **(C, D)** Representative proliferation profiles of TCR75 cells in the uterine draining lymph nodes (udLNs, top row), spleens (middle row), and non-draining lymph nodes (ndLNs, bottom row) of dams carrying syngeneic (Syn) or allogeneic (Allo) pregnancies at baseline **(C)** or after fetal injection of PBS or LPS **(D)**. **(E)** The percentage of proliferated TCR75 cells among total TCR75 cells after LPS injection. N ≥ 4 dams in each group in 4 separate experiments. ** $P \leq 0.01$, ***$P \leq 0.001$ by ANOVA with Tukey's multiple comparison test. **(F)** The percentage of apoptotic cells (Annexin V^+DAPI^-) among proliferated ($CFSE^{low}$) and non-proliferated ($CFSE^{high}$) TCR75 cells in the uterine draining lymph node (udLNs), spleens, and non-draining lymph nodes (ndLNs) (Allo pregnant, n = 4, Allo LPS, n ≥ 3 dams in 3 separate experiments). *$P < 0.05$ by Student's t-test. **(G)** Increase in the absolute number (first graph) and in the proportion of TCR75 cells among all CD4 cells after fetal intervention (PBS). For uterus, decidua and placenta, each data point represents an average of all such segments per dam. N≥3 dams in each group in 3 separate experiments. *$P < 0.05$ by Student's t-test.

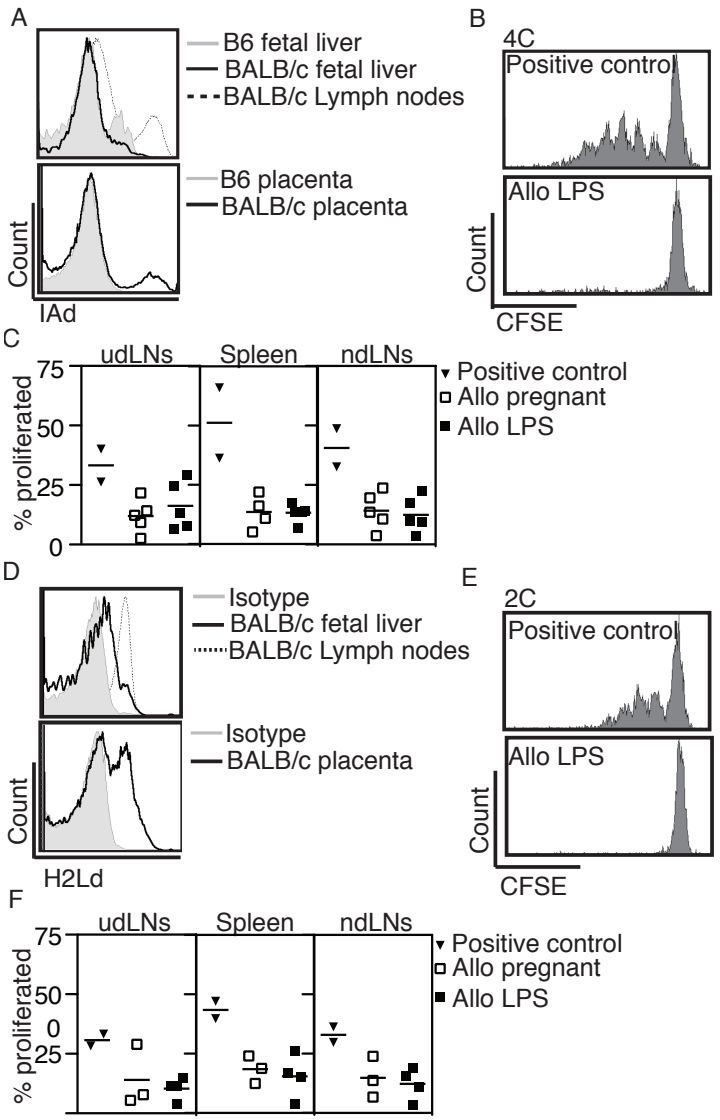
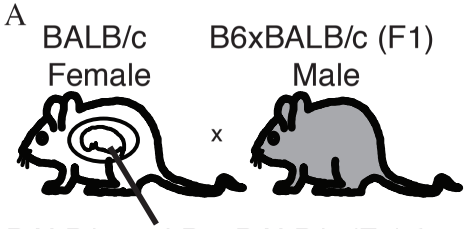


Figure 2.4. Maternal T cells that recognize the fetal alloantigen via the direct pathway do not proliferate after fetal intervention. **(A)** Expression of MHC class II antigen I-A^d, the antigen recognized by 4C mice, in BALB/c fetal liver (top) and placenta (bottom) on E13.5-E14.5 compared to that found in adult BALB/c lymph nodes (positive control) or B6 fetal tissues (negative control). **(B)** 4C ($CD4^+$ $Thy1.1^+$) lymphocytes were adoptively transferred into B6 females bred to BALB/c males and their proliferation was examined 5 days after fetal intervention with LPS. The positive control represents splenocytes harvested from a B6 mouse sensitized with BALB/c lymphocytes prior to adoptive transfer. **(C)** The percentage of proliferated $Thy1.1^+$ 4C cells among total 4C cells in the uterine draining lymph node (udLNs), spleens, and non-draining lymph nodes (ndLNs). (**D**) Expression of MHC Class I antigen H-$2L^d$ (recognized by 2C mice) in BALB/c fetal liver (top) and placenta (bottom) on E13.5-E14.5 compared to that found in adult BALB/c lymph nodes (positive control) or isotype control. **(E)** 2C ($CD8^+$ $Thy1.1^+$) lymphocytes were adoptively transferred using the same experimental design indicated above for 4C mice. (F) The percentage of proliferated 2C cells among total 2C cells in lymph nodes and spleens. Positive control n=2; Allo pregnant n≥3, Allo LPS n≥4 dams in ≥2 experiments for all experiments.

A



BALB/c and B6xBALB/c (F1) fetuses transplanted with
B6 (paternal) or C3H (third party) antigen

B

Rationale	Maternal strain	Paternal strain	Donor strain	# of fetuses injected(litters)	Overall % resorption	Genotype distribution †	Ratio of BALB/c to F1
Baseline	BALB/c	B6xBALB/c (F1)	NA	NA(9)	NA	$^{54}/_{46}$	$^{1.2}/_{1}$
Tx 3rd party antigen	BALB/c	B6xBALB/c (F1)	C3H	49(8)	36±14%	$^{54}/_{46}$	$^{1.2}/_{1}$
Tx paternal antigen	BALB/c	B6xBALB/c (F1)	B6	64(8)	55±15%*	$^{67}/_{33}$	$^{2.0}/_{1}$**
No foreign antigen	B6xBALB/c (F1)	BALB/c	B6	29(4)	22±16%	$^{52}/_{48}$	$^{1.1}/_{1}$
No maternal T cells	BALB/c. TCRα-/-	B6xBALB/c (F1)	B6	74(9)	57±15%	$^{56}/_{44}$	$^{1.3}/_{1}$

Figure 2.5. Maternal T cells exacerbate selective loss of the semi-allogeneic fetus after in utero hematopoietic cell transplantation. **(A)** Breeding scheme to detect a functional maternal immune response. BALB/c mothers are bred to F1 (BALB/c x B6) fathers and the survival of BALB/c and F1 littermates is compared after in utero transplantation with hematopoietic cells from B6 mice which expressing the paternal antigen or from third party mice (C3H) which express a different allogeneic antigen. **(B)** Overall litter resorption rate and the relative survival of the BALB/c and F1 fetuses at baseline and in various experimental conditions explained under "rationale." **†:** Ratio between the percentage of live BALB/c pups born and percentage of live F1 pups born. The percentage is calculated as the # of live pups born of the specific genotype divided by all live pups born. NA: not applicable (no fetal intervention). *= $p< 0.0001$ and **= $p<0.0005$ by c^2 test compared to no foreign antigen group.

SUPPLEMENTAL MATERIAL

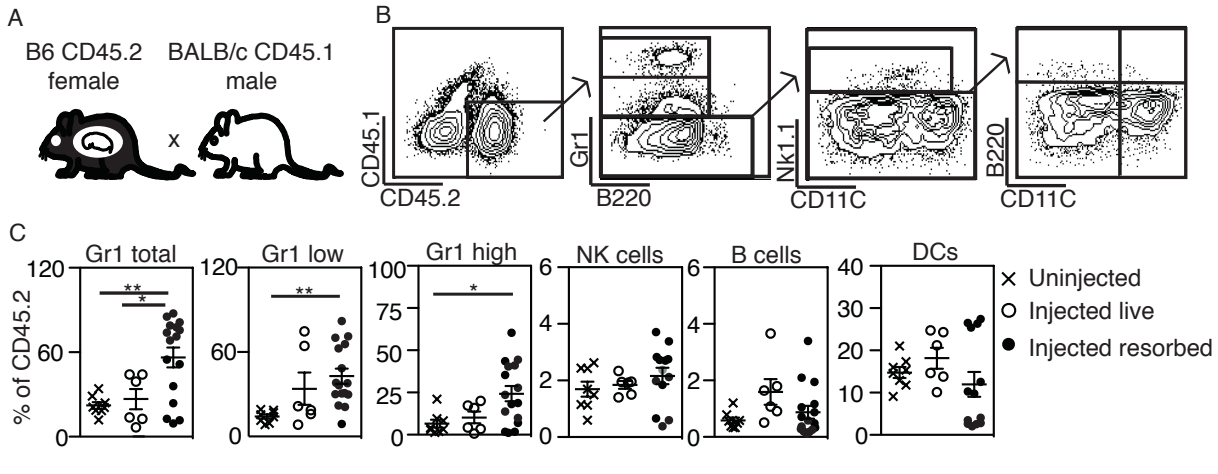


Figure 2.S1. Maternal myeloid cells accumulate in the uterus after fetal intervention. **(A)** Experimental design, **(B)** gating strategy and representative flow cytometry plots, and **(C)** percentages of various leukocyte populations in uterine segments surrounding uninjected, injected live and injected resorbed fetuses after fetal PBS injection. Monocytes: Gr1low gate, neutrophils Gr1high gate; NK cells: NK1.1+ gate; B cells: B220+ gate, DCs: CD11c+ gate. N≥6 uterine segments in n≥2 independent experiments. Each data point represents a uterine segment surrounding one fetus. *P < 0.05, ** P 0.01 by ANOVA with Tukey's multiple comparison test.

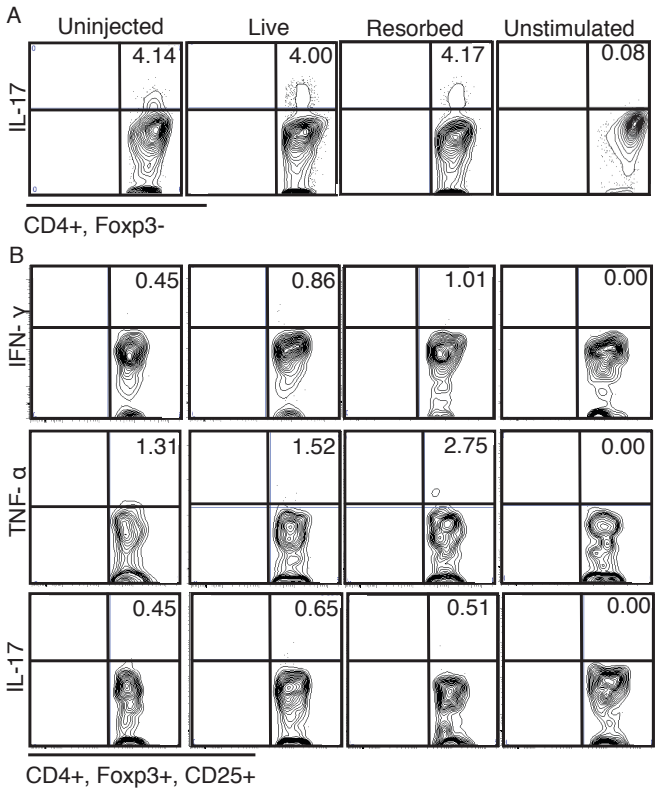


Figure 2.S2. No changes in IL-17 production by uterine T cells or Tregs. **(A)** Representative flow cytometry plots of IL-17 production by uterine CD4 Tconv cells (CD4+Foxp3-) harvested from pregnant dams 5 days after fetal PBS injection, after stimulation with PMA and ionomycin. Percentages obtained in one representative experiment from 4 independent experiments shown. **(B)** Minimal production of IFN-g, TNF-a, and IL-17 by CD4+Foxp3+CD25+ Tregs in the uterus. Percentages obtained in one representative experiment from 4 independent experiments shown.

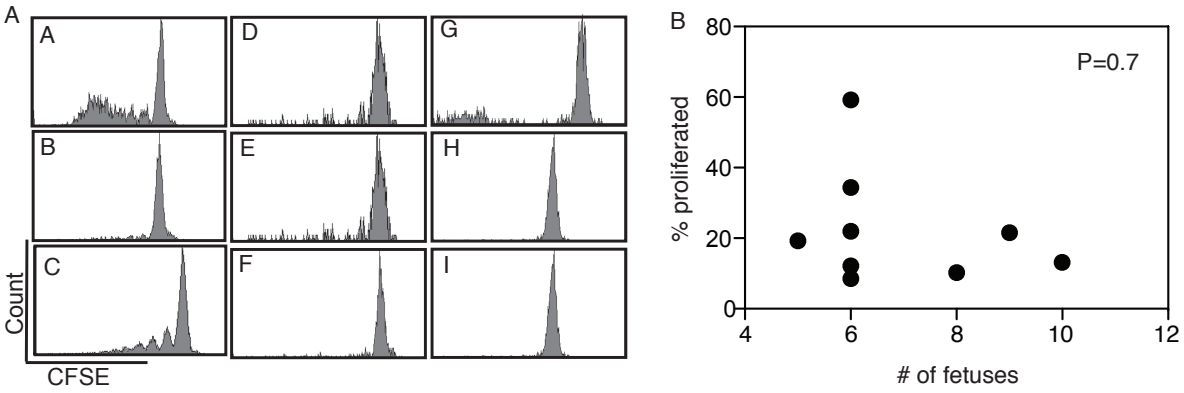


Figure 2.S3. Variation in T cell proliferation during normal pregnancy is not dependent on litter size. **(A)** Variability in the proliferation of adoptively transferred fetal antigen-specific TCR75 cells in normal allogeneic pregnancies (B6 female mated to BALB/c male) without fetal intervention. Experimental design and gating strategy are as outlined for Figure 4. CFSE profiles of TCR75 cells recovered from uterine draining lymph nodes of 9 separate dams in 6 separate experiments are shown. **(B)** A nonparametric correlation test (Spearman) was used to compare the percentage of proliferated TCR75 cells and the number of fetuses in each pregnant dam.

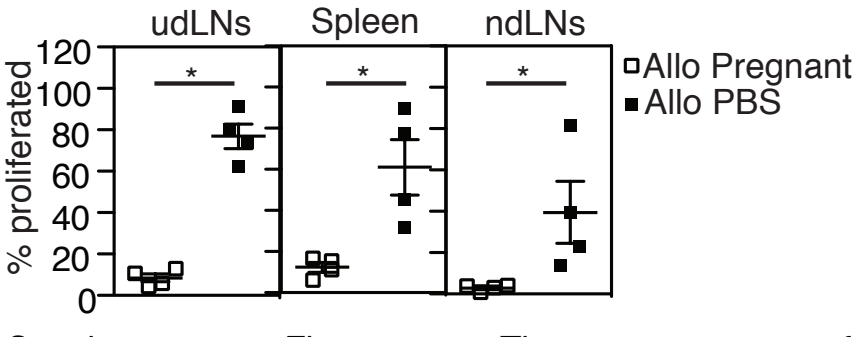


Figure 2.S4. Robust T cell proliferation in a fetal surgery model of sterile inflammation. The percentage of proliferated TCR75 cells among total TCR75 cells after PBS injection in the uterine draining lymph node (udLNs), spleens, and non-draining lymph nodes (ndLNs). N 4 dams in each group in 3 separate experiments. *P < 0.05 by Mann-Whitney test.

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CHAPTER 3

CHANGES IN MATERNAL-FETAL CELLULAR TRAFFICKING (MFCT) DURING PRETERM LABOR

ABSTRACT

Fetal surgery is a promising strategy to treat fetuses with severe congenital abnormalities but its clinical applications are often limited by preterm labor (PTL) [1, 2]. In normal pregnancy, multiple mechanisms protect the semi-allogeneic fetus from attack by maternal T cells [3-10]. Maternal microchimerism (MMc) (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 Tregs that can suppress a maternal T cell response [11]. PTL may represent a breakdown of this robust tolerance network. We hypothesize that during inflammation-associated PTL, maternal leukocytes cross the maternal-fetal interface and enter fetal circulation. Consistent with this hypothesis, we found an increased percentage of maternal microchimerism in fetal blood during PTL. The frequency of fetuses with high levels of trafficking (great than 0.5%) also increased during PTL. Finally, we show that the maternal cells trafficking into the fetus are primarily Gr1+ cells in both syngeneic and allogeneic pregnancy. During PTL in allogeneic pregnancy only, we found an increase in T cells. Our results demonstrate that trafficking of maternal cells during pregnancy is altered during PTL.

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 [12]. Surviving babies face long-term health problems, including neurodevelopmental impairments such as cerebral palsy and language and learning disabilities [13]. Although preterm labor (PTL) is multifactorial, the leading cause is inflammation and infection: 85% of cases are associated with chorioamnionitis [14].

Although multiple mechanisms protect the semi-allogeneic fetus from the maternal immune system during normal pregnancy [3-10], it is not known whether PTL entails recognition and rejection of the fetus by the maternal immune response. Maternal and fetal cells routinely traffic [15, 16] and the resultant bidirectional microchimerism may promote maternal-fetal tolerance at baseline [11]**.** Mold et al. reported that maternal cells in the fetus (Maternal michrochimerism, MMc) expose the fetus to non-inherited maternal antigens and lead to the generation of fetal Tregs that can suppress a maternal T cell response [11]. In healthy adults, maternal cells have been shown to persist as various immune cell types [17]. Maternal cells have also been identified in children with Type I diabetes [18, 19]. 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 after fetal surgery, preeclampsia, and spontaneous PTL [20, 21].

Fetal surgery to treat severe and fatal fetal congenital anomalies is limited by preterm labor [1]. During fetal intervention in mice, maternal T cells become activated to limit engraftment of in utero transplanted cell [22] and play a role in fetal loss (chapter 2). One mechanism by which maternal T cells may be involved in both limiting engraftment and inducing preterm labor during fetal surgery is by

trafficking into the fetus. Patients undergoing fetal surgery have increased levels of maternal cells in fetal blood [21]. However, trafficking in a model of preterm labor that more closely resembles human inflammation-induced PTL has not been reported.

In these experiments, we observed increased maternal microchimerism (MMc) in fetal blood during fetal intervention in mice, consistent to what has observed in humans [21]. Because a major limitation of fetal surgery is PTL, we next studied maternal-fetal trafficking in a mouse model of lipopolysaccharide (LPS)-induced PTL [23]. We demonstrate that LPS injection into the mouse uterus leads to an increase in MMc in the fetal blood. There is a specific increase in the number of T cells found in the fetus in allogeneic but not syngeneic pregnancies suggesting that maternal T cell awareness of fetal antigens may be increased during PTL. This data show altered trafficking of maternal cells into the fetus during PTL. Future studies are needed to understand the function significance of this change during PTL.

RESULTS

Maternal leukocytes trafficking increases during fetal intervention

Maternal leukocytes are present in the fetal circulation [22] and are important for tolerance induction during pregnancy [11]. In Chapter 2, we showed that maternal leukocytes play a role in mediating fetal demise during fetal intervention in mice. We next wondered whether changes in maternal-fetal cellular trafficking are associated with fetal demise during fetal intervention. We compared the number of maternal cells in fetuses from the B6.CD45.2 female and B6.CD45.1 male matings after in utero transplantation with allogeneic NOD.CD45.1 FL at E14.5. Control fetuses received PBS injection or no injection and all animals were harvested on E18.5-19.5. This experimental method, which we defined as the "leukocyte method", allowed us to detect the presence of the transplanted NOD cells ($CD45.1^{+}CD45.2^{-}$) and trafficking maternal leukocytes ($CD45.1^{-}CD45.2^{+}$) among fetal cells ($CD45.1^{+}CD45.2^{+}$) (**Figure 3.1A**). As expected, NOD donor cells were found in 95% (n=21) of fetal recipients at this early time point, supporting our interpretation that graft loss occurs gradually and is not due to technical variations in the injection method.

Analysis of maternal cells in these animals showed no maternal cell trafficking in unmanipulated fetuses (n=21) while 33% (n=33) of PBS-injected and 57% (n=21) of FL-injected pups had >0.5% maternal cells (**Figure 3.1B,** Chitest between FL and PBS p=0.15), indicating that non-specific tissue injury after fetal intrahepatic injection alters trafficking, with further increases in maternal cells after FL transplantation. Analysis of the percentage of maternal leukocytes in fetal blood in each animal revealed a trend for increased maternal cell chimerism with FL transplantation compared to PBS (**Figure 3.1C,** 1.1±0.3% PBS vs 2.2±0.7% FL, p=0.21). These results indicate either increased maternal cell trafficking into fetus or increased survival of trafficking maternal cells after fetal intervention.

Although the overall percentage of maternal cells found in the fetus was not significantly different between the two groups, we detected significant increases in both maternal T and B cells found in the fetus after allogeneic FL transplantation compared to PBS injection (**Figure 3.1D**, T cell: 12.1±2.9% PBS vs 33.8±2.3% FL, $p<0.0001$; B cell: 13.7±1.9 PBS vs 25.7±3.0% FL, $p<0.005$). Because the fetus at this gestational age has very few circulating T cells, we then determined whether the trafficked maternal cells contributed to a significant population of the T cell pool. We found maternal T cells represented 15.5±4.3% of the total circulating T cell pool in the host after FL transplantation, whereas they represented 3.5±0.8% of the total T cell population after PBS injection (**Figure 3.1E, $p<0.05$**). Thus, fetal intervention leads to a significant increase in the levels of maternal T cells in the fetal circulation, suggesting that these cells may play a functional role in the engraftment of allogeneic cells.

LPS injection into the pregnant uterus results in preterm delivery

Because fetal surgery often results in preterm labor in humans, we asked if trafficking is altered during preterm labor as well. We chose to use a classic mouse model of PTL, intrauterine LPS injection [23], to study trafficking in mice because fetal surgery results in resorption rather than preterm labor. We first did a dose response study for intrauterine LPS in syngeneic pregnancies, injecting doses of ranging from 0.05ug to 1.00ug. We found that delivery occurred earlier at concentrations higher than or equal to 0.075ug. Injection of 0.05ug resulted in delivery after 64±21 hours while higher concentrations (0.075, 0.100, 0.500, 1.00) resulted in an earlier delivery (36±12 - 40±16 hours) (**Figure 3.2A**). We also compared time of delivery after treating syngeneic to allogeneic pregnancies with 0.05ug of LPS and found no difference (Syngeneic, 64±21 hours; Allogeneic, 66±15 hours) (**Figure 3.2B**). Based on these results, we used 0.05ug of LPS to look at trafficking in fetal blood 12 hours to ensure viable pups at time of harvest.

Comparison of two methods to track maternal-fetal cellular trafficking during preterm labor

We next compared two strategies for quantifying maternal cells in fetuses (maternal microchimerism) (**Figure 3.3A, B**). We used the two methods to track maternal cells in fetal blood. In the "leukocyte method," that we previously used to identify maternal cells in fetal blood during fetal intervention, CD45.2 mothers are bred to CD45.1 fathers such that the fetuses are $CD45.1^{+}/CD45.2^{+}$ and maternal cells are identified by lack of CD45.1. In the "GFP method," [24] $GFP^{+/-}$ mothers are bred to $GFP^{-/-}$ fathers such that maternal cells can be tracked in the $GFP^{-/-}$ pups. To directly compare the two 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, **Figure 3.3A,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 of these two methods. We found that the levels of MMc were the same with each method (P = 0.76 by paired t-test), such that they may be used interchangeably in the subsequent analyses. (**Figure 3.3C**).

Increased presence of maternal cells in fetal blood 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 cells in fetal blood after 12 hours. We found low but detectable levels of maternal cells in unmanipulated fetuses (0.1±0.1%), with no increase in trafficking with intrauterine PBS injection (Syn PBS, 0.3±0.1% and Allo PBS, 0.5±0.2%) (**Figure 3.4B**). However, during LPS-induced PTL, there was a striking increase in the percentage of maternal cells in the fetus with both syngeneic and allogeneic pregnancies (Syn LPS, 2±0.7% vs uninjected, $P < 0.05$ and Allo LPS, 2±0.6% vs uninjected, $P < 0.05$ by Kruskal-Wallis test with Dunn's post-hoc comparison) (**Figure 3.4A,B**). When we specifically examined fetuses with high levels (>0.5%) of maternal microchimerism (MMc), we determined that the pups of

allogeneic matings undergoing PTL were most likely to have high MMc compared to the other groups (Allo PTL, 64%; Allo PBS, 38%; Syn PTL, 47%; Syn PBS, 27%; uninjected, 5%) (**Figure 3.4C**). In allogeneic matings, there was an increased percentage of pups with high MMc during PTL compared to PBS injection (Allo PTL, 64% vs Allo PBS, 38%, Chitest 0.03) (**Figure 3.4C**). In syngeneic matings, there was no difference in the percentage of pups with high MMc during PTL compared to PBS injection (Chitest 0.09). We also compared allogeneic and syngeneic pregnancies that were treated with LPS and did not see a difference either in the average number of trafficking cells or in the percent of pups with high trafficking (Chitest 0.16).

We analyzed the location of fetuses with high levels of trafficking and determined that in both syngeneic and allogeneic pregnancies treated with LPS these were most likely to be at the uterine horns or near the cervix (**Figure 3.4D-E**). These areas have been reported to have highest levels of blood flow [25], suggesting a link between 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 cells using markers for T cells (CD3), B cells (B220 or CD19) and granulocytes (Gr-1) (**Figure 3.5A**). Most trafficking maternal cells were Gr-1^{+}. When we compared the lineage distribution of maternal Gr1, T, and B cells found in fetal blood compared to that found in maternal blood, we determined that there is a higher concentration of Gr1 cells in fetal blood, suggesting specific recruitment of these cells into the fetus rather than a non-specific breakdown of the maternal-fetal interface (**Figure 3.5B**). Interestingly, there was a significant increase in the percentage of maternal T cells found in fetal blood during PTL in allogeneic pregnancies compared to syngeneic (Allo PTL, 13 ± 3% vs Syn PTL, 4.1 ± 1.1%, P = 0.01) (**Figure 3.5B**). Thus, although there were no differences in the overall leukocyte MMc between syn and allo 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.

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-grade™) (LPS, Alexis Biochemicals), Histopaque 1077 Ficoll (Sigma-Aldrich), Ficoll-Paque Plus (GE Healthcare). 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). NOD.CD45.1.uGFP (NOD.CD45.1) mice were generated by backcrossing B6.uGFP transgenic mice (strain 004353, Jackson Laboratories) at least 6 times to NOD (Jackson Laboratories) mice. 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 ug of LPS (or PBS) was injected into the uterus between the first and second fetus of the right horn to induce PTL [23]. Twelve hours later, fetal blood was collected into HBSS with heparin and stained for cell surface markers. The dose of LPS (0.05ug, 0.075ug, 0.100ug, 0.500ug, 1.000ug) 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. “High traffickers” were defined as fetuses showing greater than 0.5% maternal microchimerism in the peripheral blood.

Detection of maternal cells in fetal mice after fetal intervention

B6.CD45.2 mothers were bred to B6.CD45.1 fathers and the resulting pups were harvested at indicated time points. Pups were washed twice in PBS prior to decapitation in heparinized HBSS to minimize contamination with maternal blood. At the earlier gestational ages (E12-5-E15.5), blood from 2-3 pups was pooled to obtain enough sample for flow cytometry. After blood collection, pups were dissected under a dissecting microscope and individual organs were collected, dissociated using collagenase and DNase, and single cell suspensions were prepared. Red blood cells in blood and spleen samples were lysed using ACK Lysing Buffer. The cells were stained for CD45.1, CD45.2, CD3, B220 (or CD19), and Gr-1 to detect maternal cells and their lineage composition. The presence of maternal leukocytes was quantified by calculating the percentage of $CD45.2^{+}$ $CD45.1^{-}$ cells over the total $CD45^{+}$ pool. A clear population of maternal leukocytes that was >0.5% and >50 events was considered significant. In some experiments, a more detailed lineage analysis was performed using two panels of antibodies to detect lymphocytes of the adaptive (CD3, CD4, CD8, CD19) and innate (CD11c, Gr-1, NK1.1, F4/80) immune systems. To determine the effect of IUHCTx on maternal cell trafficking, fetuses were sacrificed 4-5 days after in utero transplantation of NOD.CD45.1 FL and were analyzed for the presence of donor and maternal lymphocytes by flow cytometry (LSRII, Becton Dickinson). Dead cells were gated out using DAPI. The results for uninjected, PBS-injected, and FL-injected groups were compiled from at least 3 independent litters per group.

Detection of maternal cells in fetal mice after preterm labor

BALB/c and B6 mice were bred to create syngeneic and allogeneic pregnancies. Two strategies were used to identify maternal cells in fetal blood with equivalent results (**Figure 3.3**). In our previously

published 'leukocyte method'[11], CD45.2 mothers are bred to CD45.1 fathers, such that the fetuses are $CD45.1^{+}/CD45.2^{+}$ and maternal cells are identified by lack of CD45.1 In the 'GFP method', $GFP^{+/-}$ mothers are bred to $GFP^{-/-}$ fathers such that maternal cells can be tracked in the $GFP^{-/-}$ pups (50% of pups). On E16.5, a laparotomy was performed and 0.05 ug of LPS (or PBS) was injected into the uterus between the first and second fetus of the right horn to induce PTL[15]. Twelve hours later, fetal blood was collected into HBSS with heparin and stained for cell surface markers. The dose of LPS 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. "High traffickers" were defined as fetuses showing greater than 0.5% maternal microchimerism in the peripheral blood as described previously[11].

Statistics

Comparisons involving 2 groups were evaluated using either Chi-square test (for changes in frequency) or a Student's *t* test. Comparisons between 2 or more groups were evaluated using ANOVA with a Kruskal-Wallis test with Dunn's post-hoc comparison 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).

DISCUSSION

Fetal intervention in mice results in an increase presence of maternal cells in fetal blood. In addition to baseline trafficking, we have determined that there are key changes in the composition of maternal cells in fetal blood after fetal intervention, with particular increases in the levels of maternal T cells following in utero transplantation. To study the effect of preterm labor on maternal microchimerism in the absence of fetal intervention, we used a mouse model of LPS-induced preterm labor where we demonstrated increased maternal microchimerism of Gr1+ and T cells in fetal blood. The significant increase of maternal cells 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.

Several mechanisms may result in such a finding: there may be selective recruitment of maternal cells across the placenta, increased proliferation or decreased turnover of T cells which have already crossed, or decreased homing of maternal T cells to fetal tissues with resultant increases in the circulation. The route of entry of maternal cells 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 see a correlation between fetuses with high MMc and high maternal blood flow 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 three layers, the fetal mononuclear trophoblasts and a continuous double-layer of syncytiotrophoblasts [26]. In the vasculature, capture and adhesion of recruited cells occurs through the interaction between leukocytes and the endothelium that is mediated by selectins. At the maternal-fetal interface in the placenta, leukocytes do not come into contact with the endothelium but with a mononuclear trophoblast layer and a continuous double-layer of syncytiotrophoblasts. However, in mice, E-selectin expression on

trophoblasts lining maternal blood sinuses and VCAM-1 expression on maternal blood vessels increases during mid-gestation and correlates with infiltration of neutrophils [27]. A study in humans has shown that L-selectin expression on trophoblasts could potentially enable leukocyte capture from the maternal circulation [28]. In addition, maternal T cells have been observed in fetal tissues of patients with villitis of unknown etiology [29, 30], with coordinate changes in placental chemokines [31] suggesting that maternal leukocytes can penetrate the maternal-fetal interface in the placenta.

Another alterative route where maternal cells may be gaining entry into fetal circulation is at the interface of the decidua and trophoblast giant cells in the placenta. In normal pregnancy, epigenetic silencing of chemokine genes inhibits T cell migration into the decidua [32]. 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-1α) are associated with monocyte and T cell recruitment to the pregnant uterus during the onset of labor [33, 34]. Selective recruitment of maternal leukocytes to the decidua (decidual leukocytes) has been described in mice [27] and human [35] and it has been proposed that alterations in type of cells recruited to the decidua may result in pregnancy complications The mechanisms of increased cell trafficking may be addressed using various genetic mouse models and intravital imaging techniques.

We have shown two methods of quantifying MMc are equivalent and tested them in a mouse model of pregnancy complication. Our lineage analysis has indicated that determination of lineage composition is a critical addition to quantifying overall levels. Thus, studies of human MMc should take this into account the composition of trafficked maternal cells in addition to frequency of chimerism. One weakness in our paper is that we were not able to determine whether increased MMc is secondary to increased trafficking or secondary to increased proliferation of cells after entering the fetal circulation.

Although we have shown, in chapter 2, that there is proliferation of adoptively transferred antigen-specific maternal T cells at the maternal-fetal interface, there is an overall low level of trafficking in the fetus which 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.

The finding that fetal PBS injection alone leads to maternal cell trafficking may have clinical implications in the field of fetal intervention. The amount of fetal trauma from the intrahepatic injection in our model is likely more than would be expected for human fetal surgery, which uses minimally invasive methods. Therefore, it is not known whether human fetal interventions will lead to similar alterations in trafficking of maternal cells into the fetus, although changes in the amount of fetal DNA in the mother have been described [36]. If cellular trafficking is related to maternal-fetal tolerance, alterations in trafficking may correlate with the onset of preterm labor [20, 37]. Given the growing interest in clinical fetal surgery for various anatomic anomalies, our findings may have important implications for the pathogenesis of preterm labor following fetal intervention.

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. In chapter 2, we showed that activated maternal T cells are implicated in fetal demise during fetal intervention. Here we asked if maternal microchimerism is present in a mouse model of preterm labor. Further studies are required to determine if trafficking maternal cells are responsible for the breakdown of tolerance during pregnancy and responsible for preterm labor.

Figures

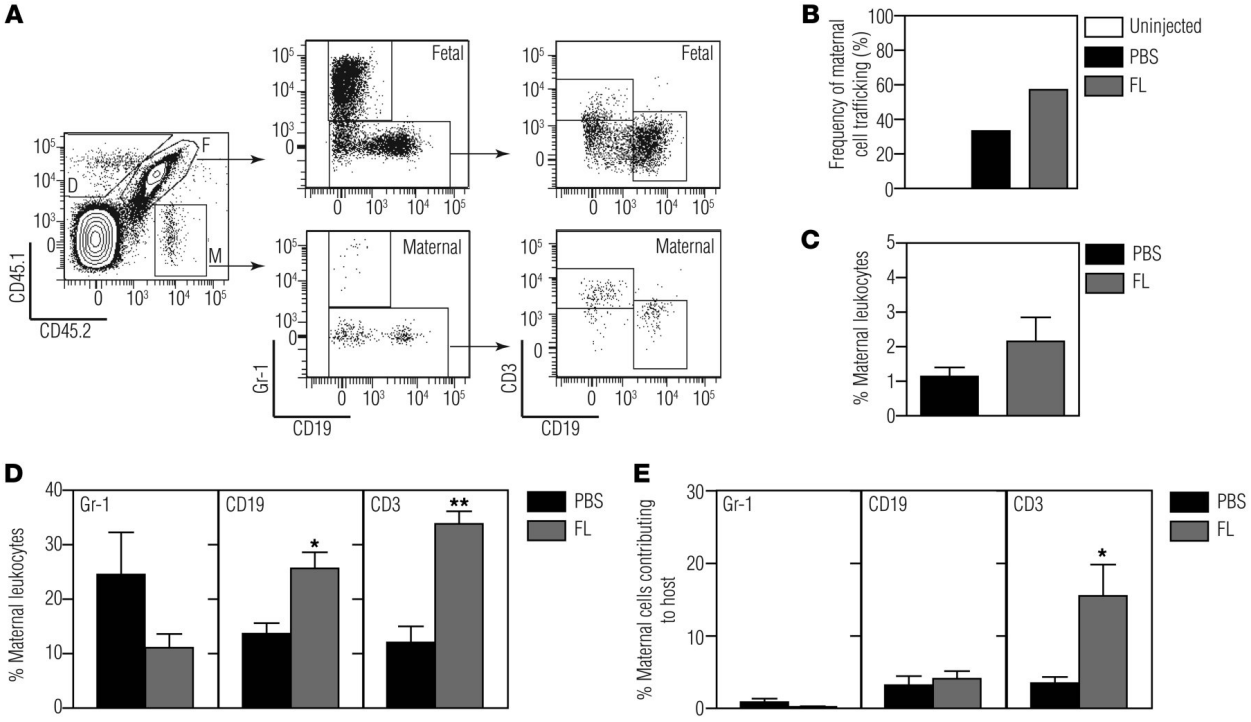

Figure 3.1. Maternal-fetal cellular trafficking after fetal intervention. B6.CD45.2 mothers were mated with B6.CD45.1 fathers and the CD45.1$^+$/CD45.2$^+$ fetuses were injected with allogeneic NOD.CD45.1 fetal liver cells or PBS on E14.5. Injected (and uninjected control) fetuses were sacrificed on E18.5-E19.5 and the number of maternal leukocytes (CD45.2$^+$) in fetal blood was quantified. **(A)** Flow cytometric analysis of donor (gate D), maternal (gate M), and fetal (gate F) leukocytes. **(B)** Frequency of fetuses with circulating maternal leukocytes (number of fetuses with circulating maternal leukocytes/total number of fetuses) after PBS injection (n=11/33, 33%), allogeneic FL injection (n=12/21, 57%), and in age-matched uninjected controls (n=0/21, 0%). **(C)** Percentage of maternal leukocytes (CD45.2$^+$ maternal leukocytes/total CD45.2$^+$ cells) in fetal circulation after PBS (n=11) and allogeneic FL injection (n=12). **(D)** Lineage analysis of trafficking maternal cells shown as percent maternal leukocytes (e.g. trafficked maternal Gr-1$^+$ cells/total trafficked maternal leukocytes). *p<0.005 by t-test; **p<0.0001 by t-test. **(E)** Lineage analysis of trafficking maternal cells shown as the percent of maternal cells contributing to each of the leukocyte subsets in fetal circulation (e.g. trafficked maternal Gr-1$^+$ cells/total number of fetal and trafficked maternal Gr-1$^+$ cells). *p<0.05 by t-test.

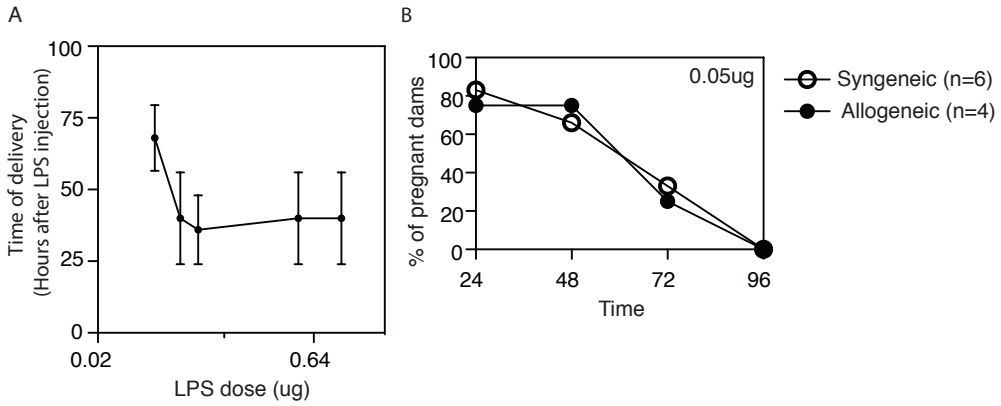

Figure 3.2: LPS injection into the pregnant uterus results in preterm delivery. (**A**) The time of delivery (in hours) after injecting various concentrations of lipopolysaccharide (LPS) into the uterus in syngeneic pregnancies (0.05ug, n=6; 0.075ug, n=3; 0.100ug, n=3; 0.500ug, n=3; 1.000ug, n=3). (**B**) The percentage of dams pregnant at 24, 48, 72 and 96 hours after injection of 0.5ug of LPS into the uterus in syngeneic (n=6) and allogeneic (n=4) pregnancies.

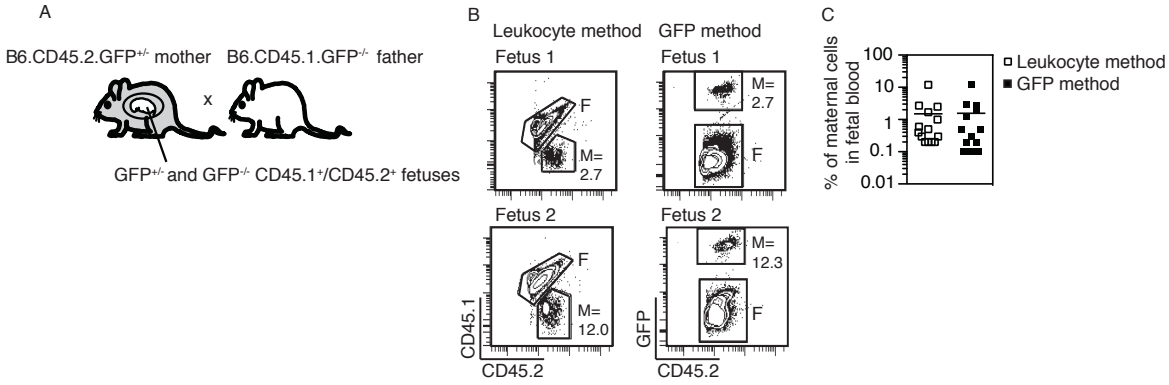

Figure 3.3: Comparison of two methods to track maternal-fetal cellular trafficking. (**A**) Breeding scheme in which $GFP^{+/-}$CD45.2 mothers are bred to $GFP^{-/-}$CD45.1 fathers such that resulting fetuses are either $GFP^{+/-}$ or $GFP^{-/-}$ and all fetuses are $CD45.1^{+}/CD45.2^{+}$. (**B**) Gating strategy to quantify maternal cells in fetal blood. In the leukocyte model [22], 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 two 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.

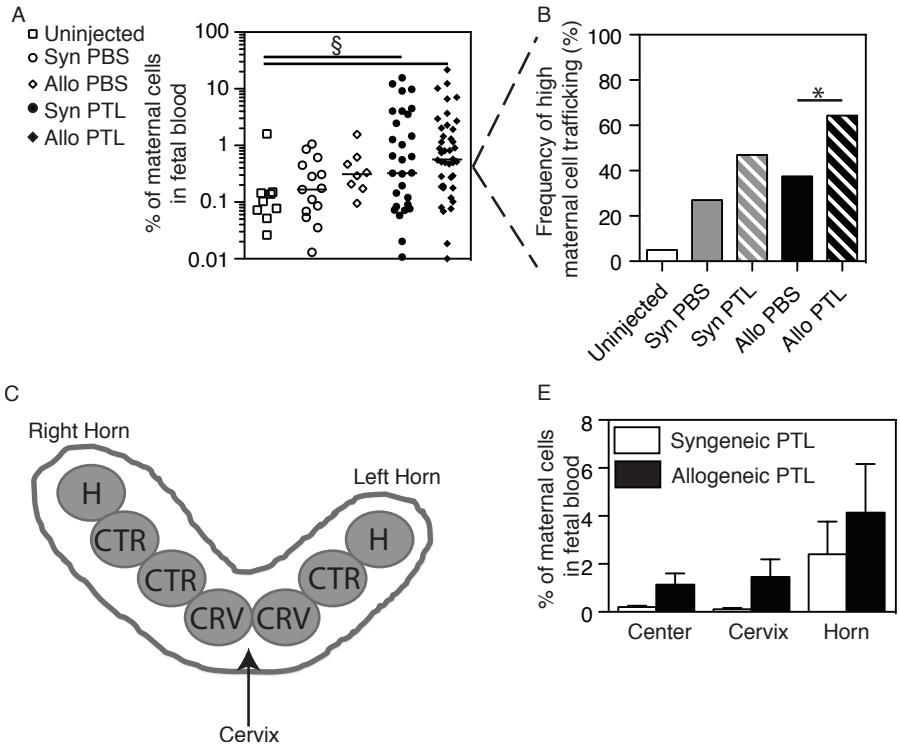

Figure 3.4: Increased presence of maternal cells in fetal blood during preterm labor at sites of highest blood flow. (**A**) The percentage of trafficked maternal leukocytes among fetal leukocytes from uninjected (n=19), PBS-injected (syngeneic (Syn PBS), n=15; allogeneic (Allo PBS), n=8), and LPS-injected fetuses (syngeneic (Syn PTL), n=32; allogeneic (Allo PTL), n=42). §P<0.05 by Kruskal-Wallis with Dunn's post-hoc comparison. (**B**) Frequency of pups with high levels (>0.5%) of maternal cells 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).

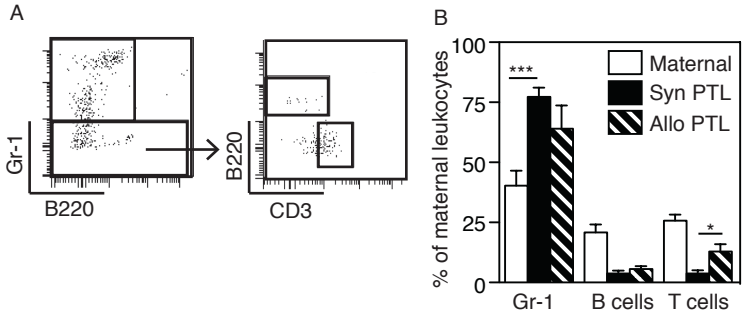

Figure 3.5: Increase in maternal Gr-1 cells in fetal blood with a specific increase in T cells during preterm labor in allogeneic pregnancy. (**A**) Gating strategy to phenotype trafficked maternal cells. Maternal leukocytes were separated into Gr-1$^+$ and Gr-1$^-$ gates. Gr-1$^-$ cells were divided 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. * P<0.05, ***P<0.001 by Student's t-test.

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CHAPTER 4

TREGS PREVENT PRODUCTION OF MATERNAL ALLOANTIBODIES TO FETAL ALLOANTIGENS

ABSTRACT

During pregnancy, the maternal adaptive immune system is exposed to fetal antigens but multiple mechanisms prevent T cell activation. However, the mechanisms affecting maternal alloantibody production against fetal antigens have not been studied. We recently showed fetal intervention results in antigen-specific T cell activation and a local shift in the T cell/Treg balance in the uterus. Here, we specifically probe the role of Tregs in the onset of a maternal humoral immune response against fetal alloantigens with and without fetal intervention. We report a significant increase in maternal alloantibody responses with Treg depletion. Fetal intervention by itself does not lead to significant antibody response. Prolonged exposure to fetal antigens (resorption, as opposed to preterm delivery) in the absence of Tregs is necessary for antibody formation and correlates with an increase in Teff cells. Finally, we show anti-fetal antibodies do not prevent future pregnancy suggesting other mechanisms play a role in protecting the pregnancy once exposure to fetal antigens occurs. These findings point out the requirement of multiple insults to disrupt the robust tolerance mechanisms protecting pregnancy.

INTRODUCTION

Pregnancy is a unique immunological state that allows for maternal tolerance of the semi-allogeneic fetus. Because the placenta is an imperfect barrier, the mother is exposed to conceptus-derived alloantigens through trafficking of fetal cells into the maternal blood [1-4]. Multiple mechanisms limit maternal T cell activation against the semi-allogeneic fetus [5-7]. We have recently shown that fetal intervention in mice disrupts maternal T cell tolerance to fetal antigens and can lead to the selective demise of the semi-allogeneic fetus within a litter [8]. Maternal regulatory T cells (Tregs) with fetal specificity play a role in establishing maternal T cell tolerance during pregnancy [9-16]. We now ask whether Treg protection is disrupted during fetal intervention.

Previous studies examining the role of Tregs during pregnancy have suggested Tregs act in an antigen-specific manner to maintain pregnancy. For example, depletion of Tregs results in elevated fetal resorption rates in allogeneic but not syngeneic pregnancies [9, 10, 13-15] and has been correlated with increased activation of effector $CD4^+$ T cells [13, 14]. Tregs also play an important role in regulating antibody production and have been shown to alleviate antibody-mediated diseases during pregnancy, such as rheumatoid arthritis [17]. Tregs acting in an antigen-specific manner have also been implicated in inhibiting alloantibody mediated organ rejection in heart transplantation in mice [18]. However, it is still unclear whether Tregs play a role in minimizing maternal antibody production against the fetus.

Prenatal exposure of the fetus to maternal antibodies is a physiological event. During pregnancy, maternal antibodies increase as a function of advancing gestation [19]. Delivery exposes the mother to more fetal antigens [20] which persist and increase with each pregnancy, such that multiparous women are more likely to have antibodies in their serum compared to nulliparous (first-time) women [21]. These antibodies cross the placenta during pregnancy [22] and augment the immunity of the newborn

[23]. In rare cases, maternal alloantibodies can cross the placenta and can be pathogenic as seen in Rh disease.

Clinical studies of pregnancy complications suggest that alloantibody formation may be linked to pregnancy complications. For example, there is a correlation between increased levels of HLA Class I and II IgG antibodies and spontaneous preterm delivery [24] especially in the setting of inflammatory conditions such as chorioamnionitis [25]. More recently, these antibodies have been shown to be fetal specific [19, 24] and develop more frequently with increasing HLA antigen mismatch between mother and fetus [26]. Additionally, positive HLA antibodies early in pregnancy correlate with decreased likelihood of live birth in patients with recurrent spontaneous abortion [27]. Although the precise mechanisms are not known, studies have shown a correlation between HLA antibodies and increased complement activation [25] and increased chemokines involved in T cell migration [25]. However, a definite causal role of antibodies in pregnancy complications has not been established.

We have recently showed that fetal intervention results in a shift in Teff/Treg ratio in the uterus [8]. Using targeted depletion of Tregs, we now ask whether Tregs regulate a maternal humoral immune response. Given that fetal intervention disrupts tolerance, we also asked whether fetal intervention can overcome Treg regulation to activate a maternal humoral immune response and if the presence of maternal antibodies has an effect on pregnancy in murine pregnancies.

RESULTS

Treg depletion and resultant increase in circulating effector T cells

To study the role of Tregs in controlling maternal antibody production, we used B6.Foxp3^{DTR} mice to deplete maternal Tregs. We bred these mice to BALB/c males and injected pregnant mothers with diphtheria toxin (DT) starting on E13.5 for 6 days (**Figure 4.1A**). In some animals, we also performed fetal intervention (injection of PBS or LPS into fetal liver) on E13.5 in the presence and absence of Tregs and all animals had a laporatomy to account for effect of surgery alone and to inspect litter size (**Figure 4.1A**). There was no adverse effect of this level of Treg depletion on maternal health (data not shown). We confirmed the extent of Treg depletion one and five days after the start of DT injection (**Figure 4.1B, C**). Interestingly, Treg depletion resulted in an increase in circulating effector T cells (defined as CD4+FoxP3-CD25+ T cells) by day 5 (**Figure 4.1D**). Treg depletion resulted in a significant percentage of fetal loss compared to normal pregnancy as previously described [12] (**Figure 4.1E**). There was a significant correlation between fetal loss and circulating effector T cells, suggesting a role for effector T cells in mediating fetal loss (**Figure 4.1F**).

Increase in maternal antibodies against fetal antigens with Treg depletion

We next asked whether Treg depletion during pregnancy affects the production of maternal antibodies against fetal alloantigens. Maternal antibodies specific for paternal BALB/c antigens were detected by culturing maternal serum with BALB/c cells to detect the presence of allo-specific IgG and IgM antibodies against the fetus by flow cytometry (**Figure 4.2A, B**). In normal allogeneic matings, we did not detect IgG or IgM antibodies in maternal serum (**Figure 4.2A-D**). When we depleted Tregs during pregnancy, there was a striking increase in IgG antibody levels in the maternal serum, suggesting a role for Tregs in inhibiting sensitization during normal pregnancy (**Figure 4.2C**). Antibody production peaked at two weeks after the start of Treg depletion (**Figure 4.2C**). These antibodies were specific for BALB/c antigen (**Figure 4.2E**). We also found a significant increase in allospecific IgM within one week of Treg

depletion compared to normal pregnancy, but the overall levels were lower than those of IgG (**Figure 4.2D, E**).

We also asked whether fetal intervention, which increases the trafficking of cells between the mother and fetus [30], and can induce maternal T cell activation [8], would also induce a humoral immune response. We injected fetuses with PBS at E13.5 and analyzed the production of BALB/c-specific IgG and IgM antibodies. With this fetal intervention alone, there was only a slight increase in IgG antibodies with no increase in IgM levels in maternal serum compared to normal pregnancy (**Figure 4.2C, D**). Treg depletion with fetal intervention resulted in a slight increase in IgG and IgM antibodies compared to fetal intervention alone (**Figure 4.2C,D**).

Effect of T cell activation and antigen exposure on alloantibody response

Because fetal PBS injection resulted in only a slight increase alloantibody production, we asked if fetal LPS Injection, which causes a stronger inflammatory response and results in a near-total fetal loss [8] would result in a more robust humoral immune response. In this model, levels of IgG antibody in maternal serum were equivalent to fetal PBS injections in the presence of Tregs (data not shown). However, maternal Treg depletion combined with fetal LPS injection resulted in preterm delivery in 6 of 9 litters. When we compared antibody levels in dams that resorbed or delivered their pups, we noted an increase in dams that resorbed the litter compared to those that delivered the litter prematurely (**Figure 4.3B**). These results suggest that continued exposure to the paternal antigen (during resorption) in the absence of Tregs is necessary for the antibody production. We also noted a strong correlation between the level of antibody found two weeks after Treg depletion and fetal LPS injection and the levels of Teff cells in maternal serum five days after depletion, suggesting a role for effector T cells in facilitating the humoral immune response (**Figure 4.3C**).

Maternal allo-antibodies do not affect future pregnancy

Maternal alloantibody formation is common in mutliporous women with unclear significant for pregnancy success. We therefore asked whether maternal sensitization in our model has an effect on the success of future pregnancy. We sensitized B6 females to BALB/c antigens by injecting them with BALB/c cells (**Figure 4.4A,B**), a treatment that leads to both formation of alloantibodies (**Figure 4.4B,C**) as well as increased percentage of BALB/c-specific T cells [30]. We then bred these B6 dams with B6, BALB/c or C3H males starting at two weeks after sensitization (one week before the peak antibody response, **Figure 4.4C**). We assessed the rate of successful pregnancy after plug formation as well as the litter size. Mice that plugged also became pregnant in all three groups indicating no adverse affect on fertility (data not shown). Moreover, there were no differences in the litter size when sensitized females were bred to allogeneic (BALB/c), syngeneic (B6) or third party (C3H) fathers (**Figures 4.4C-D**).

Interestingly, when we analyzed serum from pups of these matings at E18.5, there were lower levels of alloantibodies in Balb/c matings compared to the other two groups (**Figure 4.4E**). We speculate that this finding is secondary to binding of the antibody to fetal or placental tissues, which lowers the amount detectable in the serum.

MATERIALS AND METHODS

Reagents and Antibodies

The following reagents were used: Diphtheria toxin (DT, Sigma-Aldrich), IgG (eBioscience), IgM (ll/41, eBioscience), and Lipopolysaccharide from Salmonella abortus equi S-form (TLR-grade™) (LPS, Alexis Biochemicals).

Mice

The inbred strains, BALB/c, C3H-HeJCr, C57BL/6 (B6), were obtained from either NCI or Jackson Laboratories. B6.Foxp3^{DTR} mice [28] were obtained from Matthew Krummel (UCSF). 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. Females used were all nulliparous.

Treg depletion

B6.Foxp3^{DTR} females bred to BALB/c males received an intraperitoneal injection (ip) of diphtheria toxin (DT) on E13.5 (day zero) for six days. Mice received 15ug/kg of DT on day one followed by 5-15ug/kg for the next five days. The degree of depletion was analyzed on day one and day five after DT injection.

In utero injections

Fetal mice were injected with PBS or LPS on embryonic day E13.5 directly into the fetal liver using pulled glass micropipettes as previously described [8, 28, 29]. The pregnant dam was anesthetized, a laparotomy was performed, and the fetuses were injected through the intact uterus. For LPS injections, a dose of 0.5ug was diluted among the total fetuses in the litter [8]. A sham surgery (laporatomy without in utero injection) was performed on mice that received DT alone or normal pregnant controls. The

percentage of fetal loss was calculated by subtracting the number of live pups born from the number of fetuses counted on E13.5 and dividing by the number of fetuses on E13.5.

Alloantibody assay

B6.Foxp3^{DTR} females bred to BALB/c received an intraperitoneal injection of DT alone or with fetal intervention (PBS or LPS). Controls included pregnant mice that received a laporatomy without fetal intervention, and those that received DT injection only. Submandibular bleeding was performed weekly starting at E13.5 for six weeks. Blood was collected into serum tubes and spun at 6000rpm for 6 minutes. Serum was frozen and kept at -20°C. To detect circulating alloantibodies, serum was incubated with 2x10^6 Balb/C splenocytes and lymph nodes for 1 hour. The stained cells were analyzed using flow cytometry to determine the Mean Fluorescent Index (MFI) of IgM and IgG staining on CD19-negative cells. Serum from naive non-pregnant BALB/c mice was used as negative control, and serum from sensitized BALB/c mice was used as a positive control (sensitized mice were generated as described below). The relative MFI was normalized to that of BALB/c lymphocytes that were not exposed to serum. To test for antibody specificity against BALB/c antigens, serum from experimental animals was incubated with BALB/c, B6, and C3H splenocytes and lymph nodes.

Sensitization

B6 females were injected retro-orbitally on week zero and week one with 5x10^6 isolated BALB/c non-draining lymph node (inguinal, axillary, brachial and mesenteric) Blood was drawn weekly to confirm IgG and IgM serum levels for six weeks. Two weeks after the initial injection of BALB/c cells, females were bred to B6, C3H or BALB/c males for three weeks. Plugs were checked daily. On E18.5, females were sacrificed. The number of fetuses was counted. Blood was collected and pooled from the fetuses in the litter.

Statistical analysis

Differences between more than two groups in the mouse data were evaluated using ANOVA with Tukey's multiple comparison test (mouse data) using Graphpad Prism. A p value <0.05 was considered significant. Data was summarized as means $\pm$ SEM.

Discussion

In this study, we analyzed maternal alloantibody formation in the context of Treg depletion and fetal intervention. We determined that Treg depletion in mice significantly increases the production of maternal alloantibodies, but these antibodies do not have an effect on the success of future pregnancy. These findings suggest that Tregs regulate a maternal humoral immune response, but alternative mechanisms may be in place to protect the pregnancy against maternal alloantibodies.

We report that Tregs regulate maternal alloantibody production during pregnancy. Treg depletion during pregnancy has been correlated with an increase in activated T cells [14]. We also found an increase in the percentage of effector T cells in Treg depleted dams with a significant correlation found between antibody production and the percentage of effector T cells. Because Tregs control T cell activation, the most obvious explanation for how Tregs prevent antibody production is by limiting T cell help [31]. It has been reported that depletion of Tregs results in the expansion of a subset of helper CD4+ T cells that promote the loss of B cell anergy [32]. In humans, in vitro studies show Tregs suppress a B cell response driven by $CD57^{+}$ germinal center T cells [33].

We did not detect a significant increase in antibody production with fetal PBS injection, but we did find an increase when LPS was injected into fetal liver. This response may be due to the effect of LPS acting as a mitogen for robust B cell responses [34]. Fetal LPS injection sometimes results in fetal resorption and sometimes in early delivery, which allowed us to tease out the role of antigen exposure on antibody production. Fetal LPS injection in the absence of Tregs resulted in a maternal humoral response but only when the litter resorbed. Early delivery of the dead fetuses produced a weaker response. We speculate antibody production in these two scenarios is dependent on the amount of time the mother is exposed to fetal antigens after intervention. Compared to fetal resorption, early delivery of the entire litter

shortens the duration of maternal exposure to fetal antigens. These results suggest that antigen exposure is required for antibody production during fetal intervention.

We did not detect an additive affect of Treg depletion with fetal PBS injection on alloantibody formation. This result was surprising in light of the finding that Treg depletion alone resulted in a significant maternal IgG response against fetal antigens. We speculate that this result is secondary to lower detection of circulating antibodies in the fetal intervention group due to formation of immune complexes between free antigens released after fetal surgery and circulating antibodies. Because the alloantibody assay uses antigen to capture free antibody in maternal serum, already formed antigen-antibody complexes will not be detected. It has been shown that such complexes can form during pregnancy and they are actually a component of tolerance due to binding to follicular dendritic cells (FDCs) [35].

In our mouse study, we did not find an adverse effect of maternal alloantibodies on the future pregnancy. The observation that multiparous women become pregnant despite the presence of HLA antibodies [21] is consistent with our mouse experiments and suggests that other mechanisms play a role to protect the pregnancy. We speculate that the placenta may prevent harm to the pregnancy by acting as a “sink” to sequester alloantibodies. It has been proposed that clearance of antibody-antigen immune complexes by Hofbauer cells, placental macrophages, may prevent transmission of maternal antibodies of fetal specificity through the placenta (reviewed in [37]). Additionally, if antibodies are able to enter the placental transport system, antibodies are degraded in low pH vesicle when excess antibody cannot bind already saturated Fc receptors [36]. Together these studies suggest the placenta may protect the fetus from antibody-mediated rejection.

We studied antibody formation during pregnancy and fetal intervention. We also sensitized mice against an alloantigen and asked whether alloantibodies are able to harm the future allogeneic pregnancy.

Using fetal LPS injections, we were able to study maternal alloantibody formation in the presence and absence of maternal exposure to fetal antigen. To examine the effect of Treg depletion on fetal health, previous reports examined fetal resorption at implantation sites towards the end of gestation [12, 14, 15]. In our model, we were interested in antibody levels in maternal serum for weeks after delivery. Therefore, we counted the number of pups after birth.

In this study, we show that Tregs inhibit anti-fetal alloantibody response. If Treg protection is disrupted, however, circulating alloantibodies do not prevent future pregnancy. Future studies identifying alternative mechanisms that prevent maternal alloantibodies from harming pregnancy will help determine if these mechanisms are altered during pregnancy complications.

Figures

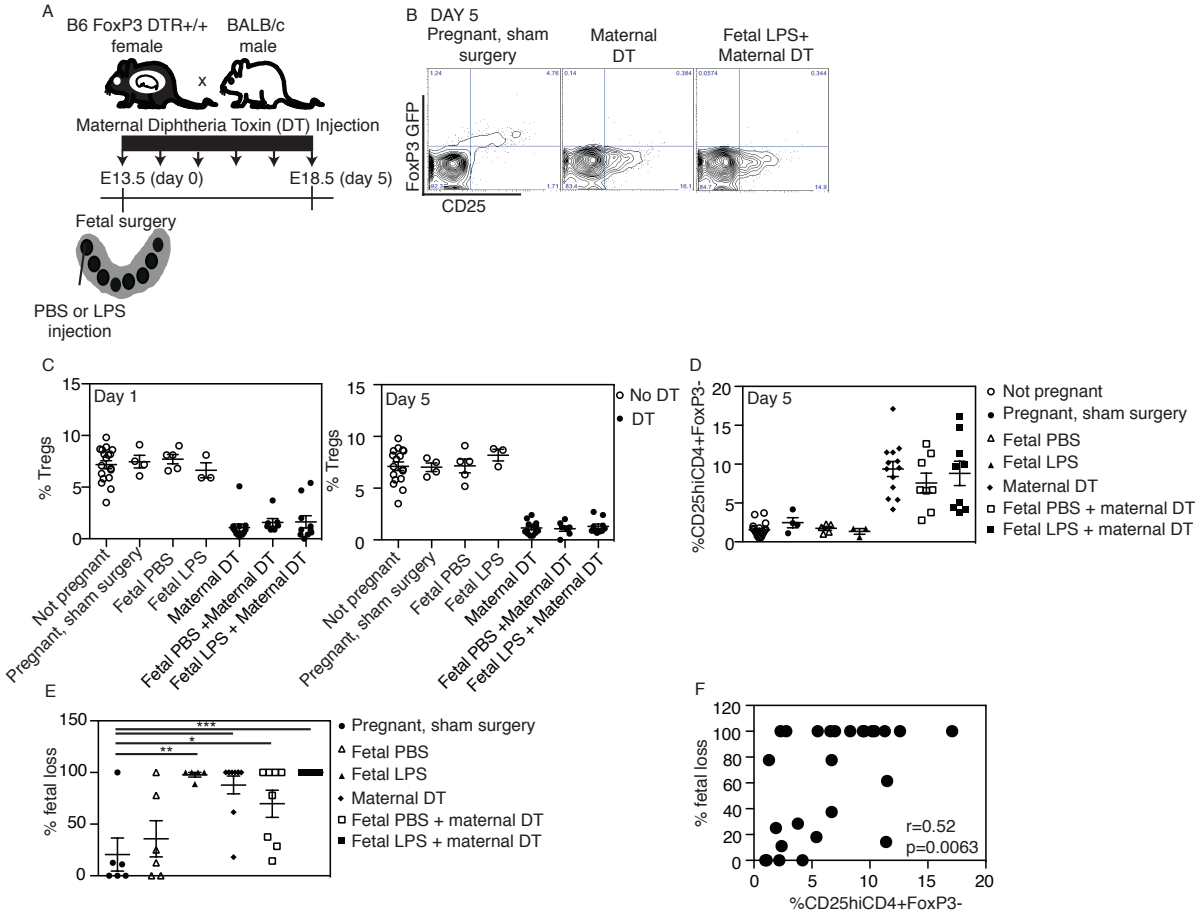

Figure 4.1. Treg depletion and resultant increase in circulating effector T cells. **(A)** Breeding scheme and methods used to deplete maternal Tregs (maternal diphtheria toxin (DT) treatment) for 6 days after fetal intervention (fetal PBS or LPS) on E13.5. **(B)** Flow cytometry plots showing successful depletion of maternal Tregs (FoxP3 GFP+CD25+) on day 5 in DT treated (center) and LPS and DT treated (right) compared to sham surgery in pregnant dams (left). **(C)** The percentage of CD25+FoxP3+ among all CD4+ T cells after DT and/or fetal intervention compared to controls (pregnant, sham surgery or fetal intervention alone) on day 1 and day 5. **(D)** The percentage of CD25+ T cells among all CD4+FoxP3- T cells on day 5 of DT treatment. **(E)** The percentage of fetal loss defined as the number of live pups born subtracted from the number of fetuses counted on E13.5 after DT and/or fetal intervention compared to controls (pregnant, sham surgery or fetal intervention alone). *$P < 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$ by ANOVA with Tukey's multiple comparison test. **(F)** A Pearson correlation was used to compare the fetal loss in pregnant (sham surgery), PBS injected, and PBS and DT injected groups and the percentage of effector T cells on day 5.

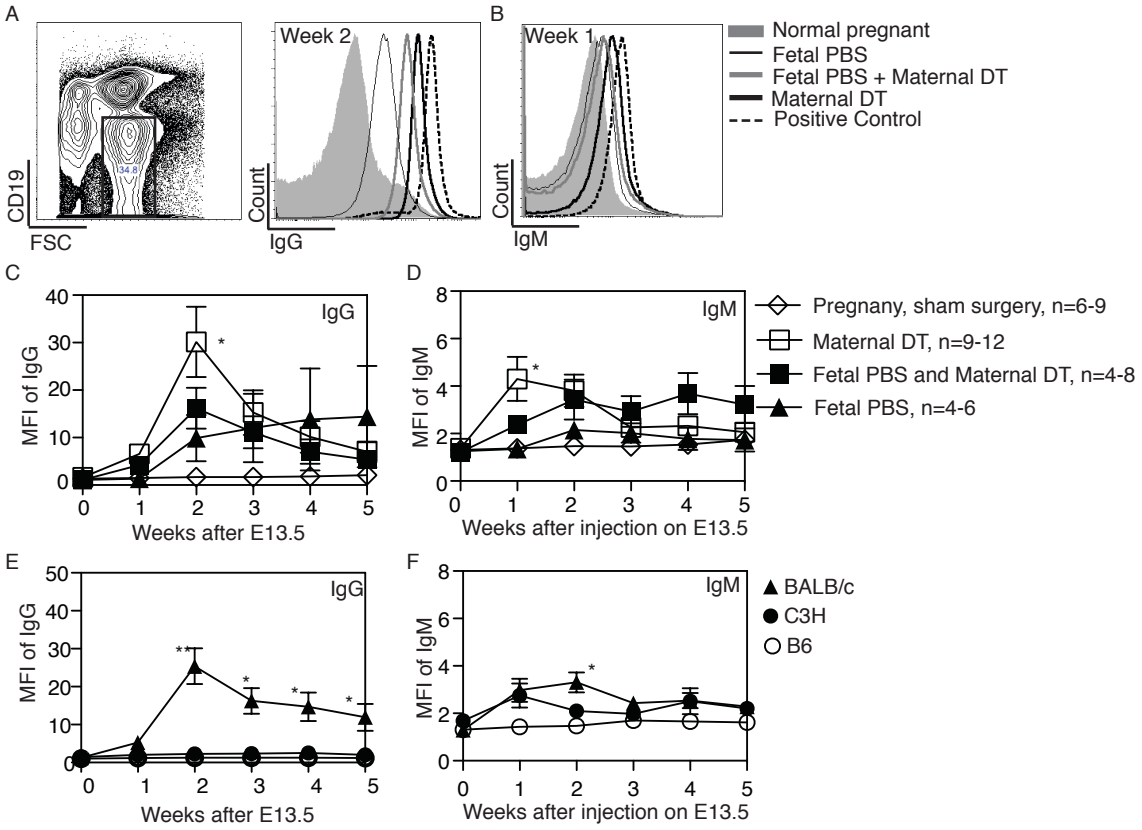

Figure 4.2. Increase in maternal alloantibodies with Treg depletion and fetal intervention. **(A-B)** Representative histogram to identify the mean fluorescence intensity (MFI) of **(A)** IgG and **(B)** IgM levels in maternal serum mixed with BALB/c LN and spleen after gating out CD19+ cells on A) week 2 or **(B)** week 1. C-D) MFI of allo-specific **(C)** IgG or **(D)** IgM in maternal serum in normal pregnant (n=7-11), fetal surgery (PBS, n=4-6), DT injection (n=4-6) and fetal surgery with DT injection (PBS, n=4-8) at weeks 0-5 post fetal surgery and/or DT injection. **P* < 0.05 between normal pregnant and DT injections by ANOVA with Tukey's multiple comparison test. E-F) Detection of **(E)** IgG and **(F)** IgM antibodies after culturing with BALB/c, B6 or C3H lymph node and spleen cells. **P* < 0.05, ** *P* ≤ 0.01 between BALB/c and B6 by ANOVA with Tukey's multiple comparison test. **(E)** **P* < 0.05 between BALB/c and C3H by ANOVA with Tukey's multiple comparison test on week 2 (not shown).

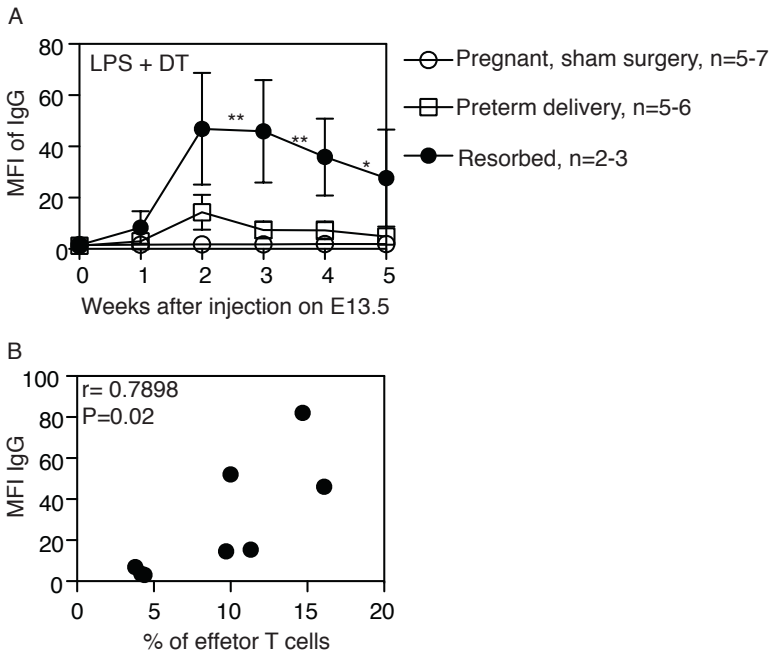

Figure 4.3. Effect of T cell activation and antigen exposure on alloantibody response. **(A)** The mean fluorescence intensity (MFI) of allo-specific IgG in maternal serum in normal pregnant (n=5-7) and fetal surgery (LPS) with DT injection (delivered early, n= 5-6; full term, n=2-3) at weeks 0-5 post fetal surgery and/or DT injection. $*P < 0.05$, $**P \leq 0.01$ between delivered early and full term by ANOVA with Tukey's multiple comparison test. **(B)** A Pearson correlation was used to compare the MFI of IgG in the fetal surgery (LPS) and DT groups two weeks after injection and the percentage of effector T cells 5 days post depletion.

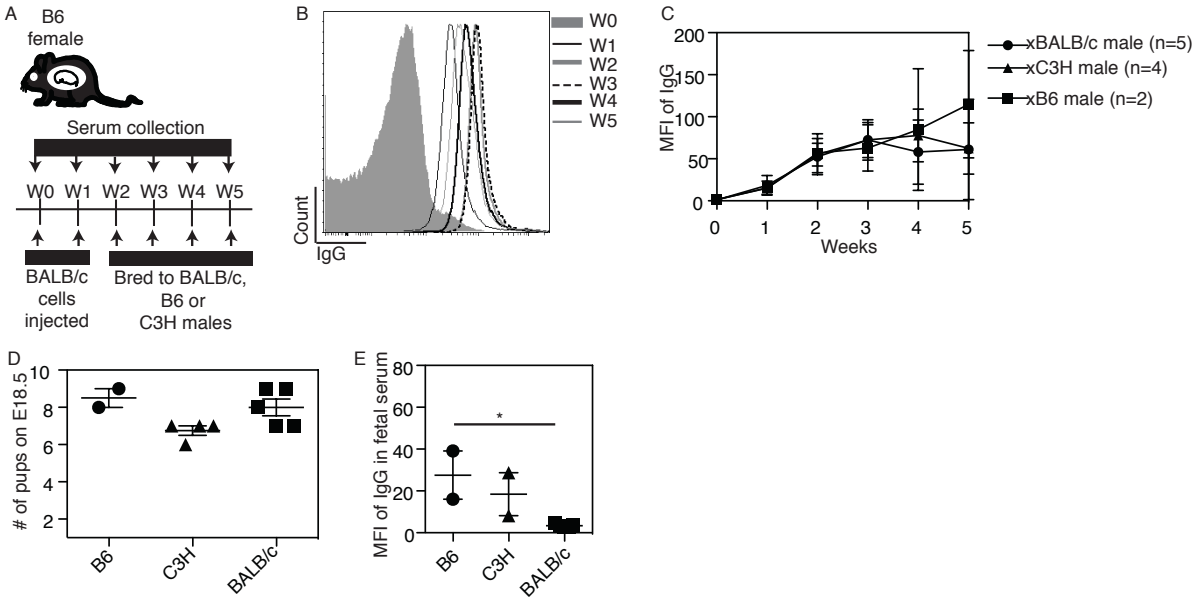

Figure 4.4. Maternal alloantibodies do not affect future pregnancy. **(A)** Schematic for breeding sensitized B6 females to BALB/c males. **(B)** Representative histogram and **(C)** mean fluorescence intensity (MFI) of IgG levels in serum of sensitized B6 females over a 5-week period. **(D)** The number of live fetuses found in the uterus on E18.5 after B6 sensitized females were bred to B6, C3H and BALB/c males. **(E)** The MFI of IgG in serum of pups born to B6 dams bred to B6, C3H and BALB/c males on E18.5. **P* < 0.05 by ANOVA with Tukey's multiple comparison test.

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CHAPTER 5

Conclusions & Future Directions

Chapter one of this dissertation outlines the various tolerance mechanisms protecting the fetus from maternal immune attack during pregnancy. Chapter two and three provide evidence for the breakdown of tolerance mechanisms during fetal intervention. Finally, Chapter four disrupts one tolerance mechanism to define its effect on the maternal immune response to the fetus and its effect on the success of pregnancy. Together the results in mice reveal tolerance mechanisms are required for a successful pregnancy and overlapping mechanisms may be in place to protect pregnancy in case one mechanism fails.

The future of mouse models studying maternal T cell awareness of the semi-allogeneic fetus

The strength of the studies presented in this thesis is that they examine maternal immune activation to the endogenous fetal antigen. Currently, the field has not identified the fetal antigen the maternal immune system is exposed to during pregnancy. Studies seeking to understand maternal immune recognition of fetal antigen during pregnancy have therefore used a model that allows them to use a known protein (ovalbumin) as a surrogate for the fetal antigen. In this model, wild-type females are bred to Act-mOVA-transgenic males, where the transmembrane form of ovalbumin (mOVA) is expressed from the β-actin promoter [1]. Therefore, ovalbumin is ubiquitously expressed in the conceptus with high levels shown in placental trophoblasts invading the decidua and maternal spiral arterioles [1]. Adoptively transferred transgenic T cells, OTI and OTII, proliferate upon exposure to ovalbumin [1]. Proliferation of OTI or OTII cells in this system represents maternal immune awareness of fetal antigen. However, the ubiquitous expression of ovalbumin may not reflect the endogenous expression of natural fetal antigens. Throughout this study, we have examined the maternal immune system to endogenously

expressing fetal antigens and found that maternal T cell proliferation in this context is lower than that observed in the ovalbumin model (Figure 2.3c versus Figure 6a of reference [1]) suggesting expression levels of fetal antigens in the ovalbumin model do not accurately represent endogenous levels. The field of reproductive immunology could benefit from studies focused on defining the origin (placental versus fetal) and identity of the antigen(s) (MHC versus tissue specific) that are responsible for activating the maternal T cells during pregnancy. The identity of the fetal or placental ("conceptus-derived") antigen will allow contribute to the growing knowledge of mechanisms in place to protect the fetus from maternal immune attack. This basic finding will also provide opportunities to develop novel models in which to more accurately study the role of the maternal immune response during pregnancy complications.

What can we learn from human studies about the role of the maternal immune system during fetal surgery induced preterm labor?

Future studies following up on this work should ask if breakdown of tolerance during fetal surgery results in preterm labor? Chapter two argues that breakdown of tolerance resulting in maternal T cell activation plays a role in mediating fetal demise in mice. This finding in mice provides support for a potential role of the maternal adaptive immune system during fetal surgery induced preterm labor. Using the mouse to study preterm labor in a model of fetal surgery is limited in that mice do not always undergo preterm labor after fetal intervention, as do humans. In mice, fetal intervention most often results in resorption of the fetus where the maternal immune system is directly exposed to fetal antigens. In humans, the lack of resorption may mean less direct exposure to fetal antigens, which may have a different outcome for maternal immune activation. Future studies in the lab are now focusing on human samples to understand the degree of reactivity of the maternal fetal peripheral blood mononuclear cells (PBMCs) to fetal antigens derived from fetal PBMCs. Using human samples, fetal

immune reactivity to maternal antigens can also be studied which was not performed in the studies outline in Chapter two in the mouse model. Unlike human fetuses, mouse fetuses have few T cells, which made it difficult to study the fetal T cell response to maternal antigens.

How are maternal cells getting into fetal blood and what is their function?

Our lab had previously shown that maternal cells are found in fetal blood during normal pregnancy in mice [2]. This finding was also found in humans and suggested to be mechanism by which the fetal immune system learns to tolerate maternal antigens during pregnancy [3]. Chapter three of this thesis work asked the question if an increased presence of maternal cells in fetal blood during fetal intervention may lead to fetal rejection and preterm labor. We examined trafficking during fetal intervention upon transplantation of allogeneic cells in utero and found enhanced maternal microchimerism in this setting. We also used a mouse model of preterm labor and found a similar result suggesting that enhanced maternal microchimerism in fetal blood is associated with fetal intervention and preterm labor in mice. These findings are in agreement with a human study that found patients undergoing open fetal surgery for myelomeningocele have elevated levels of maternal microchimerism [4].

Future studies using microscopy are needed to determine how maternal cells are gaining access to the fetal circulation. They should focus on the two maternal-fetal interfaces outlined in the introduction of this work. One interface is located between the maternal decidua and “conceptus-derived” trophoblast giant cells and the other interface is located in the placental labyrinth where maternal blood comes into direct contact with “conceptus-derived” endovascular trophoblast cells (mononuclear trophoblasts and a continuous double-layer of syncytiotrophoblasts). A histological analysis of these locations during fetal intervention and preterm labor may provide insights into where and how maternal cells are crossing into the fetus. Transcript levels of adhesion molecules and chemokines can be assessed by qRT-PCR and

in-situ hybridization while protein levels can be determined by immunofluorescent localization and immunoblot of placentas from mice undergoing preterm labor. Adhesion molecules and chemokines chosen for this study would include those implemented in neutrophil and T cell trafficking into various tissues during inflammation and found to be expressed at the maternal-fetal interface during normal pregnancy in mice and humans. Disruption of the chemokine(s) and/or adhesion molecule(s) preferentially expressed in PTL placentas using small molecule inhibitors or neutralizing antibodies would help determine which chemokine(s) and/or adhesion molecule(s) are required for maternal cell trafficking.

An understanding of how maternal cells gain access to fetal circulation can further help elucidate the function significance of enhanced maternal microchimerism during pregnancy complications. Using the small molecule inhibitor or neutralizing antibody effective in blocking maternal trafficking in a mouse model of preterm labor will determine if trafficking causes preterm labor.

A novel function for the placenta: protecting the fetus from maternal immune rejection

In Chapter four, we made the interesting discovery that Tregs prevent maternal allo-antibody production during pregnancy. However, despite the presence of these antibodies in maternal circulation, they had no effect on the pregnancy or the health of the fetus. This result suggests alternative mechanisms are in place that may prevent these antibodies from harming the pregnancy. One potential mechanism may involve the placenta. Normally during pregnancy, maternal IgG antibodies transport occurs upon binding to neonatal Fc receptor (FcRn) on the placenta [5]. IgG present in the maternal circulation is taken up by endocytosis by syncytiotrophoblast cells in the placenta. In the endosome, they are able to bind FcRn receptors in a pH-dependent manner [5] which protects them from being degraded by the acidic environment. Upon fusing with the membrane on the fetal end of the syncytiotrophoblast cells, the more basic pH promotes dissociation of IgG from the FcRn receptor [5].

High levels of antibodies results in saturation of FcRn receptors and degradation of excess antibodies [5]. Therefore, the placenta plays a key role in the regulation of the amount of antibodies that are able to cross from the mother and into the fetus. Although maternal antibodies are important for supporting the newborn's immune system in fighting infection early in life, in rare cases pathological antibodies can also harm the baby as seen in Rh disease. It is also possible the placenta may have a mechanism to capture and degrade antibodies that may harm the fetus, making diseases such as Rh disease rare. Future studies could focus on determining if maternal antibodies are binding and getting degraded by the placenta to inhibit transport across to the fetus.

During fetal intervention, maternal antibodies could not be detected in the circulation, even in the absence of Tregs. This interesting result may be due to the release of fetal antigen into the maternal circulation that binds to antibodies to form antigen-antibody immune complexes. These complexes have been shown to bind follicular dendritic cells (FDCs) to induce maternal T cell tolerance to fetal antigens [6]. These complexes can also persist for weeks after delivery and may play a role in preventing antibody-mediated immune attack of the future pregnancy.

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