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Fetal Intervention Increases Maternal T Cell Awareness of the Foreign Conceptus and Can Lead to Immune-Mediated Fetal Demise

Marta Wegorzewska,*† Amar Nijagal,*† Charissa M. Wong,*† Tom Le,*† Ninnia Lescano,*† Qizhi Tang,‡ and Tippi C. MacKenzie*†

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 postsurgical pregnancy complications has not been explored. We recently showed that fetal intervention in mice increases maternal T cell trafficking into the fetus and hypothesized that this process also may lead to increased maternal T cell recognition of the foreign conceptus and subsequent breakdown in maternal–fetal tolerance. In this study, we show that fetal intervention in mice results in accumulation of maternal T cells in the uterus and that 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 with endogenous T cells in the uterus and uterine-draining lymph nodes. Finally, we demonstrate that 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 that maternal T cells are necessary for this phenomenon. These results suggest that fetal intervention enhances maternal T cell recognition of the fetus and that T cell activation may be a culprit in postsurgical pregnancy complications. Our results have clinical implications for understanding and preventing complications associated with fetal surgery such as preterm labor. The Journal of Immunology, 2014, 192: 000–000.

Fetal surgery is a promising strategy to treat fetuses with severe or fatal congenital anatomic anomalies such as diaphragmatic hernia 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). Although 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 semiallogeneic fetus from the maternal immune system (reviewed in Refs. 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 Ref. 9). For example, it has been demonstrated that maternal T cells recognize the fetal allograft primarily using the relatively inefficient “indirect” pathway of Ag presentation (in which fetal Ag is presented by maternal APCs) and that these indirectly reactive T cells undergo clonal deletion after activation (10). Directly reactive T cells (which recognize Ag presented by fetal APCs) represent a higher percentage of alloreactive T cells (11) but are not activated in normal pregnancy. Pregnancy also is associated with an increase in maternal regulatory T cells (Tregs) (12–19) whose loss leads to elimination of the semiallogeneic fetus (13–15, 17–19). However, it is not known whether these maternal–fetal tolerance mechanisms are thwarted after fetal intervention, leading to recognition and rejection of the fetus by maternal T cells. Because fetal surgery can trigger PTL without overt infection, it is possible that inflammation from surgical trauma can activate maternal T cells.

Although patients experience PTL after fetal surgery, murine fetal intervention instead results in resorption of some of the fetuses in the litter. Resorption also has 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 semiallogeneic 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 Ag-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 Ag. These results suggest that medical interventions to inhibit maternal T cells could be beneficial in treating pregnancy complications after fetal intervention.

Materials and Methods

Reagents and Abs

The following reagents were used: Invitrogen Vybrant CFDA SE Cell Tracer Kit (CFSE; Invitrogen), Flou-Paque Plus (GE Healthcare), annexin V (BD Pharmingen), Qdot 605 streapavidin conjugate (Invitrogen), Foxp3 staining buffer set (eBioscience), DAPI (Invitrogen), LPS 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), DAPI (Roche), collagenase D (Roche), Alexa Fluor 488 goat anti-rat IgG Ab (Life Technologies), Triton X-100 (Sigma-Aldrich), BSA (Bio Scientific), goat serum (Jackson Immunoresearch Laboratories), sucrose (Fisher Scientific), Tissue-Tek OCT Compound (VWR), PMA (Sigma-Aldrich), immunogenic (Sigma-Aldrich), and brefeldin A (Sigma-Aldrich).

The following Abs for flow cytometry were purchased from BD Biosciences: CD3 (145-2C11), CD8 (53-6.7), CD19 (1D3), CD45 (30-F11), CD45.1 (A20), CD45R/B220 (RA3-6B2), H-2Kb (AF6-48.8.5), H-2Kd (SF1-1.1), H-2Kq (36-7.5), I-A^d^ (AMS-32.1), Thy1.1 (HIS51), V β8 (F23.1), V β13 (MR12-3), Ki67 (B56), NK1.1 (PK136); eBioscience: CD4 (RM4-5), CD8 (53.6.7), CD45.1 (A20), CD45.2 (A104), CD25 (PC6.5), Foxp3 (FJK-16s), IL-17A (eBio1B7), TNF-α (MP6-XT22), IFN-γ (XM12.1), CD11c (N418), and IgG2a,k isotype control (eBm2a); Southern Biotechnology Associates: CD8 (53-6.7); University of California, San Francisco (UCSF) Hybridoma Core: Gr-1 (RB6-8C5), FcR (2.4G2); BioLegend: CD25 (3C7), H-2L^d^ (28-14-8), IgG2a,k isotype control (MOPC-173); and Abcam: CD3 (RM0027-3B19).

Mice

The inbred strains, BALB/c, C3H-HeJCr, C57BL/6.CD45.2 (B6), and the F1 hybrid strain B6 × BALB/c (F1) were obtained from either the National Cancer Institute or The Jackson Laboratory. BALB/c and C57BL/6.CD45.1 were obtained from The Jackson Laboratory (UCSF), and B6-Thy1.1.CC (2C), B6-Thy1.1.4C (4C), and B6.Thy1.1.TCR75 (TCR75) mice were obtained from Dr. S. M. 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 μl/fetus in all experiments) directly into the fetal liver using pulled glass micropipettes as previously described on embryonic day (E)13.5–14.5 (22, 23). The pregnant dam was anesthetized, a laparotomy was performed, and the fetuses were injected into the intact uterus. For LPS injections, a dose of 0.5 μg was diluted among the total fetuses in the litter. We based our LPS concentration on a reported model of intrauterine injection of LPS (24) and performed titration experiments to achieve a dose that resulted in the loss of the litter within 40 h without maternal death (0.5 μg; 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 (22), and 2.5 × 10^6 cells were injected into each fetus. For experiments involving survival of the semiallogeneic and syngeneic fetuses, peripheral blood leukocytes were stained for H-2^d^ and H-2^b^ to genotype the surviving BALB/c (H-2^b^) 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 Nitróx filter. Tissues surrounding each fetus were processed separately with DNase I (5 μg/ml) and collagenase D (400 U/ml) to make a single-cell suspension. Cells also were harvested from spleens, uterine draining lymph nodes (udLN; para-aortic), and nondraining lymph nodes (ndLN; 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 nonresorbed uterine segments within a dam were pooled to obtain adequate cell numbers. After the samples were stained with the indicated Abs, they were analyzed on a LSRII flow cytometer using FACSDiva or FlowJo software.

*Intracellular cytokine stain*

Maternal lymphocytes were stimulated with PMA (70 ng/ml) and ionomycin (70 ng/ml) for 3 h, treated with brefeldin A (200 mg/ml) for 2 h, and then stained for flow cytometry.

*Proliferation of adoptively transferred fetal Ag-specific lymphocytes*

Whole lymphocytes were harvested from the spleen and lymph nodes of TCR75, 2C, or 4C mice and labeled with CFSE. A total of 1–5 × 10^6 T cells were adoptively transferred i.v. into pregnant females at E12.5, followed by injection of PBS or LPS into the fetuses 1 d 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 5 × 10^7 BALB/c splenocytes i.v. prior to adoptive transfer for 2C or 4C cells.

*Statistics*

Differences between two groups were evaluated using either a χ^2^ test (for changes in survival) or a Student *t* test (or Mann–Whitney *U* test, for nonnormally distributed data) and those among more than two groups were evaluated using ANOVA with Tukey’s multiple comparison test (or Kruskal–Wallis test for nonnormally distributed data) using Graphpad Prism. A *p* value < 0.05 was considered significant. Data are summarized as means ± SEM.

*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 PBS to study the effect of surgical trauma alone, or with LPS, to study the effect of trauma alone with a strong inflammatory stimulus on fetal survival (Table I). Baseline resorption in this allogeneic strain combination is low, and we observed increased fetal loss with PBS injection in syngeneic matings compared with 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 with syngeneic (χ^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 (χ^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 T effector cells 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 (Fig. 1A). Because some fetuses are resorbed after injection while littermates are not, we an-
alyzed the uterus surrounding resorbed fetuses (“resorbed uterus”) separately from the uterus surrounding live fetuses (“live uterus”) (Fig. 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 described previously (Fig. 1A, 1B) (22).

We first analyzed the uterine T cell composition to detect changes in effector and Treg subsets (Fig. 1B). The numbers of conventional Foxp3−CD4 T cells (Tconv) and CD8 cells increased after fetal intervention, with significant increases in resorbed uteri compared with uninjected (Fig. 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 cell subsets after fetal intervention (Fig. 1D, 1E). CD25 expression on CD4 T cells further increased in resorbed compared with live uteri, suggesting increased activation of effector cells in this setting (Fig. 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 with live uteri, such that the overall Teff/Treg ratio was significantly increased in resorbed uteri (Fig. 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 (Fig. 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 Treg balance.

We also analyzed other leukocyte populations in the uterus and found increases in the percentage of Gr-1+ myeloid cells (both Gr1low [monocytes] and Gr1high [neutrophils]) after fetal intervention (Supplemental Fig. 1). There were no differences in the percentages of NK cells, B cells, or dendritic cells between groups (Supplemental Fig. 1).

Table I. Complementary models of fetal intervention in mice

<table>
<thead>
<tr>
<th>Models of Fetal Intervention</th>
<th>Rationale</th>
<th>Percentage of Resorption</th>
<th>No. of Litters (L)/No. of Fetuses (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>Baseline</td>
<td>Syngeneic, 3.2 ± 2</td>
<td>7L/58F</td>
</tr>
<tr>
<td>PBS injection</td>
<td>Sterile inflammation</td>
<td>Syngeneic, 1.5 ± 1</td>
<td>12L/114F</td>
</tr>
<tr>
<td>LPS injection</td>
<td>Strong inflammatory stimulus</td>
<td>Syngeneic, 34 ± 7</td>
<td>20L/155F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allogeneic, 44 ± 6*</td>
<td>43L/355F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allogeneic, 79 ± 4</td>
<td>32L/252F</td>
</tr>
</tbody>
</table>

n ≥ 4 independent experiments in each group.

*χ² = 0.04 between syngeneic and allogeneic matings undergoing fetal PBS injection; χ² = NS between syngeneic and allogeneic matings in uninjected and LPS-injected groups.

FIGURE 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 d 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, Tregs, and CD8 T cells per milligram of tissue in various experimental conditions. Representative histograms (D) and the mean fluorescence intensity (MFI) (E) of CD25 on Tconv, Tregs, and CD8 T cells in uninjected, live, and resorbed uteri. (F and G) The absolute numbers of total (F) or Ki67+ (G) Teffs and CD25+ Tregs per milligram 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 by Kruskal–Wallis test with Dunn’s post hoc comparison.
Increased IFN-γ 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 udLNs after fetal PBS injection, stimulated them with PMA and ionomycin, and stained them for the intracellular cytokines IFN-γ, TNF-α, and IL-17 (IFN-γ and TNF-α in a representative experiment shown; Fig. 2A). We found a significant increase in the percentage of IFN-γ-producing CD4 T cells and a trend for increased percentage of TNF-α-producing cells in the uterus after fetal intervention (Fig. 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 (Fig. 2B). There were no changes in IL-17 production with fetal intervention (Supplemental Fig. 2). Thus, maternal T cells resident in the uterus can assume an effector phenotype after fetal intervention.

We also examined cytokine production (IFN-γ, TNF-α, 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 (Supplemental Fig. 2).

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 Ags. To determine whether maternal T cell activation after fetal intervention is Ag specific, we adoptively transferred fetal Ag-specific T cells from TCR transgenic mice and analyzed their proliferation and accumulation in maternal tissues after fetal intervention. Maternal T cells may recognize fetal Ag 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 Ag (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 Ag (H-2Kd) 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 (Fig. 3A). We first confirmed that the Ag recognized by TCR75 T cells is expressed in BALB/c fetal liver and placenta at E13.5–E14.5 (Fig. 3B), consistent with the detailed analysis of H-2Kd and H-2Dd 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 ndLNs (Fig. 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 (Supplemental Fig. 3) but may instead represent occasional spontaneous resorption.

We next asked whether Ag-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 (Fig. 3D). Proliferation was Ag specific, because there was no proliferation in syngeneic pregnancies even in the presence of LPS (Fig. 3D, 3E). This TCR model was very sensitive in detecting any fetal Ag release after fetal intervention: TCR75 proliferation was robust with both PBS and LPS injection (Fig. 3E, Supplemental Fig. 4), 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 Ags. Unlike the proliferation pattern observed in the udLNs, TCR75 cells in spleens and ndLNs were either not proliferated or highly proliferated (Fig. 3D), suggesting migration of TCR75 cells that were initially activated and proliferated in the udLNs.

In normal pregnancy, fetal Ag-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 nonproliferated TCR75 cells in the udLNs of mothers with normal pregnancy were apoptotic (Annexin V+, DAPI−), but this percentage decreased significantly during fetal intervention (Fig. 3F). Apoptosis decreased only in TCR75 cells in the udLNs but not in other lymphoid organs (Fig. 3F).

We next asked whether the adoptively transferred Ag-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 Ag-specific cells over endogenous CD4 cells (Fig. 3G). Similar increases in the proportion of TCR75 cells were seen in the udLNs but not in placenta, ndLNs or spleens (Fig. 3G). We also detected some TCR75 cells in the decidua, which is normally protected from maternal T cell infiltration (Fig. 3G) (29). Thus, fetal intervention leads to an enrichment of Ag-specific cells in the uterus.

**FIGURE 2.** Increased IFN-γ 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-γ (top panel) and TNF-α (bottom panel). Maternal tissues were harvested 5 d after fetal PBS injection in an allogeneic mating. Percentages obtained in one representative experiment shown. (B) The percentage of Tconv producing a given cytokine in uterine segments and udLNs harvested from pregnant dams with or without fetal injection of PBS. Each point represents tissues from one dam. n = 4 dams/group in n ≥ 4 independent experiments. *p < 0.05 by ANOVA with Tukey’s multiple comparison test.
and udLNs. Collectively, our results suggest that fetal intervention leads to changes in normal tolerance mechanisms that usually prevent proliferation, migration, and accumulation of Ag-specific T cells.

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

Maternal T cells also can 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 with indirectly reactive T cells (11), they are not activated in normal pregnancy, and the fetus is therefore protected from the majority of maternal Ag-specific T cells (10). We asked whether fetal intervention can increase Ag 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 Ag I-Ad, whereas 2C mice have CD8 T cells that recognize BALB/c class I Ag H-2Ld, both presented “directly” by fetal BALB/c APCs. 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 Ag-specific T cells using flow cytometry. To maximize the possibility of detecting fetal Ag exposure, we used the fetal LPS injection model, which leads to higher rates of resorption (Table I). We first analyzed the expression of the Ags recognized by these transgenic T cells in fetal tissues on E13.5–E14.5 and determined that I-Ad (recognized by 4C) is present in the placenta but not in the fetal liver (Fig. 4A), whereas H-2Ld (recognized by 2C) is present both in the placenta and in the fetal liver (Fig. 4D). We found no increase in the proliferation of 4C

[FIGURE 3. Fetal intervention results in activation, proliferation, and accumulation of Ag-specific maternal T cells. (A) Experimental design and gating strategy to track proliferation of adoptively transferred fetal Ag-specific TCR75 (CD4+Thy1.1+) cells after fetal intervention. TCR75 proliferation is detected as dilution of CFSE. (B) Expression of MHC class I Ag H-2Kd in BALB/c fetal liver (top panel) and placenta (bottom panel) on E13.5–E14.5. Adult BALB/c lymph nodes (top panel) and an isotype Ab (top and bottom panels) were used as controls. n ≥ 3 in each group in three separate experiments. (C and D) Representative proliferation profiles of TCR75 cells in the udLNs (top row), spleens (middle row), and 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 four separate experiments. *p < 0.05, **p = 0.01 by Kruskal–Wallis test with Dunn’s post hoc comparison. (F) The percentage of apoptotic cells (Annexin V+DAPI+) among proliferated (CFSElow) and nonproliferated (CFSEhigh) TCR75 cells in the udLNs, spleens, and ndLNs (Allo pregnant, n = 4, Allo LPS, n ≥ 3 dams in three separate experiments). *p < 0.05 by Student 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 three separate experiments. *p < 0.05 by Student t test.]
which exposes the mother to a higher dose of the Ag 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 antifetal immune response could be read out as increased survival of semiallogeneic pups compared with syngeneic litters. We bred BALB/c females to B6 × BALB/c (F1) males such that half of the resulting fetuses expressed the foreign paternal Ag H2-Kd and were semiallogeneic to the mother, whereas half were syngeneic to the mother (F1 and BALB/c pups, respectively) (Fig. 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 (Fig. 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 semiallogeneic 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 semiallogeneic pups. However, after in utero transplantation with hematopoietic cells from B6 mice, which carry the paternal Ag, we observed a striking decrease in the percentage of surviving semiallogeneic fetuses, resulting in a 2:1 ratio of syngeneic to semiallogeneic pups. As another control, we performed breedings in which the mother was B6 × BALB/c F1, such that neither the fetuses nor the transplanted B6 cells are allogeneic to the mother. Consistent with our hypothesis, resorption rates were significantly lower in this group compared with the experimental (paternal Ag 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 Ag only when the same paternal Ag 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 semiallogeneic pups after fetal intervention. We bred BALB/c.TCRαD3−/− females (which lack T cells) to B6 × BALB/c F1 males and performed in utero transplantation with B6 hematopoietic cells. We found that the survival of semiallogeneic fetuses was not affected by in utero transplantation when the mother lacks T cells (1:3:1 ratio; Fig. 5B). We have noted high overall rates of surgical complications in immunodeficient dams (including Rag knockout 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 Ag.

**Discussion**

In this study, we tested the hypothesis that fetal intervention perturbs maternal–fetal tolerance, leading to activation of fetal Ag-specific maternal T cells. We first showed that fetal PBS injection leads to higher rate of fetal resorption in allogeneic matings compared with 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-γ. Using an adoptive transfer model, we demonstrated that fetal intervention results in activation and proliferation of Ag-specific maternal T cells, which escape apoptosis and accumulate in the uterus. We also showed that maternal T cells can exacerbate demise of semiallogeneic fetuses after in utero transplantation of additional paternal Ag. The finding that maternal T cells become activated after fetal intervention and can
compared with no foreign Ag group. NA, Not applicable (no fetal intervention).

In utero transplantation with hematopoietic cells from B6 mice, which express the paternal Ag, or from third-party mice (C3H), which express a different allogeneic Ag. (*compared after in utero transplantation with hematopoietic cells from B6 mice, which express the paternal Ag, or from third-party mice (C3H), which express a different allogeneic Ag. (A) Breeding scheme to detect a functional maternal immune response. BALB/c mothers are bred to F1 (BALB/c × B6) fathers and the survival of BALB/c and F1 littersmates is compared after in utero transplantation with hematopoietic cells from B6 mice, which express the paternal Ag, or from third-party mice (C3H), which express a different allogeneic Ag.

In normal pregnancy, multiple overlapping mechanisms keep maternal T cells in check such as lack of direct Ag presentation (10), physical entrapment of dendritic cells in the uterus (34), chemokine gene silencing (29), and dominant suppression by Tregs (12–19). In our adoptive transfer experiments, we detected TCR75 proliferation only when fetal alloantigen is present and not in settings to show that fetal alloantigens are critical to the maternal immune response. We also have observed increased trafficking of maternal T 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 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 Teff less sensitive to Treg suppression (37).

In many models of pregnancy complications, it is difficult to distinguish the effects of nonspecific inflammation from a true Ag-specific immune activation. Resorption in our fetal intervention model is multifactorial and includes a component of nonspecific inflammation because 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 with 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 among animals, comparing the survival of syngeneic and semiallogeneic pups within the same litter allows a more accurate quantification of an allospecific immune response. It is interesting that despite 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 OVA observed at baseline (10) and postinfection (36) and provide further information regarding the response to an MHC Ag expressed physiologically. We also have performed a detailed analysis of immune cells in the uterus and udLNs to show that there is Ag-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 Ags 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 after fetal surgery (4), and its true incidence is likely higher than the reported rate because 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. Although it has been suggested that the indirect pathway of Ag 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, because 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 decidua 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 at postinfection-induced immune ac-
tivation (36). Although the mouse fetoplacental unit is signifi-
cantly different from the human, it is likely that clinical fetal
surgery also would expose maternal T cells to fetal and placental
AgS and enhance maternal T cell recognition of the fetus. Fur-
thermore, 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 ac-
tivation 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 ac-
tivated 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 PTU.

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Supplementary Figure 1: (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.
Supplementary Figure 2: (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-γ, TNF-α, and IL-17 by CD4+Foxp3+CD25+ Tregs in the uterus. Percentages obtained in one representative experiment from 4 independent experiments shown.
Supplementary Figure 3: (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.
Supplementary Figure 4: The percentage of proliferated TCR75 cells among total TCR75 cells after PBS injection in the uterine draining lymph node (udLN), spleen, and non-draining lymph nodes (ndLN). N ≥ 4 dams in each group in ≥ 3 separate experiments. *P < 0.05 by Mann-Whitney test.