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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 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). 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 allreactive 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 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 semiallo-
genic 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 pheno-
type. Furthermore, maternal T cells can exacerbate selective de-
mise 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), Fc Gil-Paque PS (GE Healthcare), annexin
V (BD Pharmingen), Qdot 605 streptavidin conjugate (Invitrogen), Foxp3
staining buffer set (eBioscience), DAPI (Invitrogen), LPS from Salmomella
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 gmo (Molecular
Probes), Tritton X-100 (Sigma-Aldrich), BSA (Fish Scientific), goat serum (Jackson Immunoresearch Laboratories), sucrose
(Fisher Scientific), Tissue-Tek OCT Compound (VWR), PMA (Sigma-
Aldrich), ionomycin (Sigma-Aldrich), and brefeldin A (Sigma-Aldrich).

The following Abs for flow cytometry were purchased from BD Bio-
sciences: CD3 (145-2C11), CD8 (53-6.7), CD19 (1D3), CD45 (30-F11),
CD45.1 (A20), CD45R/B220 (RA3-6B2), H-2Kb (AF6-88.5), H-2Kd (SF1-
1.1), H-2K (36-7-5), I-A<sup>d</sup> (AMS-32.1), Thy1.1 (HIS51), V<sub>B</sub>8 (F23.1),
V<sub>B</sub>13 (MR12-3), K<sub>67</sub> (B6K), NK1.1 (PK136); eBioscience: CD4 (RM4-5),
CD8 (53-6.7), CD45.1 (A20), CD45.2 (104), DC25 (PC5.6.5), Foxp3 (FJK-
16b), IL-17A (eBio17B7), TNF-α (XMG1.2), CD11c (N418), and IgG2a isotype control (eBM2a); Southern Biological Associates: CD8 (53-6.7); University of California, San Francisco (UCSF)
Hybrimaon Core: Gr-1 (RB6-8C5), FcR (2.4G2); BioLegend: CD25 (3C7),
H-2<sup>d</sup> (28-14-8), IgG2a, isotype control (MOPC173); and Abcam: CD3
(TM0027-3B19).

Mice

The inbred strains, BALB/c, C57BL/6, C3H-HeJCr, C57BL/6.CD45.1 (B6), and the
F<sub>I</sub> hybrid strain B6×BALB/c (F<sub>I</sub>) were obtained from either the National Cancer Institute or The Jackson Laboratory. BALB/c CD45.1 was obtained from
the University of California, San Francisco (UCSF), and B6.Thy1.1.2C (2C), B6.Thyl.1.1.4C
(4C), and B6.Thy1.1.1TCR75 (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 pro-
tocol. 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 micro-
pipettes as previously described on embryonic day (E)13.5–14.5 (22, 23).
The pregnant dam was anesthetized, a laparotomy was performed, and the
uterus from the fetus and placenta; deciduas could be further separated from
the placenta 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 µg/ml) and collag-
ense D (400 U/ml) to make a single-cell suspension. Cells also were har-
vested from spleens, uterine draining lymph nodes (udLNs; para-aortic), and
nondraining lymph nodes (ndlLNs; inguinal, axillary, brachial, and mes-
enteric). 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 num-
bers. 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 ion-
omycin (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 1×10<sup>6</sup> 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
uterine 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<sup>5</sup> 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<sup>2</sup>
-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 posttest, for nonnormally distributed data) using Graphpad Prism. A p value < 0.05 was considered significant.

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 (al-
logeneic) males and injected the fetuses with PBS to study the effect of surgical trauma alone, or with LPS, to study the effect of trauma along 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 com-
pared with syngeneic (χ<sup>2</sup> = 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 stim-
ulus, there was near-total resorption in most experiments, which
precluded discerning a difference between syngeneic and allogeneic
matings (χ<sup>2</sup> = 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 func-
tional 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 ALB/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 littersmates 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/255F</td>
</tr>
</tbody>
</table>

\[ n \geq 4 \text{ independent experiments in each group.} \]
\[ * \chi^2 = 0.04 \text{ between syngeneic and allogeneic matings undergoing fetal PBS injection; } \chi^2 = \text{NS between syngeneic and allogeneic matings in uninjected and LPS-injected groups.} \]

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 \( n \geq 7 \) uterine segments in \( \geq 2 \) independent experiments and each point represents a uterine segment surrounding one fetus. \( \* p < 0.05, \* \* p \leq 0.01, \* \* \* p \leq 0.001 \) 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-2Kb) 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-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 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 placentas, 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.
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 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 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 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.

![Image](http://www.jimmunol.org/)
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, because 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 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 decreased survival of semiallogeneic pups compared with syngeneic littersmates. 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). Un.injected 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.TCRα−/− 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

FIGURE 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 Ag I-A^d, the Ag recognized by 4C mice, in BALB/c fetal liver (top panel) and placenta (bottom panel) on E13.5–E14.5 compared with 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 d 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 udLNs, spleens, and ndLNs. (D) Expression of MHC class I Ag H-2L^d (recognized by 2C mice) in BALB/c fetal liver (top panel) and placenta (bottom panel) on E13.5–E14.5 compared with 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.
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 Ag presentation (10), physical entrapment of dendritic cells in the uterus (34), chemokine gene silencing (29), and dominant suppression by Tregs (12–19). Fetal intervention may increase Ag presentation to maternal T cells through the release of fetal Ag into the maternal circulation. 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 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 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 settings to show that fetal Ags are critical to the maternal 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 in 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
37. Darrasse-Jeze, G., D. Klatzmann, F. Charlotte, B. L. Salomon, and J. L. Cohen. 2006. CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus re-
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 (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.