JCI The Journal of Clinical Investigation

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*J Clin Invest.* 2009;119(9):2590-2600. https://doi.org/10.1172/JCI38979.

**Research Article** **Immunology**

The lack of fetal immune responses to foreign antigens, i.e., fetal immunologic tolerance, is the most compelling rationale for prenatal stem cell and gene therapy. However, the frequency of engraftment following in utero hematopoietic cell transplantation (IUHCT) in the murine model is reduced in allogeneic, compared with congenic, recipients. This observation supports the existence of an immune barrier to fetal transplantation and challenges the classic assumptions of fetal tolerance. Here, we present evidence that supports the presence of an adaptive immune response in murine recipients of IUHCT that failed to maintain engraftment. However, when IUHCT recipients were fostered by surrogate mothers, they all maintained long-term chimerism. Furthermore, we have demonstrated that the cells responsible for rejection of the graft were recipient in origin. Our observations suggest a mechanism by which IUHCT-dependent sensitization of the maternal immune system and the subsequent transmission of maternal alloantibodies to pups through breast milk induces a postnatal adaptive immune response in the recipient, which, in turn, results in the ablation of engraftment after IUHCT. Finally, we showed that non-fostered pups that maintained their chimerism had higher levels of Tregs as well as a more suppressive Treg phenotype than their non-chimeric, non-fostered siblings. This study resolves the apparent contradiction of induction of an adaptive immune response in the pre-immune fetus and confirms the potential of actively […]



# Maternal alloantibodies induce a postnatal immune response that limits engraftment following in utero hematopoietic cell transplantation in mice

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**The lack of fetal immune responses to foreign antigens, i.e., fetal immunologic tolerance, is the most compelling rationale for prenatal stem cell and gene therapy. However, the frequency of engraftment following in utero hematopoietic cell transplantation (IUHCT) in the murine model is reduced in allogeneic, compared with congenic, recipients. This observation supports the existence of an immune barrier to fetal transplantation and challenges the classic assumptions of fetal tolerance. Here, we present evidence that supports the presence of an adaptive immune response in murine recipients of IUHCT that failed to maintain engraftment. However, when IUHCT recipients were fostered by surrogate mothers, they all maintained long-term chimerism. Furthermore, we have demonstrated that the cells responsible for rejection of the graft were recipient in origin. Our observations suggest a mechanism by which IUHCT-dependent sensitization of the maternal immune system and the subsequent transmission of maternal alloantibodies to pups through breast milk induces a postnatal adaptive immune response in the recipient, which, in turn, results in the ablation of engraftment after IUHCT. Finally, we showed that non-fostered pups that maintained their chimerism had higher levels of Tregs as well as a more suppressive Treg phenotype than their non-chimeric, non-fostered siblings. This study resolves the apparent contradiction of induction of an adaptive immune response in the pre-immune fetus and confirms the potential of actively acquired tolerance to facilitate prenatal therapeutic applications.**

## Introduction

One of the predictions of Burnet and Fenner's theory of immunity (1) is that prenatal exposure to foreign antigens prior to the development of the immune system should lead to tolerance rather than immunization. Billingham, Brent, and Medawar experimentally confirmed this prediction by inoculation of murine fetuses with cellular material from another mouse strain, which led to what they termed "actively acquired tolerance" (2). Additional support for the concept was provided by observations in numerous species of hematopoietic chimerism and associated tolerance in dizygotic twins that share placental circulation (3–8). Finally, mechanistic insight into tolerance for self-antigens (and, by inference, foreign antigens), and the central role of the thymus in this process, has been provided by numerous studies, primarily in TCR transgenic mice, over the past 2 decades (9, 10).

The potential for strategies based on actively acquired tolerance to facilitate organ or cellular transplantation was immediately appreciated (2) but has not been clinically achieved. One such strategy is in utero hematopoietic cell transplantation (IUHCT), an approach that has, as of yet, unfulfilled promise for the treatment of congenital hematologic disorders (11). The assumption that fetal tolerance will be permissive of allogeneic IUHCT is a primary rationale for this strategy and follows naturally from the classic observations outlined above. The primary events required for tolerance of self-antigen occur in the developing thymus and consist of positive- and negative-selection events that result in the clonal deletion of developing T cells with high-affinity recognition of self-antigen as well as the maintenance of a repertoire of T cells reactive to foreign antigen. The assumption has been that introduction of allogeneic cells by IUHCT, prior to completion of the thymic processing of self-antigen, would mimic self-antigen and result in clonal deletion of alloreactive lymphocytes and secondary permanent donor-specific tolerance.

We recently demonstrated in a murine model of IUHCT that there is an unequivocal and dramatic difference in the frequency of engraftment in allogeneic compared with congenic recipients (12). This observation strongly suggests the presence of an adaptive immune response as a barrier to engraftment after IUHCT and challenges the assumption of fetal tolerance as a facilitator of IUHCT. If the observed difference in frequency of chimerism is due to an adaptive immune response, we hypothesized that chimeric and non-chimeric recipients of allogeneic IUHCT would have quantitative differences in their allospecific humoral and effector T cell response. In the present study, we confirm the presence of an adaptive immune response in murine allogeneic recipients of IUHCT that lose their chimerism after IUHCT and the absence of that response in animals that maintain hematopoietic chimerism. Unexpectedly, we also demonstrate a maternal immune response after IUHCT that appears after delivery of the pups. Furthermore, we show that the immune response in the recipients is entirely dependent on breast feeding from the immunized mother, and that the period of loss of chimerism corresponds to the appearance of maternal alloantibodies. We

**Conflict of interest:** The authors have declared that no conflict of interest exists.
**Citation for this article:** *J. Clin. Invest.* **119**:2590–2600 (2009). doi:10.1172/JCI38979.

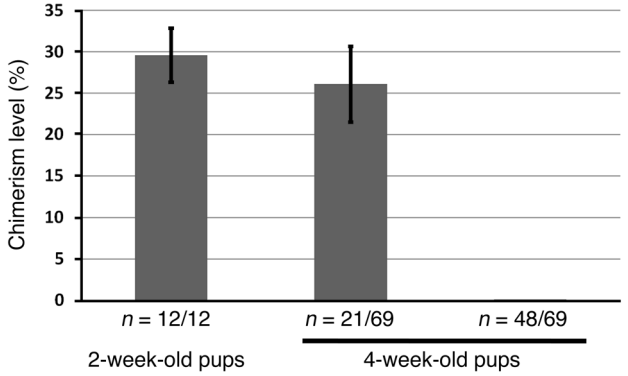


**Figure 1**
Peripheral blood chimerism levels at 2 and 4 weeks of age. Donor cell chimerism was assessed as the percentage of CD45$^+$ cells that were GFP$^+$ by flow cytometry, with chimerism being defined as more than 1% GFP$^+$. Data are mean ± SEM.

further show that transfer of maternal serum to fostered pups is sufficient to induce loss of chimerism, supporting an indirect mechanism by which transfer of maternal alloantibodies in breast milk induces a postnatal, allospecific immune response in the chimeric pup. Finally, we show that non-fostered pups that maintain their chimerism have higher levels of Tregs, as well as a more suppressive Treg phenotype, compared with their non-chimeric, non-fostered siblings. These findings explain the apparent contradiction of activation of an immune response in the pre-immune fetal recipient and confirm, in the absence of maternal immunization, the potential efficacy of strategies based on actively acquired tolerance for facilitation of allogeneic cellular or organ transplantation.

## Results

*Non-chimeric pups exhibit an increased frequency of alloreactive T cells.* To elucidate the role of an effector T cell response in the loss of allogeneic chimerism after IUHCT, we performed allogeneic IUHCT at E14 from MHC-H2K$^{b+}$GFP$^+$ C57BL/6TgN(act-EGFP)OsbY01 mouse donors (referred to as "B6GFP mice") into MHC-H2K$^{d+}$ BALB/c recipient fetuses. In this model, we have previously demonstrated that while all recipients are initially engrafted, 70% of recipients lose their donor chimerism between 2 and 4 weeks of age (5 weeks after IUHCT) (12). We confirmed our previous findings of 100% chimerism at 2 weeks of age. In a separate cohort of recipients, peripheral blood hematopoietic chimerism was determined by flow cytometry at 4 weeks of age, and the pups were divided into 2 groups, chimeric and non-chimeric (Figure 1). The effector T cell response to donor cells was measured in each group using the in vivo mixed lymphocyte reaction (MLR) (13). While quantitative, this method does not provide a true frequency of alloreactive cells, but rather allows a sensitive and quantitative assessment of the relative frequency of allospecific T cells between 2 comparable groups. After determination of chimerism, lymphocytes from BALB/c recipient mice were harvested, stained with CFSE dye, and adoptively transferred to CB6F1 (H2K$^{b+/d+}$) recipients, generating a parent into F1 MHC mismatch in which BALB/c T cells allospecific for MHC-H2K$^{b+}$GFP$^-$ C57BL/6 (referred to as "B6") donor cells would proliferate against the F1 host. F1 recipients were euthanized at 24, 48, 72, and 96 hours after the adoptive transfer, and CFSE-stained CD4$^+$ H2K$^{b-}$ lymphocytes were analyzed by flow cytometry. Cell division began after 24 hours following adoptive transfer, and maximal discernible proliferation consisting of 8 rounds of cell division was present by 96 hours (Figure 2, A and B). To evaluate background proliferation, BALB/c lymphocytes were adoptively transferred into syngeneic BALB/c recipients and demonstrated negligible proliferation (0.02%) in this system (data not shown). The relative frequency of alloreactive T cells was calculated at 96 hours (13) (see Methods), and the frequency of alloreactive T cells in non-chimeric mice was 6.32% ± 0.92% as compared with 1.63% ± 0.66% in chimeric mice ($P$ = 0.006), indicating the presence of an allospecific cellular response in the non-chimeric pups (Figure 2C). Lymphocytes from naive and B6-immunized BALB/c mice were also assayed as negative (4.47% ± 0.45%; $n$ = 5) and positive (8.57% ± 0.33%; $n$ = 5) controls, respectively. These data are consistent with the expected frequency of between 1% and 10% of peripheral T cells that recognize an alloantigen (14). Alloreactive lymphocytes from chimeric pups were also present at a significantly lower frequency than were alloreactive lymphocytes from naive pups ($P$ < 0.001), suggesting a mechanism of partial clonal deletion of allospecific lymphocytes with associated donor-specific tolerance.

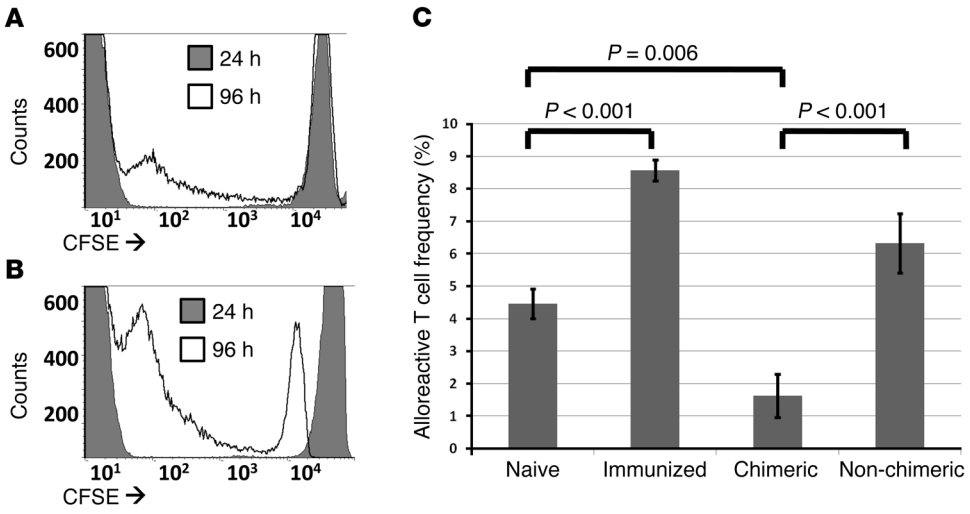


**Figure 2**
T cell alloreactivity in vivo. Flow cytometry of adoptively transferred CD4$^+$H2K$^{b-}$ CFSE$^+$ lymphocytes from (**A**) chimeric pups and (**B**) non-chimeric pups. Each tracing is representative of 5 independent experiments. (**C**) Frequency of alloreactive T cells in naive (negative control), immunized (positive control), chimeric, and non-chimeric BALB/c pups 5 weeks after B6 IUHCT. Data are mean ± SEM.

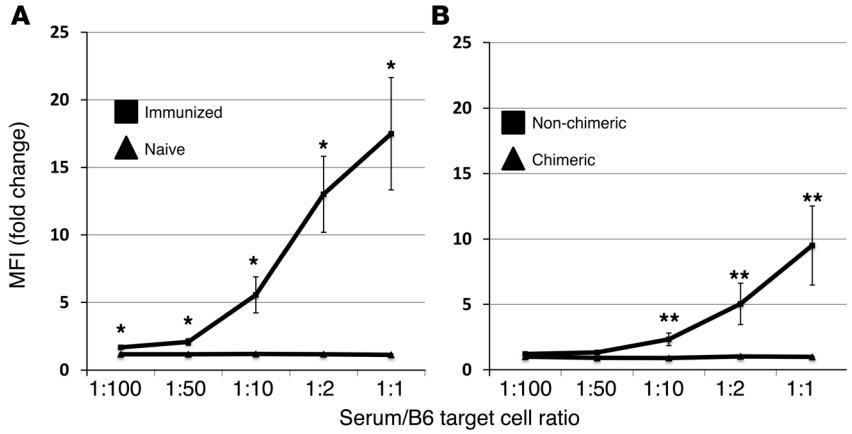


**Figure 3**
Alloantibody assay in chimeric and non-chimeric mice. (**A**) Control mice showed a significant difference in alloantibody formation at all ratios of serum to target cells. (*$P < 0.01$). (**B**) Non-chimeric BALB/c pups ($n = 20$) showed evidence of alloantibody formation as compared to chimeric pups ($n = 10$) at ratios of 1:1, 1:2, and 1:10 (**$P < 0.001$). Data are mean ± SEM.

Analysis of $CD8^+$ lymphocytes revealed a higher frequency of alloreactive lymphocytes with a $CD8^+/CD4^+$ cell ratio of 2:1 but a similar pattern of proliferation on in vivo MLR. To confirm that our analysis included all of the transferred alloreactive cells, we analyzed all of the hematopoietic compartments of the assay mice and found that the $CFSE^+$ cells were restricted to the spleen and lymph nodes, with no $CFSE^+$ lymphocytes detected in the peripheral blood, bone marrow, and thymus (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38979DS1). We also measured serum cytokine levels in non-chimeric pups relative to those in naive pups and IUHCT mothers. While levels of IL-4 were undetectable in all groups, levels of IL-2, IL-10, and IFN-γ were elevated in the non-chimeric pups and immunized mothers compared with naive controls ($P < 0.01$), with the greatest difference seen in IFN-γ, thus indicating a predominantly Th1 profile in the non-chimeric pups (Supplemental Figure 1). To assess the potential contribution of anergy or suppression to our findings of tolerance in fostered and non-fostered chimeric pups, we assessed reactivity to third party donors and the effect of the addition of IL-2 to in vitro MLR. Both groups demonstrated normal reactivity to third party cells, excluding anergy as a mechanism for tolerance. However, donor-specific tolerance to B6 cells was partially overcome with the addition of IL-2, supporting the presence of suppression by regulatory populations (15) (Supplemental Figure 2).

*Non-chimeric pups have circulating alloantibodies*. For assessment of the humoral immune response, serum was isolated from 4-week-old chimeric and non-chimeric BALB/c pups after IUHCT. After incubation of serum with B6 target cells and subsequent staining with anti-IgG secondary antibody, the magnitude of the humoral response was determined by the mean anti-IgG fluorescence intensity using flow cytometry. Naive BALB/c serum and serum from B6 immunized BALB/c mice were used as negative and positive controls, respectively, and showed a significant difference ($P < 0.01$) at all serum to target cell ratios (Figure 3A). At a 1:1 ratio of serum to target cells, the fold increase in anti-IgG immunofluorescence was 9.50 ± 3.02 ($n = 20$) in non-chimeric mice as compared with 1.00 ± 0.04 ($n = 10$) in chimeric mice ($P < 0.001$), demonstrating clear evidence of anti-donor alloantibody formation in the non-chimeric pups compared with the chimeric pups (Figure 3B).

*Injected dams are sensitized against allogeneic donor cells*. The presence of an immune response in non-chimeric pups suggested that either the fetus was not tolerant or the maternal immune system had been sensitized and had directly or indirectly caused the loss of chimerism in the pups. We therefore analyzed injected dams for evidence of sensitization to donor cells after IUHCT. We utilized the in vivo MLR assay described above to assess the

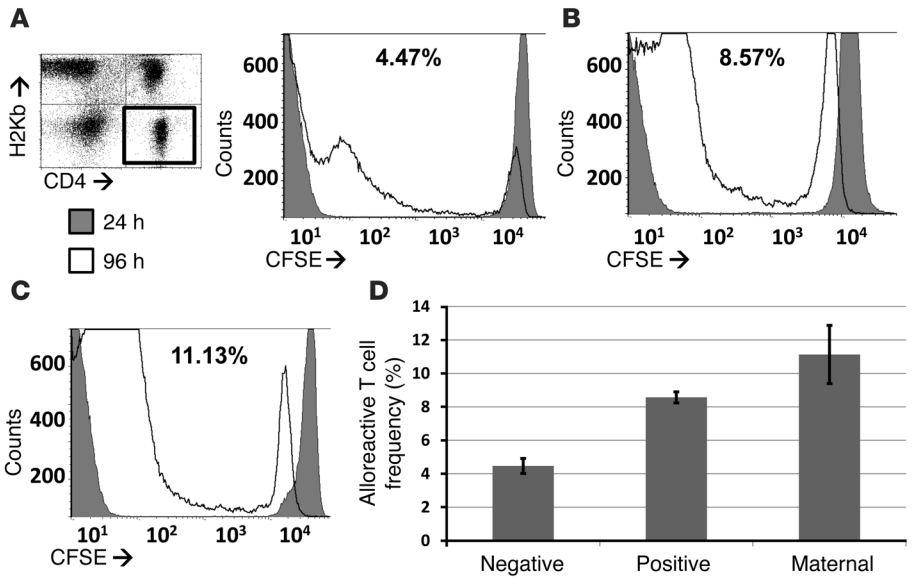


**Figure 4**
Maternal T cell alloreactivity in vivo and frequency of alloreactive T cells. (**A**) Flow cytometry of $CD4^+H2K^{b-}$ $CFSE^+$ lymphocytes. (**A** and **B**) Histogram overlay representing CFSE profile at the 24-hour time point (gray histogram) and 96-hour time point (white histogram) for (**A**) naive (negative control) and (**B**) immunized (positive control). (**C**) Histogram overlay for injected dams. (**D**) Frequency of maternal alloreactive T cells (11.13% ± 1.74%) compared with positive and negative controls. Data are mean ± SEM. Each tracing represents at least 5 independent experiments.

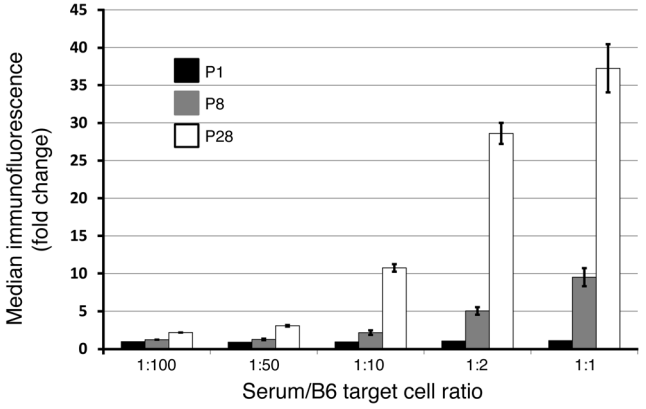


**Figure 5**
Maternal alloantibody assay. Median anti-IgG immunofluorescence representing maternal alloantibodies at 1, 2, and 5 weeks after IUHCT. Data are median ± SEM.

frequency of alloreactive maternal T cells and found that there was no statistically significant difference in the frequency of alloreactive T cells between injected dams and immunized positive controls (Figure 4), which was consistent with maternal sensitization by donor cells after IUHCT.

We next analyzed the maternal humoral response. Alloantibodies were found in maternal serum but did not appear until approximately 2 weeks after IUHCT, when the pups were 1 week of age (Figure 5). This suggested that if in fact the maternal immune system was causative in the adaptive immune response in the pups, this response would occur during the postnatal period, implicating maternal breast milk as a likely mode of transmission. Interestingly, antibody levels increased dramatically between 2 and 5 weeks after IUHCT, which corresponds with the time period when chimerism is lost (12). Upon analysis of anti-B6–specific maternal alloantibody by class and subclass, we saw a predominance of IgG2a and, to a lesser extent, IgM and IgG2b (Figure 6). This correlated with the anti-B6–specific alloantibody profile in non-chimeric pups and suggested a mechanism of maternal-fetal antibody transmission, as IgG2a and IgG2b are known to be preferentially transferred in rodent breast milk (16). Additionally, we found that the magnitude of the maternal humoral response strongly influenced the loss of chimerism in pups, as non-chimeric pups, on average, were found to have been exposed to significantly higher levels of maternal alloantibodies than chimeric pups ($P = 0.0002$) (Supplemental Figure 3).

The 2 most likely sources of maternal sensitization are leakage of donor cells into the maternal peritoneal cavity during injection and exposure to injected cells upon re-absorption of aborted fetuses. By keeping all injected dams in separate cages and counting the number of fetuses that were injected and then subsequently born, we were able to gain some insight into the mechanism of maternal sensitization. Statistically significant correlations were noted between the magnitude of the maternal humoral response and both the number of aborted fetuses per litter ($n = 24$, $r = 0.547$, $P = 0.0057$) and the total number of fetuses injected per litter ($n = 23$, $r = 0.546$, $P = 0.0070$) (Supplemental Figure 4). Only 3 of 24 litters were born without fetal loss, and 2 of those 3 injected dams still developed alloantibodies, suggesting that while fetal loss is strongly associated with maternal immunization, it is not required.

To better define this relationship, we next performed a series of experiments designed to differentiate between the effects of i.p. leakage of donor cells and re-absorption of donor cells within aborted fetuses. To explore the effect of i.p. leakage, we performed a midline laparotomy and then injected an appropriate volume of B6 donor cells onto the uterine horns to mimic leakage of either 10% or 25% of the volume normally injected during our standard IUHCT. To test exposure to fetal antigens during fetal loss, we utilized an F1 model (BALB/c female crossed with B6 male), in which we performed a midline laparotomy without IUHCT, with transient occlusion of the uterine blood supply, resulting in miscarriage of 10 of 10 fetuses. Upon analyzing maternal serum for evidence of anti-B6 alloantibodies, we found that both i.p. leakage of donor cells and exposure to fetal antigens during fetal loss were sufficient to generate maternal alloantibodies (Figure 7). Therefore, the source of maternal immunization appeared to be the combined effect of leakage of cells into the maternal peritoneal cavity during IUHCT and reabsorption of cells following fetal loss. This mechanism is consistent with previously described mechanisms of reproductive immunology in which paternal antigens are able to induce a pathologic immune response (17, 18).

*Postnatal exposure to maternal breast milk results in loss of chimerism.* Having shown that the injected dams are sensitized against donor cells during IUHCT, we next attempted to ascertain whether the immune response in pups was in fact induced by maternal influence or whether both mother and fetus had been independently sensitized. The timing of the maternal humoral response allowed isolation of the pups from postnatal maternal influence through the use of non-injected foster dams. Upon substitution of non-injected foster dams, we found that 100% (22/22) of fostered pups maintained their chimerism (Table 1), with stable levels of chimerism 6 months after IUHCT (Supplemental Figure 5). In contrast, only 30.4% (21/69) of pups nursed by dams injected on E14 maintained their chimerism at 5 weeks after IUHCT, consistent with previous data (12). This striking result demonstrates that in the absence of a maternal immune response, fetal immunologic tolerance is uniformly permissive of hematopoietic engraftment across MHC barriers after IUHCT and definitively identifies postnatal exposure to maternal breast milk as a requirement for loss of chimerism. Immunologic analysis of the fostered pups confirmed an

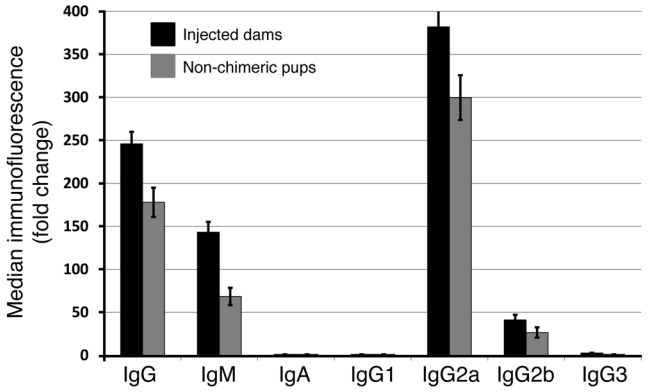


**Figure 6**
Alloantibody class and subclass. Serum from injected BALB/c dams and non-chimeric BALB/c pups was analyzed for anti-B6 alloantibody at the time of loss of chimerism (5 weeks after IUHCT). Data are median ± SEM.

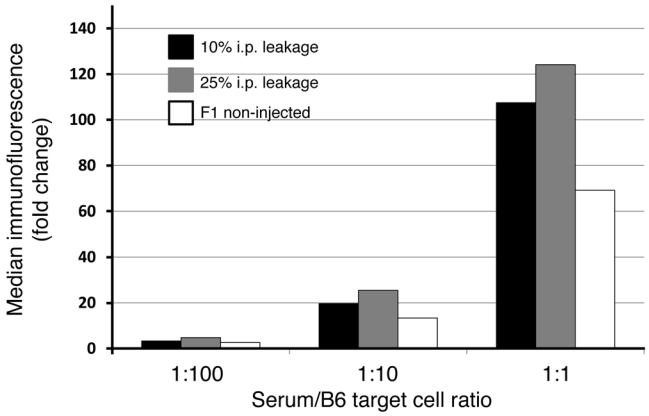


**Figure 7**
Maternal immunization. Evidence of anti-B6 alloantibodies in BALB/c dams that did not undergo IUHCT, after i.p. injection of 10% or 25% of the standard B6 IUHCT cell dose, as well as one F1 dam (BALB/c female × B6 male) following fetal loss and re-absorption of 10 F1 fetuses.

absence of alloantibodies as well as donor-specific tolerance, as represented by a frequency of alloreactive T cells of 0.81% ± 0.33% ($n$ = 5), which was significantly lower than that seen in naive mice ($P$ = 0.0002), again consistent with at least partial clonal deletion of donor-reactive cells. The mean level of chimerism in fostered pups was 30.96% (range, 10.24%–73.96%), which was not significantly different from the level of chimerism in chimeric pups nursed by dams injected on E14.

To further confirm the findings of the fostering experiment, we bred BALB/c males with B6 females and performed E14 IUHCT using B6GFP donor cells. Because donor cells were of maternal origin, we hypothesized that there would be no maternal sensitization and that, similar to the fostering experiment, all of the pups would maintain their chimerism. On analysis, we found no evidence of maternal antibody formation or T cell alloreactivity, and all pups (20/20) were chimeric (Supplemental Figure 5). This finding further supported our conclusion that the donor cells were responsible for maternal immunization and that it was the maternal immune response that caused loss of chimerism in the pups.

*Maternal lymphocytes are not required for loss of chimerism.* There are 2 possible explanations for our data: the maternal immune cells might be directly involved in ablation of chimerism, or transfer of maternal antibodies might indirectly induce the immune response in the pups. We hypothesized that if maternal cells are directly participating in donor cell rejection, then maternal cells should be present in the lymphocyte population from non-chimeric pups and should proliferate in an in vitro MLR assay. To allow discrimination of maternal cells in this assay, we bred BALB/c females with B6 males and performed IUHCT on E14 using B6GFP donor cells. We then isolated lymphocytes from the spleen, lymph nodes, peripheral blood, bone marrow, and thymuses of non-chimeric 4-week-old pups after IUHCT and performed an in vitro MLR assay using CFSE labeling to assess proliferation against irradiated B6 stimulator cells. The utilization of the F1 model allowed us to gate on only $H2K^{b-}$ cells, excluding all cells except those of maternal origin. Congenic B6 cells were used as a negative control and showed no evidence of bystander proliferation (Figure 8A). Our data show proliferation by $CD4^+H2K^{b+/d+}$ T cells from non-chimeric pups (Figure 8B) as well as proliferation by $CD4^+H2K^{b-}$ T cells from dams injected on E14 (Figure 8C). However, we found no proliferation of $H2K^{b-}$ cells in the non-chimeric pups (Figure 8D), showing that maternal-fetal cellular trafficking is minimal and direct maternal cell-mediated ablation of donor cells is not responsible for loss of chimerism. Examination of the bone marrow, peripheral blood, lymph node, and thymus compartments excluded sequestration of maternal cells, as these tissues also lacked $H2K^{b-}$ lymphocytes (Supplemental Figure 6).

Although this experiment suggested that primary ablation of donor cells by maternally derived lymphocytes was unlikely, it did not rule out the possibility that a low frequency of maternally derived lymphocytes were serving in a costimulatory or antigen-presenting capacity. Therefore, in order to prove that transfer of maternal lymphocytes was not required for immune activation, we isolated serum from injected dams and administered it to fostered pups after IUHCT. Interestingly, i.p. administration of maternal serum resulted in a 50% frequency of chimerism (3/6 pups chimeric), while oral administration of maternal serum resulted in complete loss of chimerism (0/3 pups chimeric) (Table 2). These experiments established the fact that serum alone was capable of transferring the maternal immune response to pups and that transfer of maternal cells was not required.

*$CD4^+CD25^+$ Tregs are more prevalent in non-fostered chimeric pups and exhibit a more suppressive phenotype.* The existence of a maternally derived immune response still failed to explain the fact that many litters contained both chimeric and non-chimeric pups, despite ingestion of the same breast milk. Although previous studies had shown a correlation between the level of maternal humoral response and a loss of chimerism, this pattern alone does not explain how some pups in a litter can be chimeric while others in the same litter are non-chimeric. We hypothesized that enhanced peripheral regulatory mechanisms controlled by Treg populations resulted in suppression of the alloimmune response in these mice. To investigate this hypothesis, we first measured the levels of Tregs in naive, fostered, and non-fostered chimeric and non-chimeric pups, and we found that the non-fostered chimeric pups had higher levels of $CD4^+CD25^+$ Tregs ($P < 0.001$) in all tissue compartments except the spleen ($P$ = 0.06) (Figure 9). These cells were confirmed by FACS to be more than 85% $FoxP3^+$. We next analyzed the functional suppressive ability of these cells in vitro, and we found that the non-fostered chimeric Tregs exhibited an enhanced ability to suppress effector T cell alloimmune reactivity ($P$ = 0.001), as compared with naive, fostered, and non-fostered non-chimeric Tregs (Figure 10).

## Discussion

Given the current understanding of the mechanistic basis of fetal tolerance, one would anticipate that with appropriate timing and mode of antigen administration, consistent donor-specific tolerance would be achievable by a primary mechanism of clonal deletion of donor-reactive lymphocytes. However, historically that has not been the case. Even in Billingham, Brent, and

**Table 1**
Effect of maternal breast feeding on the frequency of chimerism after IUHCT

| Mouse | Chimeric | Non-chimeric |
|---|---|---|
| IUHCT-injected dam | 30.4% (21/69) | 69.9% (48/69) |
| Non-injected foster dam | 100% (22/22) | 0% (0/22) |

Mice were analyzed 5 weeks after IUHCT.

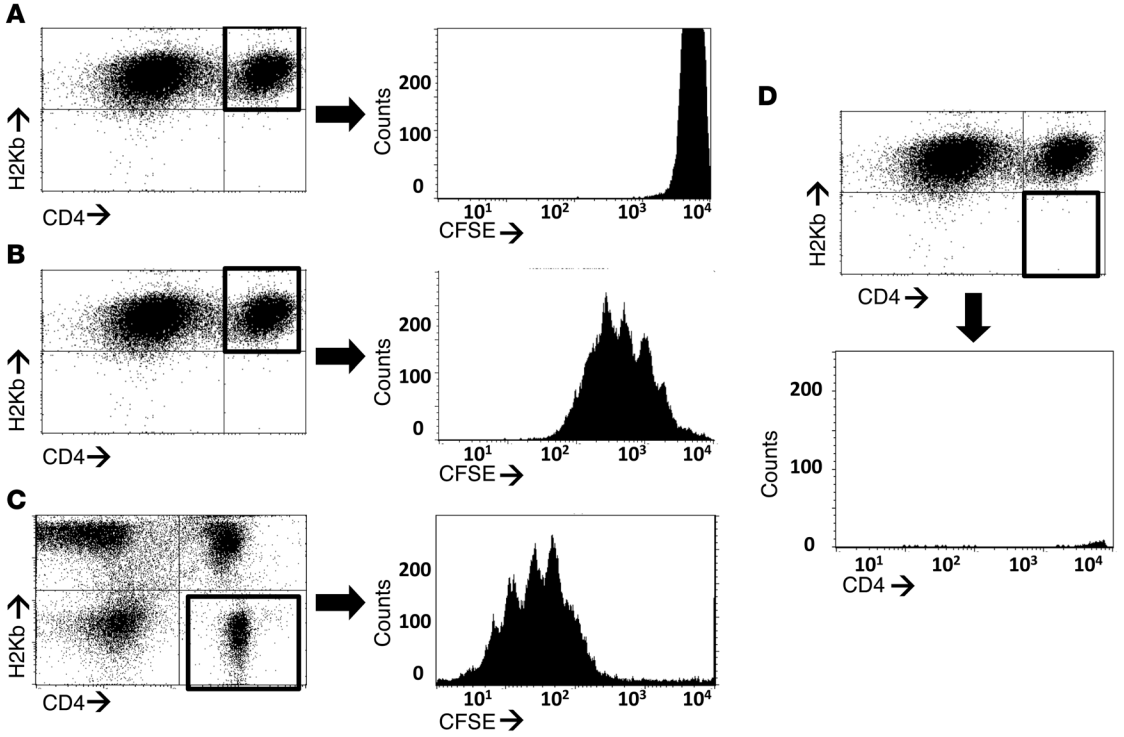


**Figure 8**
Analysis of the origin of alloreactive lymphocytes in non-chimeric pups. (**A**) $CD4^+H2K^{b+}$ lymphocytes were harvested from congenic B6 mice. (**B**) $CD4^+H2K^{b+}$ lymphocytes were harvested from F1 neonatal recipients. (**C**) $CD4^+H2K^{b-}$ lymphocytes were harvested from E14 injected BALB/c dams. (**D**) A negligible number of $H2K^{b-}$ maternal cells were derived from F1 recipients. Each histogram is representative of at least 8 independent experiments.

Medawar's original report (2), only 3 of the 5 *CBA* strain mice born after prenatal injection of cells at E15 from *A* strain mice demonstrated evidence of tolerance. They concluded from that experiment and their larger experience with many injections in fetal and neonatal mice that "the conferment of tolerance is not of an all-or-nothing character; every degree is represented." Subsequent studies by many investigators on fetal and neonatal tolerance have documented the entire spectrum of immune response from donor-specific tolerance to immunization. Part of that confusion can historically be explained by the lack of recognition of mechanistic differences between fetal and neonatal tolerance in mice. Thus, observations made in neonatal mice demonstrating the ability to mount mature immune responses given an appropriate presentation of antigen have been used as an argument against the validity of actively acquired tolerance (19–21). These findings, as well as observations of immunization in large animal models (22, 23) and humans (24) after relatively late administration of antigen, can be easily attributed to missing the window of opportunity for central tolerance induction. More difficult to explain are failures of engraftment and associated donor-specific tolerance induction after IUHCT when the procedure has been performed at an early developmental time point (prior to the emergence of mature lymphocytes in the thymus and peripheral circulation), when one would anticipate appropriate thymic processing of antigen. In the mouse, this period exists prior to E17 (25). In the murine model of allogeneic IUHCT, when transplants have been performed at E14–E15, numerous studies have reported failure of engraftment or of only microchimerism (26–34), inconsistent or absent tolerance induction (26, 31, 35), or immunization to alloantigen after IUHCT (30, 33). Similarly, studies of organ transplantation after IUHCT with minimal levels of chimerism in large animal studies have shown an absence of tolerance (36), incomplete tolerance (37), or donor-specific tolerance (38). Interpretation of the results from both the murine and large animal studies has been complicated by marginal levels of engraftment or concerns about inconsistent delivery of donor cells to the fetus.

The most convincing argument that engraftment in the fetus is not limited by an immune barrier was the early observation in the murine model by ourselves and others that there was no significant engraftment advantage for congenic versus allogeneic cells (28, 31, 34, 39). However, in retrospect, those studies were misleading because of the minimal levels of chimerism present and a low, and sometimes transient, frequency of chimerism, which made interpretation difficult. We were able to overcome the low levels of chimerism in the model by increasing the number of donor cells to achieve consistent levels of chimerism easily measurable by flow cytometry. With higher levels of chimerism we demonstrated consistent association of donor-specific tolerance with levels of donor hematopoietic chimerism of greater than 1%–2% as determined by skin grafting or the ability to boost postnatal engraftment with a minimal conditioning bone marrow transplant from the donor strain (40–42). Chimerism and tolerance were associated with reduced frequencies of donor-specific lymphocytes, consistent with a mechanism of partial clonal deletion, supporting the absence of an adaptive immune barrier to IUHCT (26, 41). However, although

**Table 2**
Ability of immunized maternal serum to induce loss of chimerism

| Mouse | Chimeric | Non-chimeric |
|---|---|---|
| Non-injected foster dam | 100% (22/22) | 0% (0/22) |
| Non-injected foster dam plus i.p. serum from IUHCT-injected dam | 50% (3/6) | 50% (3/6) |
| Non-injected foster dam plus oral serum from IUHCT-injected dam | 0% (0/3) | 100% (3/3) |

Mice were analyzed 5 weeks after IUHCT.

higher cell doses increased the level of chimerism in chimeric pups, it did not increase the frequency of chimerism, and we could not explain why, despite consistent injection techniques, a minority of the recipients were chimeric. We therefore reexamined congenic versus allogeneic engraftment using much higher cell doses, which were permitted by an intravascular injection technique (43), and performed tracking experiments following engraftment at early and late time points (12). This study revealed a marked difference in the frequency (but not the level) of chimerism in congenic versus allogeneic recipients and made the clear observation that all animals were initially engrafted (confirming equal delivery of donor cells), but that engraftment was lost between 2 and 4 weeks of age in most of the allogeneic recipients, which strongly suggests that there was in fact an immune barrier to IUHCT, contradicting the assumption that actively acquired tolerance could facilitate allogeneic engraftment.

The results of this study explain this apparent contradiction and provide a mechanism for the inconsistencies observed in murine studies of IUHCT. Specifically, the ability to achieve a 100% frequency of chimerism through the substitution of non-injected foster dams or the use of maternally derived donor cells confirms that it is maternal immunization, rather than a fetal immune barrier, that results in loss of engraftment after IUHCT. Furthermore, this effect can be reproduced by postnatal oral or i.p. administration of maternal serum alone and is mediated by recipient effector T cells rather than those of the mother. These observations support a mechanism whereby passive transfer of maternal alloantibodies via breast milk induces a postnatal cellular and humoral immune response in the recipient.

Traditionally, maternal-fetal antibody transmission has been thought to act transiently, through direct binding of maternal antibody to fetal antigen, however there is increasing evidence that maternal antibody is capable of inducing a durable and pathogenic T cell response. In the mouse, the bulk of passively acquired maternal antibody is derived from breast milk (44). Antibody transport occurs at the level of the neonatal duodenum and jejunum, where enterocytes expressing a surface membrane receptor (FcγR) bind the Fc region of IgG and facilitate transcytosis of immunoglobulins (16).There have been a number of recent studies documenting de novo T cell–mediated immune responses triggered by maternal antibodies. Greeley et al. (45) demonstrated prevention of diabetes in NOD mice through the elimination of maternal autoantibodies, establishing a direct connection between maternal-fetal antibody transmission and T cell–mediated autoimmune disease in progeny. Setiady et al. (46) more recently demonstrated transmission of autoimmune ovarian disease via the same pathway, whereby maternal autoantibodies induce a pathogenic neonatal T cell response.

A mechanism connecting humoral and cellular responses involves immune effector cells that express FcγRs (47). This family of receptors has activating and inhibitory functions and varies in its distribution within monocytes, macrophages, and neutrophils, which may display activating or inhibitory Fcγ receptors. NK cells only express the activating FcγIIIaR. In vitro studies have shown that antibody can facilitate the uptake of its cognate antigen into APCs and that antibodies are also capable of activating antigen-specific T cells through the interaction of the immune complex and FcγR on dendritic cells (48–50). Additionally, the epitope specificity of a given antibody has been shown to influence the specificity and magnitude of the T cell response induced by that antibody (51). Finally, an allospecific antibody can directly activate NK cells via the mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC). Therefore, the possible consequences of the passive transfer of maternal allospecific antibody may include direct antibody cytotoxicity, ADCC, antigen-antibody complex processing by APCs with immune activation of T cells, and inflammation, which would enhance antigen presentation and a cascade of other signals driving an adaptive immune response.

In this study we have not formally ruled out the innate immune system as a potential barrier to engraftment. Recent studies of both autoimmune disease (52) and allogeneic IUHCT (53) have established that murine neonatal NK cells are not only functional but also important for modulation of T cell reactivity. In the context of IUHCT, NK cells have been implicated in loss of minimal chime-

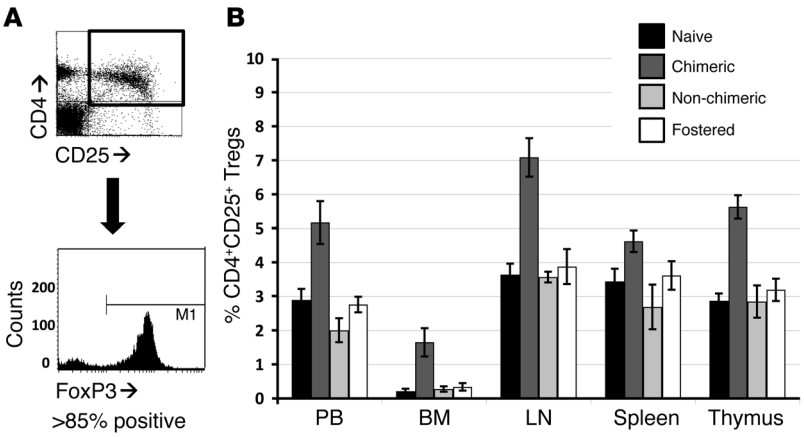


**Figure 9**
Treg distribution. (**A**) CD4⁺CD25⁺ lymphocytes were shown to be more than 85% FoxP3⁺. (**B**) The percentage of CD4⁺CD25⁺ lymphocytes was measured in peripheral blood (PB), bone marrow, lymph nodes (LN), spleen, and thymus. The percentage of chimeric cells was significantly higher ($P < 0.01$) in all compartments except the spleen ($P = 0.06$). The histogram is representative of at least 5 independent experiments. Data are mean ± SEM.

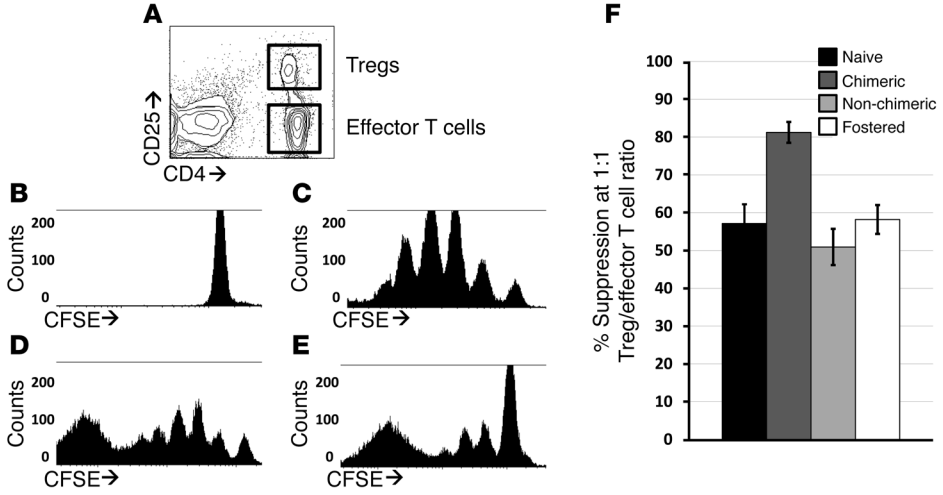


**Figure 10**
Treg suppression assay. (**A**) $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ effector T cells were sorted. (**B** and **C**) Controls consisted of (**B**) unstimulated and (**C**) stimulated effector T cells. (**D** and **E**) Suppression was measured after a 1:1 addition of (**D**) non-chimeric and (**E**) chimeric Tregs. Each histogram is representative of at least 5 independent experiments. (**F**) The percentage suppression at a 1:1 Treg/effector T cell ratio. Data are mean ± SEM.

rism (<1.8%) despite the presence of T cell tolerance. Our findings of transfer of maternal alloantibodies would suggest that a possible mechanism for this observation is activation of donor MHC class I–specific NK cells by maternal alloantibody via an ADCC mechanism in a milieu of low frequencies of donor cells. Levels of chimerism in our model of intravascular injection far exceed the threshold level of initial chimerism postulated to be required for host NK cell tolerance and subsequent durable engraftment (53). The fact that 100% of our foster-reared pups maintained stable engraftment demonstrates that fetal NK cells are not a barrier to the levels of engraftment seen in this study and supports previous data (54, 55) suggesting that the milieu of high levels of donor cells during NK cell development may modify their receptor profile and reduce the frequency of donor-reactive NK cells, negating their effect.

An intriguing initial observation in this study was the fact that pups not reared by foster mothers that lose their chimerism were often exposed to the same breast milk as pups that remained chimeric. One explanation is that the magnitude of the maternal immune response, and therefore the dose of alloantibody transferred, determines the likelihood of chimerism. At the extremes this appears to hold true. Two dams that underwent IUHCT and had minimal humoral response delivered pups that maintained chimerism, whereas high levels of humoral response were uniformly associated with no chimeric pups. Statistically, there appears to be a negative correlation between level of maternal antibody and chimerism in pups, whether analyzed by individual pup or by comparing litters with either 0 or at least 1 chimeric pup. However, the magnitude of an individual mother's response was not an explanation for how individual pups within the same litter could be chimeric or non-chimeric.

Self-reactive T cells are known to escape thymic deletion in significant numbers due to inadequate or late presentation of antigen in the thymus, and to be controlled by regulatory mechanisms, including Treg populations, which are essential for the prevention of autoimmune disease (56, 57). It is also known that maternal-fetal cell trafficking in humans results in the generation of tolerogenic fetal Tregs (58). This suggested that donor cells would induce Tregs in our chimeric pups and that these would potentially counteract a low-level alloimmune response. Therefore, we examined the level and suppressive capacity of $CD4^+CD25^+$ Tregs in each group of pups and found that there does appear to be a more robust Treg response in the non-fostered chimeric pups. Our data support the hypothesis that after IUHCT, tolerance occurs by a primary mechanism of clonal deletion that is supplemented by the generation of Tregs to suppress donor-reactive cells that escape thymic deletion. In our fostered pups, this mechanism is uniformly successful in maintaining a tolerant state. However, the transfer of allospecific antibodies induces an allogeneic response that may overwhelm Treg suppression, resulting in a loss of engraftment. We speculate that in the context of maternal immunization and breast-feeding, it is the balance of immune-activating and regulatory influences that determines whether a given pup remains chimeric.

Finally, although we view the identification and characterization of this immune response to be potentially critically important to overcoming the barriers to successful IUHCT, the importance of host cellular competition is not to be overlooked. Indeed, despite the delivery of what would be considered massive doses of donor cells ($2 \times 10^{11}$ cells/kg fetal weight) in fostered allogeneic recipients or in the congenic model, levels of donor chimerism can be variable and, in many animals, low (12). In fact, our current view from studies in the murine model is that the level of engraftment is limited by donor cell dose, donor cell competitive capacity, and host cell competition. However, given an adequate dose of donor cells, it appears that the frequency of allogeneic engraftment (or the corollary, loss of engraftment) is a function of the adaptive immune barrier as characterized in this study.

The applicability of these findings to other species and specifically humans remains a major question. We recognize that there are species-specific differences in gestational length and maternal-fetal lymphocyte and antibody trafficking that could result in an entirely different sequence of events after clinical IUHCT. If maternal sensitization is in fact relevant to the outcome of human IUHCT, it likely occurs via a different mechanism. In humans, antibodies in breast milk do not enter the neonatal circulation because gut closure occurs precociously (16). In light of the longer gestation period and the fact that the murine FcγR is similar to the placental receptor responsible for active placental transfer of IgG in humans, the more likely route of transmission in large animal models is via the placenta during the late second and third trimesters of pregnancy. Whether maternal sensitization occurs in humans and whether this would result in pre- or perinatal rejection of donor cells via direct or indirect mechanisms needs to be investigated in relevant large ani-

mal models. In any case, an obvious strategy to avoid any potential maternally derived immune barrier would be the use of maternal donor cells when appropriate.

To our knowledge, this is the first study documenting maternal immunization and its consequences after IUHCT. Multiple mechanisms have been implicated that contribute to maternal-fetal tolerance, both at the placental interface (59) and systematically (60). The important observation from this study is that, at least in the context of IUHCT, one cannot assume that the normal mechanisms responsible for maternal-fetal tolerance will prevent a maternal immune response against donor cells. Future experimental and clinical studies of allogeneic IUHCT need to consider and assess the importance of the maternal immune response. Our results may also have implications for prenatal gene therapy, in which potentially immunogenic transgene or viral proteins are injected by various routes into the fetus.

A second and equally important observation of this study is that, in the absence of maternal influence, engraftment and long-term chimerism uniformly occur across full MHC barriers. This confirms the absence of an adaptive immune barrier in the pre-immune fetus and validates the potential for practical application of actively acquired tolerance to facilitate allogeneic cellular and/or organ transplantation. Our findings may account for much of the inconsistency in previous studies, including perhaps the inconsistent tolerance observed in the classic study of Billingham, Brent, and Medawar (2).

## Methods

*Mice*. BALB/c ($H2K^{d+}$) mice time-dated at 14 days of gestation were used as fetal IUHCT recipients. Six- to 8-week-old MHC-$H2K^{b+}GFP^+$ C57BL/6TgN(act-EGFP)OsbY01 mice were used as bone marrow donors (provided by M. Okabe, Genome Information Research Center, Osaka University, Osaka, Japan). Adult CB6F1 ($H2K^{b+/d+}$) mice were used as in vivo MLR hosts. C57BL/6 (referred to as B6 - $H2K^{b+}$, $GFP^-$) cells were used as MLR stimulators. Except as noted, animals were purchased from Charles River Laboratories and bred in our colony as previously described (12). The experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia and followed guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals.

*Flow cytometry*. Fluorochrome-labeled mAbs and isotype controls were purchased from BD — Pharmingen. Anti-mouse Ig secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. Nonspecific FcγR binding was blocked by the mAb against mouse FcγR 2.4G2. Conjugated mAbs with irrelevant specificities served as negative controls. Propidium iodide staining was used to exclude dead cells in dual color flow cytometry. Flow cytometry was performed on a FACSCalibur, and cell sorting was performed on a FACSAria (Becton Dickinson).

*In utero transplantation*. Whole donor bone marrow was harvested from 6- to 8-week-old B6GFP mice. Low-density mononuclear cells were separated by Ficoll gradient centrifugation and resuspended at a concentration of $1 \times 10^6$ cells/μl. On day E14 of gestation, a midline laparotomy was performed under isoflurane anesthesia and the uterine horns were exposed. The vitelline vein was identified with a dissecting microscope, and each fetus was injected with $2 \times 10^7$ whole bone marrow cells (20 μl of cells at a concentration of $1 \times 10^6$ cells/μl). A successful intravenous injection was confirmed by visualization of clearance of the blood in the vein by the injectate and the absence of extravasation at the site of injection. The uterus was returned to the maternal peritoneal cavity and the abdomen closed with 2 layers of absorbable 4-0 Vicryl suture. To determine chimerism status, peripheral blood from E14 injected mice was obtained at 4 weeks of age by retroorbital puncture. Mononuclear cells were isolated by Ficoll gradient centrifugation. Donor cell chimerism was assessed as the percentage of $CD45^+$ cells that were $GFP^+$ by flow cytometry, with chimerism being defined as more than 1% $GFP^+$. Analysis of allogeneic donor cell chimerism using the $H2K^b$ marker after IUHCT of B6GFP ($H2K^{b+}$) cells into BALB/c ($H2K^{d+}$) fetuses demonstrated good correlation with GFP expression, confirming that GFP reliably represented all donor cells present in chimeric mice.

*T cell alloreactivity in vivo*. Spleen and lymph nodes (axillary, inguinal, cervical, para-aortic, and mesenteric) were harvested from 4-week-old, E14 injected chimeric and non-chimeric BALB/c mice, and mononuclear cells were isolated using ACK lysing buffer. Positive control BALB/c mice were immunized with an i.p. injection of approximately $2 \times 10^7$ B6 cells at days 0 and 7, and cells were harvested on day 14. Mononuclear cells from each BALB/c mouse were stained with CFSE dye and injected into the tail vein of a CB6F1 mouse ($H2K^{b+/d+}$). The spleen, lymph nodes, peripheral blood, bone marrow, and thymus of the CB6F1 mouse was harvested at 24, 48, 72, or 96 hours, and mononuclear cells were isolated and stained with anti-CD4 (L3T4, APC), anti-CD8 (Ly-2, PerCP), and anti-$H2K^b$ (AF6-88.5, PE) antibodies. The $CD4^+H2K^{b-}$ or $CD8^+H2K^{b-}$ cells were then analyzed for CFSE fluorescence by flow cytometry, and the frequency of alloreactive T cells in vivo was quantified as described by Suchin et al. (13). Briefly, the frequency of alloreactive T cells ($F$) was defined as the number of cells that had divided ($P^{div}$), divided by the total number of cells ($P^{tot}$). $P^{tot}$ was further defined as the number of cells that had successfully engrafted at 24 hours, and $P^{div}$ was further defined as $\Sigma M_n/2^{n-1}$, where $M_n$ represents the number of cells ($M$) in a given CFSE peak $n$, and $n-1$ represents the number of cell divisions that those cells have undergone. For example, cells in the second CFSE peak ($n = 2$) had, by definition, undergone 1 cell division, and therefore 100 cells in this peak were derived from $100 / 2^{2-1}$ original cells, or 50 cells. The total number of original cells that had divided ($P^{div}$), divided by the total number of original cells that have engrafted ($P^{tot}$), yielded the frequency ($F$).

*T cell anergy assays and cytokine ELISAs*. Lymphocytes were harvested from non-fostered chimeric and fostered chimeric BALB/c spleens, CFSE stained, and resuspended in RPMI-10 media in the presence of irradiated splenocytes from BALB/c (congenic), B6 (donor allogeneic), or Swiss Webster (third-party allogeneic) mice. Anti-CD3ε and anti-CD28 were added at concentrations of 1.0 μg/ml to each tube, and recombinant mouse IL-2 was added at a concentration of 10 ng/ml. For cytokine ELISAs, serum was obtained from peripheral blood and analyzed for IL-2, IL-4, IL-10, and IFN-γ using mouse Ready-SET-Go! ELISA kits (eBioscience).

*Alloantibody assay*. Peripheral blood from E14 injected chimeric and non-chimeric BALB/c mice was obtained at 4 weeks of age by retroorbital puncture, and serum was isolated via centrifugation. Positive control BALB/c mice were immunized with an i.p. injection of approximately $2 \times 10^7$ B6 cells at days 0 and 7, and serum was collected on day 14. Serum was also obtained from maternal blood on P1, P8, and P28. Splenocytes were isolated from adult B6 mice for use as allogeneic target cells, and $1 \times 10^6$ spleen cells were pre-incubated with 5 μl of anti-CD16/32b Fc block and then incubated for 45 minutes with BALB/c serum at serum/splenocyte ratios of 1:100, 1:50, 1:10, 1:2, and 1:1. The cells were washed twice to remove excess serum and then incubated for 45 minutes with secondary antibody against mouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b, or IgG3 to detect the presence of bound antibodies. B cells were stained with anti-CD19 and excluded by gating because these cells exhibit a large amount of nonspecific binding. Cells were analyzed by flow cytometry for median fluorescence. Median fluorescence, representing the relative concentration of alloantibodies in serum, was compared with negative control (no serum) and expressed as a fold increase.

*Materno-fetal cell trafficking (in vitro MLR)*. BALB/c females were bred with B6 males, and fetuses were injected with B6 donor cells according to

standard IUHCT protocol. Spleens, lymph nodes, peripheral blood, bone marrow, and thymuses were harvested from F1 pups at 4 weeks of age. Lymphocytes were isolated by ACK lysis, CFSE stained, and resuspended in RPMI-10 media at a concentration of $1 \times 10^6$/ml (responder cells). B6 lymphocytes were isolated from the spleen by ACK lysis, irradiated (2,000 rad), and resuspended in RPMI-10 media at a concentration of $1 \times 10^6$/ml (stimulator cells). From each population of cells, 500 μl was placed into a 5-cc FACS tube and incubated for 72 hours. Anti-CD3ε (145-2C11, purified) and anti-CD28 (37.51, purified) were added at a concentration of 1.0 μg/ml to each tube. $H2K^{b-}CD4^+$ cells were then harvested and analyzed by flow cytometry for a CFSE profile.

*Transfer of maternal serum to fostered pups.* Standard IUHCT with intravenous injection of B6GFP cells into E14 BALB/c fetuses was performed, and pups were fostered at birth. Fostered pups then received maternal serum from dams injected on E14 via either oral (100 μl pure serum twice per week for 4 weeks) or i.p. (100 μl pure serum 3 times per week for 4 weeks) administration, and they were analyzed for peripheral blood chimerism at 4 weeks of age.

*Treg distribution and suppression.* Peripheral blood, bone marrow, lymph nodes, spleen, and thymuses were harvested from 4-week-old naive, chimeric, non-chimeric, and fostered BALB/c pups. Lymphocytes were isolated and stained for Tregs with the Mouse Regulatory T Cell Staining Kit 2 (eBioscience). For suppression assays, spleen and lymph nodes were harvested and lymphocytes were sorted on a FACSAria for $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ effector T cells. These cells were resuspended at a concentration of $1 \times 10^6$ cells/ml in RPMI-10 media and then combined at Treg/effector T cell ratios of 1:8, 1:4, 1:2, and 1:1. Effector T cells were stimulated with irradiated B6 lymphocytes, and anti-CD3ε and anti-CD28 were added at concentrations of 1.0 μg/ml to each tube. Effector T cells were analyzed for a CFSE profile on a FACSCalibur. The percentage suppression was calculated using the precursor frequency (PF) method described by Brusko et al. (61). Briefly, percentage suppression = 100 × (1 – [PF (Treg + Teff) / PF (Teff)]). The precursor frequency was calculated as described above, by analyzing the CFSE profile; PF (Treg + Teff) is the effector T cell PF in the presence of Tregs; and PF (Teff) is the effector T cell PF in the absence of Tregs.

*Statistics.* One-way ANOVA was used for analysis of the frequency of alloreactive T cells (Figure 2), Treg distribution (Figure 9), Treg suppression (Figure 10), and serum cytokine levels (Supplemental Figure 1). The Mann-Whitney *U* test was used for analysis of the alloantibody assay because population variance was not equal (Levene's test, <0.05) (Figure 3). The Fisher's exact test was used for analysis of the frequency of chimerism (Tables 1 and 2) because the data were categorical. The correlation between maternal humoral response and the numbers of injected and aborted fetuses was performed by calculating the Pearson's correlation coefficient (Supplemental Figure 4). In the remaining experiments, the significance of differences among groups was determined by use of the Student's *t* test. Results with a *P* value less than 0.01 were considered to be significant. Calculations were performed using SPSS version 15.0 (SPSS Inc.).

## Acknowledgments

This work was supported by grant RO1 HL64715 from the NIH (to A.W. Flake) and by funds from the Ruth and Tristram C. Colket Jr. Chair of Pediatric Surgery and the Albert M. Greenfield Foundation (to A.W. Flake). We thank Marcus Davies for his assistance with the statistical analyses and Keith Alcorn and Christina Hughes for assistance with breeding and technical procedures. We also thank David Stitelman, Courtney Quinn, Aimee Kim, Todd Heaton, and Jessica Roybal for their assistance with bone marrow harvests.

Received for publication February 19, 2009, and accepted in revised form June 3, 2009.

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