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Understanding the fetal immune response to *in utero* stem cell transplantation

A. Specific Aims

With improvements in prenatal diagnosis and genetic testing, we can now diagnose a wide variety of congenital disorders that are amenable to stem cell based therapies. For example, many hematologic and immunologic disorders can be potentially cured by stem cell transplantation (reviewed in [1]). However, the current treatment of these disorders relies on postnatal bone marrow transplantation, which can carry significant morbidity[2]. Treatment of these diseases *in utero*, prior to the maturation of the immune system, offers an innovative approach. With *in utero* hematopoietic stem cell transplantation (IUHSCTx), allogeneic cells transplanted before the immune system matures may be perceived as "self," thereby avoiding a host immune response (reviewed in [3]). However, levels of engraftment after IUHSCTx have been low in many animal models and clinically, this approach has only been successful in the setting of severe combined immunodeficiency[4]. We hypothesize that the fetus is capable of mounting either a tolerogenic or an immunogenic response to stem cell transplantation and that the nature of the response determines the levels of engraftment. In a mouse model of IUHSCTx, it is possible to obtain engraftment of transplanted hematopoietic stem cells (HSCs), leading to multilineage chimerism across a full major histocompatibility barrier. However, there are strain-dependent variations in engraftment, and fetal transplantation does not always lead to chimerism and tolerance. Our goal is to study the effects of IUHSCTx on the nascent fetal immune system in engrafted and non-engrafted animals after IUHSCTx and to determine the mechanisms by which the fetal immune system responds to *in utero* allogeneic stem cell transplantation.

Specific Aim 1: To study the host T-cell response to allogeneic IUHSCTx. We hypothesize that if allogeneic IUHSCTx leads to engraftment and mixed chimerism, tolerance is established by a combination of deletion or anergy of alloreactive T effector cells together with the formation of donor-specific regulatory T cells. If IUHSCTx is not successful, the animal is sensitized, leading to an increase in alloreactive T effector cells. We will test the frequency and function of host alloreactive T effector cells and regulatory T cells in chimeric and non-chimeric animals following IUHSCTx.

Specific Aim 2: To induce dominant transplantation tolerance by cotransplantation of donor-specific Tregs and allogeneic HSCs into fetal recipients. Based on the known function of Tregs to inhibit immune activity and maintain tolerance, our hypothesis is that cotransplantation of donor-specific Tregs with donor HSCs will improve host engraftment after IUHSCTx.

B. Background and Significance

Currently, the standard treatment for congenital hematopoietic stem cell disorders is postnatal bone marrow transplantation. The treatment efficacy using this approach is often limited by transplantation complications, such as graft versus host disease and graft rejection, by the availability of few HLA-matched donors, and by the morbidity of host myeloablation preceding transplantation (reviewed in [2]). The induction of donor-specific tolerance to transplanted allogeneic stem cells without long-term immunosupression would therefore have important clinical applications.

Stem cell transplantation into the early gestational fetus offers an innovative approach for tolerance induction. Theoretically, introduction of allogeneic cells during the period of thymic education to self antigens may result in donor-specific tolerance[3]. This approach could be useful for inherited stem cell disorders such as thalassemias (some fatal *in utero*), for non-hematopoietic stem cell disorders (muscular dystrophy, inborn errors of metabolism), and even for tolerance induction for organ transplants for congenital renal and cardiac anomalies. While this concept is promising, levels of engraftment (and tolerance induction) have been variable in many animal models. Consequently, it has been difficult to study the mechanisms of central and peripheral tolerance induction after fetal transplantation or to carry out translational studies in this field.

Experiments in animal models have been promising but clinical success is limited. Animal models of IUHSCTx have shown that the fetal environment offers considerable advantages for the success of stem cell transplantation over the postnatal setting. For example, fetal lambs can accept HSCs with long term chimerism,[5] and fetal mice can be tolerized to stem cells from fully allogeneic mice in certain strain combinations using this approach.[6] In the setting of organ transplant, swine that become chimeras after IUHSCTx of fully allogeneic bone marrow demonstrate donor-specific tolerance to a kidney allograft [7]. However, there are barriers to engraftment of prenatally transplanted stem cells which remain poorly understood. Clinically, *in utero* cellular transplantation has only been successful for fetuses with SCID, in which there is no adaptive immune response and there is a clear survival advantage for transplanted cells. Fetuses with other diseases have been transplanted in the past with limited success (reviewed in [1]) IUHSCTx is therefore far from being applied clinically until we can adequately understand the fetal immune response to stem cell transplantation.

A mouse model of IUHSCTx can be a valuable tool for studying the fetal immune response to transplantation. In this model, we can transplant HSCs derived from fetal liver (FL) or bone marrow and achieve multilineage engraftment of donor hematopoietic cells for the lifetime of the animal. Chimeric animals are tolerant to skin grafts from the donor and their levels of engraftment can be increased with a second postnatal transplant from the same donor (reviewed in [3]).

However, not all injected animals become chimeric and there is evidence that even in the fetal setting, an adaptive immune response may limit engraftment. In a recent study comparing engraftment of syngeneic cells to allogeneic cells, 100% of syngeneic animals engrafted, whereas only 30% of allogeneic animals engrafted, indicating that the immune system serves as an important barrier.[8] However, detailed comparisons of the differences in repertoire of effector and regulatory T cells in chimeric and non-chimeric animals have not been done and are the goals of this project.

Regulatory T cells (Tregs) have been implicated as critical regulators of the immune system, responsible for maintaining immune self tolerance [9]. Through the production of inhibitory cytokines and/or direct inhibition with cell to cell interactions, Tregs function to inhibit immune activity and thereby maintain self tolerance. Furthermore, recent evidence has demonstrated the ability of Tregs to promote tolerance following allogeneic postnatal bone marrow transplantation[10]. Little is known, however, regarding their involvement in allogeneic IUHSCTx and this proposal will address the application of Tregs to improve host engraftment after IUHSCTx.

Recently developed tools in immunology are also crucial to our ability to study the allo-immune response between certain strain combinations. The frequency of alloreactive cells in a normal animal is only 2-10% at baseline, making it difficult to isolate and study this small population. However, we now have access to T cell receptor transgenic (TCR Tg) mouse models in which all of the T cells are designed to recognize one particular alloantigen. In particular, TCR Tg mice in which T cells recognize an alloantigen presented either by donor antigen-presenting cells (APC) (direct pathway) or by host APC (indirect pathway) are useful tools for the study of transplantation immunology. These mice can be used to generate defined populations of effector or regulatory T cells against one particular donor in order to study the fate of these cells in the fetal environment. Studying the activation or suppression of these cells will allow us to determine, for the first time, how alloreactive T cells respond to IUHSCTx.

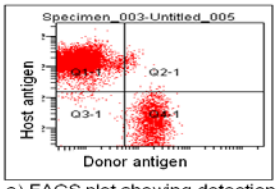
C. Preliminary Studies

We have established the previously described fetal mouse model for IUHSCTx in our laboratory. We harvested fetal liver mononuclear cells (FLMCs) from 14 day gestation fetuses (term= 21 days) and introduced 2-5 million cells/fetus using pulled glass micropipettes directly into the recipient fetal liver through the intact uterus. We allowed the pregnancies to carry to term and analyzed the pups for chimerism in their peripheral blood by staining for both donor and host leukocyte antigens and counting the cells by fluorescence-activated cell sorting (FACS) starting at 4 weeks post injection.

We have performed these FLMC transplants in both syngeneic (C57/B6 (B6)-Ly5.1 donor into B6 recipient) and allogeneic (BALB/c into B6 or B6 into BALB/c) strain combinations and have obtained chimeras (Figure 1). We have observed, as has been described, that chimerism is stable over the weeks tested. Furthermore, we have analyzed the lineage of donor-derived cells after *in utero* transplantation by FACS and determined that chimerism is multi-lineage (the transplanted cells differentiate into T cells, B cells, and granulocytes and regulatory T cells). However, not all injected mice become chimeric and the ratio of chimeric animals to injected animals is significantly lower for allogeneic transplantation than for syngeneic transplantation. This finding is consistent with previously published work[8] and is likely secondary to an adaptive immune response and not to technical differences in cell delivery. Therefore, although it is possible to establish chimerism, the process is not consistent in all recipients and clinical applications will likely require some immune modulation.

We have also performed transplants into Rag KO recipients (Rag KO mice do not develop mature T and B cells) to determine whether engraftment will be different in a host lacking an adaptive immune response (Figure 1). As expected, rates of chimerism were similar to syngeneic controls. Interestingly, absolute levels of chimerism were much higher in these animals, likely secondary to the availability of increased hematopoietic niches available for engraftment.

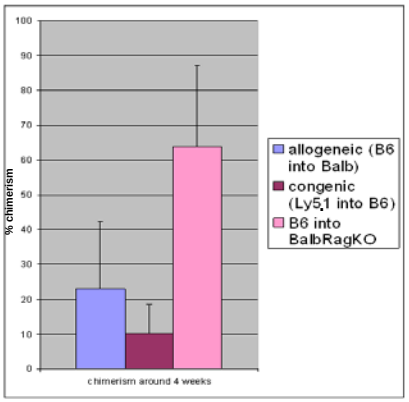
Figure 1



a) FACS plot showing detection of circulating donor cells in chimera.

Donor/Host	# Chimeric/ # born (%)	% chimerism
B6-Ly5.1 into B6 (congenic)	6/7 (86%)	10 ± 8.4
B6 into Balb (allogeneic)	9/34 (27%) P<.005	23 ± 19
B6 into Balb RagKO (immunodeficient)	9/11 (82%) P=NS	64 ± 23

b) Summary of outcomes in congenic, allogeneic, or immunodeficient fetal transplants.



c) Comparison of chimerism levels in different strain combinations

D. Research Plan

Aim 1: To study the host T-cell response to allogeneic IUHSCTx by studying the levels of donor-reactive T cells and regulatory T cells in chimeric and non-chimeric animals after allogeneic IUHSCTx. **Hypothesis:** Successful IUHSCTx with chimerism will lead to immunological tolerance as seen by a decrease in allo-reactive T effector cells and the induction of allo-specific T-regs, whereas lack of chimerism will lead to sensitization and an increase in donor-reactive T effector cells.

Experimental approach:

1a. Characterizing the cytokine profile of host allo-reactive T-cells after IUHSCTx. To study the cytokine profile after IUHSCTx in

wild type animals, B6 fetuses will be injected with BALB/c FLMCs at 14 days postcoitum. Pregnancies will be allowed to carry to term and neonatal pups will be evaluated for chimerism (Figure 2). T effector cells will then be isolated from the following groups of animals: injected chimeras, injected non-chimeras, uninjected mice (negative control), uninjected BL6 mice that have been sensitized by BALB/c skin grafts (positive control). Effector T cells from recipient mice will be subjected to the following conditions: no stimulation, direct stimulation (donor antigen, donor APC), indirect stimulation (donor antigen, donor APC), and stimulation from an independent antigen (C3H antigen). After exposure to these conditions, IFN-γ, IL-4, and IL-2 production will be measured by Elispot assays.

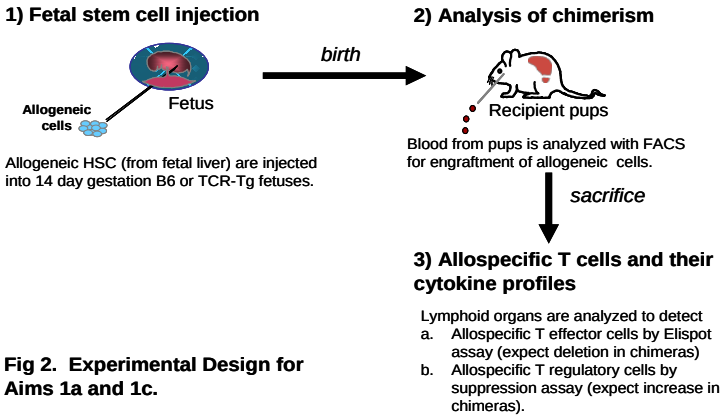


Fig 2. Experimental Design for Aims 1a and 1c.

1b. Frequency and suppressive function of Tregs in host animals after IUHSCTx. Tregs are known regulators of the immune system yet little is known regarding their role after IUHSCTx. Our hypothesis is that there is a higher frequency of Tregs in chimeric mice after IUHSCTx. To address this hypothesis, we will utilize FoxP3-GFP mice that are available to us through Dr. Abul Abbas' laboratory. FoxP3 is a Treg specific transcription factor that when activated in these mice, results in the formation of GFP[11]. After transplanting allogeneic FLMCs into FoxP3-GFP mice, we will evaluate GFP signal in peripheral blood and lymphocytes from recipient mice. To determine if the Tregs present in chimeric and non-chimeric mice can suppress the allogeneic T effector cell response, we will transplant wildtype allogeneic FLMCs into B6 wildtype recipients. Host lymphocytes will be harvested and will be purified into Tregs ($CD4+CD25+CD62L^{high}$) and T effector (CD4+CD25- or CD8+) cells. In the presence of either direct or indirect stimulation, Tregs will be added to T effector cells in a series of dilutions to determine if there are allo-specific Tregs that are able to suppress T effector cell proliferation. Proliferation will be compared among chimeric, non-chimeric, and non-injected mice using CFSE.

1c. Donor reactive T effector cells in chimeric and non-chimeric mice in a mouse model of known antigen specificity. In order to further characterize the fetal immune system response to IUHSCTx, we will utilize TCR Tg recipient mice, whose T-cells respond to a specific alloantigen. In collaboration with Dr. Sang-Mo Kang's laboratory in the Division of Transplant Surgery, we have two separate TCR-Tg mice on a B6 background that recognize BALB/c antigen using either the direct pathway (4C mouse) [12] or the indirect pathway (TCR75 mouse) [13]. FLMCs from BALB/c mice will be transplanted into the fetuses of 4C and TCR75 mice and we will analyze the ratio of Teff cells to Tregs in these animals compared to uninjected controls (Figure 2). We hypothesize that there will be a decrease in Teff cells in chimeras and a corresponding increase in Tregs. We will also measure the cytokine response of the host T-effector cell population by measuring levels of IFN-γ (Th1 cytokine), IL-4 (Th2 cytokine), and IL-2 using Elispot assays, and determine the ability of isolated Tregs from chimeric and non-chimeric mice to suppress host cell stimulation by alloantigen *in vitro*.

Aim 2: Inducing dominant transplantation tolerance by cotransplantation of donor-specific Tregs and allogeneic FLMCs. **Hypothesis:** Cotransplantation of donor-specific Tregs will improve host engraftment and chimerism after IUHSCTx.

Experimental approach:

We will culture Tregs from 4C and TCR75 mice, both of which have known antigen specificity to BALB/c antigen. These cells will be co-transplanted with BALB/c HSCs into fetal B6 mice and surviving animals will be evaluated for levels of chimerism of BALB/c cells, as well as for survival and cytokine profiles of the transplanted Tregs. We will also evaluate the host's immune repertoire for T effector cells and Tregs. The injected Tregs will carry a congenic marker, Ly5.1, to distinguish them from the host T cells. This will allow us to determine whether the presence of our transplanted, BALB/c-specific Tregs will also induce proliferation of host Tregs, even if the transplanted Treg population is lost. Control animals will receive Tregs from an irrelevant TCR Tg mouse, in which the T cells do not recognize BALB/c antigen.

IUHSCTx is a promising method for treating a variety of prenatally diagnosed stem cell disorders. Understanding the fetal immune response to transplantation and devising methods to modulate this response to promote donor-specific tolerance is central to the success of this field. Our laboratory has the tools, animal models, and necessary collaborations with immunologists to elucidate the immune response to IUHSCTx and the mechanism(s) of allo-specific immune tolerance after stem cell transplantation. Once we understand the mechanisms to induce tolerance reliably, IUHSCTx can be applied to treat hematopoietic and non-hematopoietic stem cell disorders, and to improve graft survival after organ transplantation.

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