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Donor MHC Class I Expression Determines the Outcome of Prenatal Transplantation

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Abstract

Background/Purpose—The failure of in utero transplantation in immune-competent recipients suggests the existence of a fetal immune barrier. The importance of donor MHC class I expression in the induction of prenatal tolerance remains undefined. We hypothesized that donor cell MHC class I expression facilitates engraftment in prenatal allogeneic recipients rather than promoting immune rejection.

Methods—B6.Ly5.2 (class I⁺) or B6.TAP^{-/-} (class I⁻) murine fetal liver cells were transplanted into age-matched allogeneic fetal recipients. Survival to weaning and subsequent growth was assessed. Engraftment rates and peripheral blood chimerism levels were measured serially.

Results—The presence or absence of class I expression did not affect survival or growth of recipients and no GVHD developed. Allogeneic recipients of B6.Ly5.2 cells exhibited significantly higher levels of donor hematopoietic chimerism when compared to recipients of B6.TAP^{-/-} cells (27 ± 10% vs. 11 ± 8%, p=0.004) which deteriorated further over time.

Conclusions—Donor class I MHC antigen expression is essential for stable long-term engraftment and maintenance of donor-specific tolerance. Further studies are needed to better characterize the role of the fetal innate immune system in prenatal allotransplantation.

Keywords

in utero transplantation; hematopoiesis; allogeneic; MHC class I; mice

INTRODUCTION

In Utero Cellular Transplantation (IUCT) has been clinically successful in cases involving immunodeficiency diseases [1,2]. However, broad application of IUCT has been hindered by the minimal engraftment observed in the prenatal treatment of diseases in which there is no pathologic immunodeficiency [3]. For these reasons, the existence of an uncharacterized fetal immune barrier has been postulated [4–6]. In a recent study, Peranteau et al found that prenatally transplanted allogeneic hematopoietic cells engrafted poorly when compared to cells harvested from immunologically matched donors [4]. The authors reasoned that an adaptive

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immune response from the host led to the rejection of the MHC-mismatched donor cells due to their immunogenicity.

This conclusion contradicts previous studies documenting the development of clonal T cell deletion and anergy in prenatal allogeneic recipients in which only microchimerism exists (<0.1% peripheral blood chimerism) [7,8]. Additionally, in studies of murine IUCT, we have observed that allogeneic disparity between certain strain combinations results in *higher* levels of multi-lineage donor chimerism when compared to immunologically matched combinations (manuscript in preparation). If this is true, then allogeneic MHC expression may facilitate engraftment in the complex immunologic setting of allogeneic disparity. Therefore, in this study, we hypothesized that an absence of class I expression by the donor might paradoxically lead to increased rejection in allogeneic recipients following prenatal transplantation. To answer this question, we performed a series of prenatal transplantation experiments utilizing an allogeneic murine strain combination in which chimerism levels are reliably higher than in immunologically matched combinations. Here we demonstrate that MHC class I expression by donor cells is essential to achieve durable engraftment in the allogeneic setting and ultimately determines the outcome of prenatal transplantation.

MATERIALS AND METHODS

Mice

Breeding stock for inbred strains of mice B6.Ly5.2 ($H2^b$, Ly5.2⁺), SJL/J ($H2^s$, Ly5.1⁺) (The Jackson Laboratories, Bar Harbor, ME), Balb/c ($H2^d$, Ly5.2⁺) (Charles River Laboratories, Wilmington, MA), and B6.TAP^{-/-} ($H2^b$, Ly5.2⁺) (Taconic, Germantown, NY), were purchased and bred in our colony. CSJLF1 (Balb/c×SJL/J F1, $H2^{s/d}$, Ly5.1⁺Ly5.2⁺) mice were bred in our colony. Animals were mated and inspected daily for introital plugging. Day of plug was noted as day zero for time-dated pregnancies. All animals were housed in the Laboratory Animal Resource Center of the University of Wisconsin-Madison School of Medicine and Public Health which is an AALAC approved facility. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin (#M01687), and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Fetal Liver Harvest

Donor fetal liver light density mononuclear cells (LDMCs) were harvested from fetuses at 14 days post coitum (dpc). Under isofluorane anesthesia and using sterile technique, a midline laparotomy was made in the pregnant dam and the uterine horns were exposed. Fetuses were surgically excised and allowed to exsanguinate in a petri dish containing cold sterile phosphate buffered saline (PBS) (GIBCO-BRL, Gaithersburg, MD). Fetal livers were individually dissected in a separate petri dish then dispersed into a single cell suspension by gentle passage through a syringe over a 70µm nylon mesh strainer. LDMCs were isolated from the sample using Ficoll density separation (Histopaque 1077; Sigma, St Louis, MO) after centrifugation at 900×g for 15 minutes at 4° C. Cells were washed with sterile PBS before counting and assessing for viability using trypan blue exclusion.

In Utero Transplantation

Recipient fetal mice were injected at 14 dpc. A midline laparotomy was created in the recipient dam and the uterine horns exposed. Each fetus was transplanted with 10⁶ fetal liver LDMCs in 5 µl of PBS by intraperitoneal injection (IP) through a translucent uterine wall using a 100-µm beveled glass micropipette. The abdomen was then closed with two layers of absorbable 5-0 suture. Pregnant dams were individually housed until delivery.

Determination of donor cell chimerism

Recipient fetuses were allowed to progress to delivery and weaning at 3 weeks of age. At the time of weaning and at serial time points thereafter, peripheral blood samples were obtained via retroorbital puncture. LDMCs were isolated using Ficoll density gradient separation. The following monoclonal antibodies were used for determination of chimerism (purchased from BD Pharmingen, San Diego, CA, unless otherwise specified): anti-Ly5.1/5.2 (30-F11, eBioscience, San Diego, CA) fluorescein isothiocyanate (FITC), anti- $H2^b$ (AF6-88.5) phycoerythrin (PE), anti-Ly5.2 (104) FITC, anti-Ly5.1 (A20) PE. Dead cells were excluded using propidium iodide (PI). Percent non-erythroid hematopoietic chimerism was defined as: (fraction of donor cells ($Ly5.2^+$ or $H2^b$) $\times$ 100)/(total $Ly5.1^+$ + $Ly5.2^+$ cells).

Lineage Analysis of Transplanted Cell Phenotype

Spleen biopsies were performed at 12 weeks of age and a donor and host lineage analysis was conducted. A single cell suspension was created and LDMCs were isolated as previously described. The following monoclonal antibodies were used for determination of hematopoietic lineage: anti-CD3 (145-2C11) FITC, anti-B220 (RA3-6B2, eBioscience, San Diego, CA) FITC, and anti-Gr-1 (RB6-8C5) FITC. Cells were counterstained with anti- $H2^b$, anti- $H2^d$ (SF1-1.1), or anti-Ly5.1 PE and analyzed using dual-color flow cytometry. At least 5,000 gated donor cells were analyzed.

Statistical Analysis

Data are graphically represented as the mean of the respective group $\pm$ 1 standard deviation. Statistical comparisons between groups were performed using a two-tailed t test assuming unequal variances. Chi squared analysis was used for non-parametric variables. P-values less than 0.05 were considered significant.

RESULTS

The presence or absence of MHC class I expression by the donor cells does not affect survival or lead to graft versus host disease

Four murine strains were selected to evaluate the allogeneic host response to MHC class I expression following IUCT (Figure 1). The B6.Ly5.2 strain carries the $H2^b$ haplotype and is fully allogeneic with Balb/c recipients which bear the $H2^d$ haplotype. The B6.TAP^{-/-} donor strain was chosen for the experimental group because it is deficient in the *transporter associated with antigen presentation* (TAP) and therefore does not express any MHC class I antigen but are otherwise identical with the parent B6.Ly5.2 strain. Because the B6.TAP^{-/-} ($Ly5.2^+$) cells do not express class I, the donor population could only be identified by their Ly5 expression; therefore, CSJLF1 mice ($Ly5.1^+/Ly5.2^+$) were used as an allogeneic recipient strain rather than the Balb/c ($Ly5.2^+$). The B6.TAP^{-/-} donor cells were thus identified in the setting of an allogeneic host as the $Ly5.2^+ Ly5.1^-$ population.

A total of 27 age-matched fetuses were transplanted, of which 17 animals survived for analysis. Survival was assessed at the time of weaning at three weeks of age. As illustrated by Figure 2A, mean survival was 64% with little variation between the groups. The presence or absence of class I expression by the donor cells did not seem to affect survival rates of the Balb/c or CSJLF1 recipients. Surviving recipients were closely monitored postnatally for signs and symptoms of graft versus host disease (GVHD). Animals were weighed at 3, 6, and 12 weeks of age. Figure 2B summarizes the animals' weights at these time points. As shown, recipients of each donor cell type exhibited stable and similar weight gain as they matured. The presence or absence of class I expression in by the donor cells did not affect weight gain or result in

clinical signs of GVHD such as runting, ragged or lost fur, hunched appearance, diarrhea, or decreased level of activity.

Absence of donor MHC class I expression results in graft rejection following prenatal transplantation into allogeneic recipients

Following prenatal transplantation, the recipient dams were allowed to deliver. The pups were weaned at 3 weeks of age (4 weeks after injection) and peripheral blood chimerism was assessed at serial time points thereafter. Balb/c recipients of B6.Ly5.2 fetal liver cell transplants were selected as controls. Previous experience with this fully allogeneic strain combination has resulted in reliable long-term hematopoietic engraftment without GVHD. As expected, 80% of the recipients in this strain combination demonstrated detectable levels of engraftment at weaning (Figure 3A). Despite a transplantation strain combination that was fully allogeneic, and thus susceptible to a host cytotoxic response, rejection of the donor cells did not occur in these animals and they maintained high levels of peripheral blood chimerism for over 12 weeks (Figure 3B). Conversely, when an identical number of B6.TAP^{-/-} cells, which do not express class I antigens, were transplanted into allogeneic CSJLF1 recipients, significantly lower levels of peripheral blood chimerism were evident at 3 weeks of age ($11 \pm 8\%$ vs. $27 \pm 10\%$, $p=0.004$, Figure 3B). Although this group displayed slightly lower engraftment rates than the control group (66% vs. 80%, $p=0.589$), CSJLF1 recipients of B6.TAP^{-/-} cells demonstrated a slow rejection of the donor cells as evidenced by their rapidly decreasing peripheral blood chimerism levels as the animals matured. Surprisingly, the absence of allogeneic class I expression did not permit the B6.TAP^{-/-} cells to escape immunologic rejection, but instead rendered them more susceptible to it.

Animals in both groups underwent splenic biopsy at 12 weeks of age for hematopoietic lineage analysis. The percentage of splenic leukocytes from the B and T cell lineages was determined by the B220⁺ and CD3⁺ fractions, respectively. The granulocyte population was identified using the lineage marker Gr-1. Balb/c recipients of B6.Ly5.2 donor cells demonstrated balanced multi-lineage chimerism consistent with stem cell engraftment and indicative that GVHD was not the cause of high chimerism levels seen in these animals (Figure 4A). The CSJLF1 recipients of B6.TAP^{-/-} cells also demonstrated multi-lineage engraftment indicating that the loss of chimerism observed in these animals was not likely due to an underlying defect in competitive engraftment capacity of the B6.TAP^{-/-} strain progenitor cells (Figure 4B). We did observe a relative paucity of B cell lineage by the B6.TAP^{-/-} donor cells in the CSJLF1 recipients of unclear etiology.

DISCUSSION

The bias of IUCT that has existed for many years is that transplantation prior to the development of the adaptive immune system results in long term donor-specific tolerance. However, the limited success of IUCT in clinical cases that do not involve immunodeficiency diseases forces a closer examination of this central dogma [9]. Recently, it has been postulated that an allogeneic barrier to the development of fetal tolerance may exist due to differences in donor cell MHC expression [4]. The present study challenges the existing paradigm and attempts to define the significance of donor MHC class I expression in the regulation of the host fetal immune response to allogeneic transplantation.

Using allogeneic IUCT strain combinations, we demonstrated that the presence of foreign donor class I antigen is both necessary and beneficial for engraftment. Balb/c recipients transplanted with fully allogeneic B6.Ly5.2 cells demonstrated durable long-term engraftment without evidence of graft versus host disease. Conversely, the prenatal transplantation of B6.TAP^{-/-} cells was associated with surprisingly low levels of initial chimerism that gradually

deteriorated. Taken together, these results indicate that class I deficient cells are subject to rejection by allogeneic fetal recipients as their immune system matures.

The paradoxical rejection of the B6.TAP^{-/-} cells combined with the high levels of chimerism seen in the B6.Ly5.2 to Balb/c strain combination can be best explained by a differential innate immune response to these donor cells. It is unlikely that rejection of the B6.TAP^{-/-} cells occurred because of a T cell mediated host response. The lack of class I expression by the donor cells should facilitate enhanced engraftment by escape from the host CTL response [10]. T cell tolerance to other transplantation antigens should develop similarly in either strain combination.

Previous studies using adult murine transplantation models have evaluated the host innate immune response to transplantation in mixed allogeneic chimeras. The investigators found that established mixed Balb/c → B6.Ly5.2 chimeras rejected beta-2-microglobulin-deficient bone marrow cells but not Balb/c or B6.Ly5.2 cells concluding that NK cells were specifically tolerant to both the donor and the host [11]. Subsequent studies revealed alterations in host inhibitory class I specific Ly49 receptors in mixed chimeras suggesting NK cell education by donor class I antigens was needed to stabilize engraftment [12].

NK cells clearly respond to altered class I ligand expression and may represent an innate immune barrier to prenatal transplantation even during human fetal development [13–15]. Murine Ly49 receptor expression occurs postnatally and may be responsible for the progressive rejection of the transplanted B6.TAP^{-/-} cells which occurred between 4–6 weeks of age [16–18]. This explanation is incomplete, however, as the differences in chimerism were clearly evident even at the 3 week time point in our cohort. Ly49-negative fetal NK cells can respond to class I antigens and may also function as an immunologic barrier to allogeneic transplantation in the early gestational fetus [19–22].

It is possible that the failed engraftment of B6.TAP^{-/-} cells could be the result of a defect in the biology of their hematopoiesis rather than a lack of donor-specific tolerance. However, no deficiencies in the homing or differentiation of B6.TAP^{-/-} hematopoietic cells have been previously described. Furthermore, the observed initial engraftment rates were similar to recipients of B6Ly5.2 cells suggesting that the differences in chimerism did not result from defects in cell homing or migration. A splenic lineage analysis demonstrated multi-lineage B6.TAP^{-/-} hematopoiesis within the chimeric CSJLF1 host except for a relatively lower proportion of lineage-committed B cells. The etiology for this disparity remains unclear and is currently under investigation.

These studies not only highlight the necessity of allogeneic donor class I expression in preventing rejection but also emphasize their potential to facilitate engraftment. The potential exists for a facilitation of engraftment by donor T or NK cells. The concept of NK cell inhibitory receptor mismatched prenatal transplantation thus remains untested. Landmark studies by Ruggeri et al. have demonstrated that NK cell alloreactivity can be employed in a donor-versus-recipient direction to decrease rejection without increasing the incidence of GVHD following postnatal allogeneic bone marrow transplantation [23,24]. It is conceivable that the donor NK cells played a crucial role in facilitating engraftment in the B6Ly5.2 to Balb/c strain combination. Since higher chimerism levels were already present at the initial 3 week time point, the B6Ly5.2 fetal NK cells may have already possessed this potential by that time. The mechanisms regulating the fetal NK response to allotransplantation remain poorly explored, yet offer a plausible explanation for the rejection or facilitation of engraftment following IUCT in both clinical and animal studies.

Our experiments seem to indicate that allogeneic disparity in the setting of prenatal transplantation can be beneficial in certain instances. Decreasing the overall antigenicity of

donor cells by eliminating MHC class I expression does not appear to increase engraftment rates, but rather leads to the rejection of prenatally transplanted cells. Further studies are needed to clarify the interactions of class I antigens with the fetal innate immune system and the subsequent response to prenatal allotransplantation. The possibility of NK cell mediated regulation of engraftment provides clinical relevance for application of NK cell inhibitory ligand-mismatched transplantation to the setting of in utero transplantation.

REFERENCES

1. Flake AW, Roncarolo MG, Puck JM, et al. Treatment of X-linked severe combined immunodeficiency by in utero transplantation of paternal bone marrow. *New England Journal of Medicine* 1996;335:1806–1810. [PubMed: 8943162]
2. Wengler GS, Lanfranchi A, Frusca T, et al. In-utero transplantation of parental CD34 haematopoietic progenitor cells in a patient with X-linked severe combined immunodeficiency (SCIDX1). *Lancet* 1996;348:1484–1487. [PubMed: 8942778]
3. Flake AW, Zanjani ED. In utero hematopoietic stem cell transplantation: ontogenic opportunities and biologic barriers. *Blood* 1999;94:2179–2191. [PubMed: 10498587]
4. Peranteau WH, Endo M, Adibe OO, et al. Evidence for an immune barrier after in utero hematopoietic-cell transplantation. *Blood* 2007;109:1331–1333. [PubMed: 17023584]
5. Shaaban AF, Kim HB, Gaur L, et al. Prenatal transplantation of cytokine-stimulated marrow improves early chimerism in a resistant strain combination but results in poor long-term engraftment. *Exp Hematol* 2006;34:1278–1287. [PubMed: 16939821]
6. Muench MO, Pott Bartsch EM, Chen JC, et al. Ontogenic changes in CD95 expression on human leukocytes: prevalence of T-cells expressing activation markers and identification of CD95-CD45RO + T-cells in the fetus. *Developmental & Comparative Immunology* 2003;27:899–914. [PubMed: 12880639]
7. Kim HB, Shaaban AF, Milnerx R, et al. In utero bone marrow transplantation induces donor-specific tolerance by a combination of clonal deletion and clonal anergy. *Journal of Pediatric Surgery* 1999;34:726–729. [PubMed: 10359172]discussion 729–730
8. Kim HB, Shaaban AF, Yang EY, et al. Microchimerism and tolerance after in utero bone marrow transplantation in mice. *Journal of Surgical Research* 1998;77:1–5. [PubMed: 9698523]
9. Westgren M. In utero stem cell transplantation. *Seminars in Reproductive Medicine* 2006;24:348–357. [PubMed: 17123230]
10. Wolpert EZ, Petersson M, Chambers BJ, et al. Generation of CD8+ T cells specific for transporter associated with antigen processing deficient cells. *Proceedings of the National Academy of Science U.S.A* 1997;94:11496–11501.
11. Zhao Y, Ohdan H, Manilay JO, et al. NK cell tolerance in mixed allogeneic chimeras. *Journal of Immunology* 2003;170:5398–5405.
12. Manilay JO, Waneck GL, Sykes M. Altered expression of Ly-49 receptors on NK cells developing in mixed allogeneic bone marrow chimeras. *International Immunology* 1998;10:1943–1955. [PubMed: 9885916]
13. Grzywacz B, Kataria N, Sikora M, et al. Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells. *Blood* 2006;108:3824–3833. [PubMed: 16902150]
14. Dorfman JR, Raulet DH. Acquisition of Ly49 receptor expression by developing natural killer cells. *Journal of Experimental Medicine* 1998;187:609–618. [PubMed: 9463411]
15. Lian RH, Maeda M, Lohwasserx S, et al. Orderly and nonstochastic acquisition of CD94/NKG2 receptors by developing NK cells derived from embryonic stem cells in vitro. *Journal of Immunology* 2002;168:4980–4987.
16. Kim S, Iizuka K, Kang HS, et al. In vivo developmental stages in murine natural killer cell maturation. *Nature Immunology* 2002;3:523–528. [PubMed: 12006976]
17. Raulet DH. Development and tolerance of natural killer cells. *Current Opinions in Immunology* 1999;11:129–134.

18. Veinotte LL, Wilhelm BT, Mager DL, et al. Acquisition of MHC-specific receptors on murine natural killer cells. *Current Opinions in Immunology* 2003;23:251–266.
19. Fraser KP, Gays F, Robinson JH, et al. NK cells developing in vitro from fetal mouse progenitors express at least one member of the Ly49 family that is acquired in a time-dependent and stochastic manner independently of CD94 and NKG2. *European Journal of Immunology* 2002;32:868–878. [PubMed: 11870631]
20. Manoussaka M, Georgiou A, Rossiter B, et al. Phenotypic and functional characterization of long-lived NK cell lines of different maturational status obtained from mouse fetal liver. *Journal of Immunology* 1997;158:112–119.
21. Manoussaka MS, Smith RJ, Conlin V, et al. Fetal mouse NK cell clones are deficient in Ly49 expression, share a common broad lytic specificity, and undergo continuous and extensive diversification in vitro. *Journal of Immunology* 1998;160:2197–2206.
22. Toomey JA, Shrestha S, de la Rue SA, et al. MHC class I expression protects target cells from lysis by Ly49-deficient fetal NK cells. *European Journal of Immunology* 1998;28:47–56. [PubMed: 9485185]
23. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002;295:2097–2100. [PubMed: 11896281]
24. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 1999;94:333–339. [PubMed: 10381530]

Figure 1. Schematic representation of prenatal transplantation groups with relevant strain information

IUCT experiments were conducted using fully allogeneic donor-recipient strain combinations chosen to evaluate the significance of donor MHC class I antigen expression. Recipients were analyzed for the presence of donor cell engraftment beginning at 3 weeks of age.

Figure 2. Prenatal transplant recipient survival and growth

(A) Survival to weaning was similar despite differences in donor class I expression between the donor cell strains ($p=NS$). (B) Animals in both groups exhibited similar and stable weight gain showing no signs of wasting secondary to GVHD ($p=NS$).

A

Donor Cell Engraftment Rate at 3 Weeks	
B6.Ly5.2 → Balb/c	80%
B6.TAP ^{-/-} → CSJLF1	66%
p = NS	

B

Figure 3. Engraftment rate and peripheral blood chimerism levels in allogeneic recipients

Following delivery and weaning, animals were assessed serially for peripheral blood chimerism by retroorbital puncture. Recipients with detectable engraftment (greater than 0.3%) at three weeks were considered chimeric at weaning. (A) The engraftment rates for both groups are summarized in the table and demonstrate no significant difference ($p=NS$). (B) CSJLF1 recipients of B6.TAP^{-/-} cells demonstrated significantly lower levels of peripheral blood hematopoietic chimerism at 3 weeks of age, which progressively deteriorated over time. The Balb/c recipients of B6.Ly5.2 donor cells exhibited high-level initial chimerism that remained stable for greater than 12 weeks.

Figure 4. Multi-lineage hematopoietic chimerism in host splenocytes

Lineage analysis of chimera splenocytes was conducted via splenic biopsy at twelve weeks of age. (A) Balb/c recipients of B6.Ly5.2 cells demonstrated balanced multi-lineage donor and host hematopoiesis. (B) CSJLF1 recipients also demonstrated multi-lineage donor and host hematopoiesis. A relative paucity of lineage committed B cells (B220) was observed in the B6.TAP^{-/-} donor cells ($p < 0.01$).