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Fetal allotransplant recipients are resistant to graft-versus-host disease

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Abstract

In utero hematopoietic cell transplantation (IUHCT) is an experimental treatment for congenital hemoglobinopathies including Sickle cell disease and thalassemias. One of the principle advantages of IUHCT is the predisposition of the developing fetus toward immunologic tolerance. This allows for engraftment across immune barriers without immunosuppression and, potentially, decreased susceptibility to graft-versus-host-disease (GVHD). We demonstrate fetal resistance to GVHD following T cell-replete allogeneic hematopoietic cell transplantation compared to the neonate. We show that this resistance is associated with elevated fetal serum interleukin-10 conducive to regulatory T cell induction. Finally, we demonstrate that adoptive transfer of regulatory T cells (Tregs) from IUHCT recipients to neonates uniformly prevents GVHD, recapitulating the predisposition to tolerance observed after fetal allotransplantation. These findings demonstrate fetal resistance to GVHD following hematopoietic cell transplantation and elucidate Tregs as important contributors.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

JSR designed the research study, conducted the experiments, acquired the data, analyzed the data, wrote the manuscript, and edited the manuscript.

LEM designed the research study, conducted the experiments, acquired the data, and edited the manuscript.

JDS, BEC, SKB, AD, and BMW conducted the experiments, acquired the data, and edited the manuscript.

HL acquired the data and edited the manuscript.

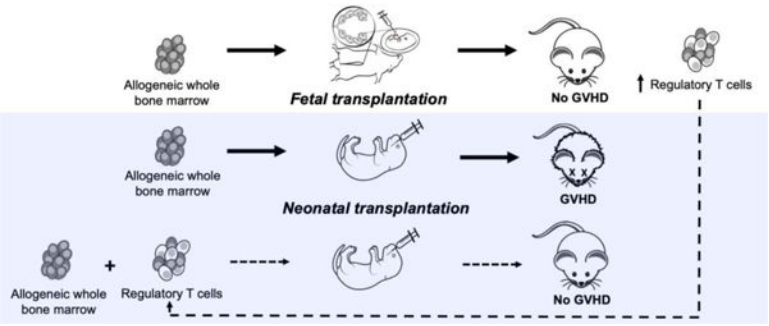
SPL, CGF, and AIBSD designed the research study, conducted the experiments, and edited the manuscript.

AWF analyzed the data.

WHP designed the research study, analyzed the data, and edited the manuscript.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.



INTRODUCTION

In utero hematopoietic cell transplantation (IUHCT) is an experimental treatment for inherited immune, metabolic, and hematologic disorders with potential advantages over postnatal hematopoietic cell transplantation (HSCT). In preclinical studies, IUHCT achieves long-term alloengraftment without myeloablation, immunosuppression, or the need for an HLA-matched donor[1–3]. Graft-versus-host disease (GVHD) is a significant cause of morbidity and mortality after postnatal HSCT[4–7] and is the one of the greatest theoretical risks of IUHCT[8]. In addition to allowing allogeneic donor cell engraftment, the tolerogenic fetal environment may also effect host-reactive donor T cells and reduce the risk of graft-versus-host disease (GVHD). In the current study, we evaluate if the fetus is less susceptible to GVHD following allogeneic HSCT and, if so, by what mechanisms.

Central tolerance, characterized by the deletion of donor-reactive host and host-reactive donor T cell clones, has been shown to be fundamental to the long-term tolerance observed following IUHCT[3,9–11]. Central tolerance alone, however, is insufficient to explain the apparent absence of GVHD following either IUHCT with T cell-replete allografts or postnatal donor lymphocyte infusion (DLI) in previous IUHCT recipients[8,12,13]. Peripheral tolerance, specifically regulatory T cell (Treg)-mediated suppression of host-reactive donor T cells, may be an important secondary contributor to tolerance following IUHCT that contributes to GVHD resistance[11,14,15].

We have recently shown that regulatory T cells (Tregs), including host-derived $CD4^+CD25^+FoxP3^+$ Tregs and donor-derived $CD4^+CD49b^+LAG\text{-}3^+$ type 1 regulatory T cells (Tr1 cells) are more prevalent in the peripheral tissues of IUHCT recipient mice than in uninjected controls[15]. In the current study, we hypothesize that fetal recipients of allogenic IUHCT are less susceptible to GVHD and that Tregs induced by transplantation into the tolerogenic fetal environment contribute to this GVHD resistance. To test this hypothesis, we established a neonatal mouse model of GVHD following allogeneic HSCT and compared the incidence of GVHD between fetuses and neonates receiving the same inoculum of T cell-replete allogeneic bone marrow. Using this approach, we demonstrate a reduction in GVHD in fetal recipients of allogeneic HSCT. Furthermore, we demonstrate that fetal resistance to GVHD is transferable to the neonate through adoptive transfer of Tregs induced by allogeneic IUHCT. These studies support a role of the tolerogenic fetal environment in fostering true reciprocal tolerance after allogeneic transplantation.

METHODS

Mice

Balb/cJ (Balb/c, CD45.2, H2k^{d}, Jackson Cat#000651) and C57BL/6J (B6, CD45.2, H2k^{b}, Jackson Cat#000664) mice were purchased from Jackson Laboratories and maintained in our colony. C57BL/6TgN(act-EGFP)OsbY01 (B6^{GFP}, CD45.2, H2k^{b}) mice were provided by Dr. M. Okabe (Osaka University, Osaka, Japan) and maintained in our colony. Experimental protocols were approved by the Institutional Animal Care and Use Committee and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In utero hematopoietic cell transplantation

Whole bone marrow (WBM, ~3% CD3^{+} T cells) was harvested from 6- to 8-week-old B6 and B6^{GFP} males and females as previously described[16]. Briefly, tibias, femurs, and iliac bones were dissected and flushed with sterile Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) (Mediatech, Manassas, VA). The WBM was then filtered through a 70μm nylon mesh filter and layered over sterile Ficoll-Paque PLUS (Ficoll, GE Healthcare Bio-Sciences, Pittsburgh, PA) to isolate the low density mononuclear cell (LDMC) layer. Cells were counted and ≥95% viability confirmed by trypan blue exclusion and resuspended in sterile PBS for injection at a concentration of 1×10^6 LDMCs/μL and a dose of 10×10^6 cells/recipient.

Fetuses of time-dated pregnant mice were injected at 14 day post-coitum (DPC) with WBM cells to generate B6→Balb/c and B6^{GFP}→Balb/c chimeras as previously described[17]. Briefly, under isoflurane anesthesia, a midline laparotomy was performed to expose the uterine horns. The vitelline vein was injected using a 100μm beveled glass micropipette, a dissecting microscope, and programmable microinjector (IM-300 Microinjector; MicroData Instrument Inc, S. Plainfield, NJ). After IUHCT, the fetuses were returned to the peritoneal cavity, and a 2-layer abdominal closure was performed with 4–0 Vicryl suture (Ethicon). To account for the effect on engraftment of maternal antibodies transferred via the breastmilk to newborn pups[18], all litters transplanted before birth were fostered immediately after birth with uninjected Balb/c dams.

Neonatal transplantation

Transplantation was performed after birth at 20 DPC via injection into the facial vein using a 100μm beveled glass micropipette and programmable microinjector, as above. 10×10^6 B6^{GFP} WBM cells were injected per neonate. As maternal sensitization only occurs after in utero injection, neonates were not fostered.

Assessment of graft-versus-host disease

Mice were assessed for survival, weight gain, GVHD phenotype score, and histologic evaluation of GVHD-prone organs. Assessment was performed at weeks 1–4, during which period GVHD occurs in this model. The GVHD phenotype score ranging from 0–10 was calculated based on the presence of hunched posture, ruffled fur, fur loss, skin integrity (desquamation/ulceration), and diarrhea as previously described[19]. Zero to 2 points were

assigned for each sign with aggregate scores corresponding to overall disease severity: 0–2 (no GVHD), 3–4 (mild GVHD), 5–7 (moderate GVHD), and 8–10 (severe GVHD). A severe GVHD phenotype was considered lethal disease, and animals so affected were euthanized. At 3 weeks of age, representative animals from each group were sacrificed. Liver, skin, and bowel were harvested, fixed in formalin, and stained with hematoxylin and eosin for histology.

Animals were considered to have developed GVHD under the following conditions: 1) death prior to 4 weeks of age, or 2) weight gain >2 standard deviations below the mean of age-matched uninjected controls plus GVHD phenotype score >2. Using this definition, 3 outcomes are possible in this model:

Outcome 1: Donor cell engraftment with GVHD

Outcome 2: Donor cell engraftment without GVHD

Outcome 3: Donor cell rejection (or missed injection) without GVHD

As persistent donor cells are necessary for GVHD to develop in this model, mice with Outcome 3 (defined as 0% donor cell chimerism at 4 weeks of age) were excluded when assessing the overall incidence of GVHD in each group, calculated as follows: (Outcome 1) / (Outcome 1 + Outcome 2).

Assessment of donor cell chimerism

Mice were bled at 4 weeks of life by retro-orbital venipuncture using Heparinized Micro-hematocrit Capillary Tubes (Kimble Chase Cat#40C505) under isoflurane anesthesia. Red cell lysis was performed with BD Pharm Lyse (BD Biosciences Cat#555899). Cells were stained with CD45-PE (Biolegend Cat#103106), $H2k^{b}$-APC (eBioscience Ref#17–5958-82), and $H2k^{d}$-PerCP/Cy5.5 (Biolegend Cat#116618) at 1:100 for 25 minutes at 4°C. Donor cell chimerism was assessed among $CD45^{+}$ cells and was calculated as ($H2k^{b+}GFP^{+}$ / ($H2k^{b+}GFP^{+}$ + $H2k^{d+}$)) × 100 for mice injected with $B6^{GFP}$ BM and as ($H2k^{b+}$ / ($H2k^{b+}$ + $H2k^{d+}$)) × 100 for mice injected with B6 BM.

Adoptive transfer of T cells from chimeric mice

In order to determine if Tregs induced by IUHCT could suppress GVHD after neonatal transplantation, spleens were harvested from 4-week-old male and female B6→Balb/c chimeric mice. Cells were resuspended in staining buffer for CD4 positive selection by magnetic activated cell sorting (MACS) using CD4 (L3T4) Microbeads, mouse (Miltenyi Biotec Cat#130–117-043) and LS columns (Miltenyi Biotec Cat#130–042-401). Post-selection $CD4^{+}$ purity >90% was confirmed by flow cytometry. Cells were resuspended at a concentration of 5×10^{5} cells/μL and co-injected with WBM cells into 20 DPC neonates at a dose of 5×10^{6} $CD4^{+}$ cells in 20uL total volume.

In order to determine whether donor- or host-derived cells more effectively prevented GVHD, $CD4^{+}$ splenocytes from chimeric mice were separated by MHC class I ($H2k^{b}$ vs. $H2k^{d}$). Cells were first stained with $H2k^{b}$-biotin (eBioscience Ref#13–5958-82) or

H2k^{d}-biotin (eBioscience Ref#13–5957-82) at a dilution of 1:100 at a final concentration of 5 × 10^{4} cells/μL for 25 minutes at 4°C. Cells were washed and incubated with Anti-Biotin Microbeads (Miltenyi Biotec Cat#130–090-485) at a dilution of 1:50 at a final concentration of 1 × 10^{5} cells/μL for 15 minutes at 4°C. Depletion was performed using LS columns. Positive selection of CD4^{+} cells was then performed as described above using MS columns (Miltenyi Biotec Cat#130–042-201). Cells were confirmed to be >99.5% H2k^{b-} or H2k^{d-} by flow cytometry. Cell doses were chosen based on the relative frequency of the subpopulations in a 4-week-old chimeric mouse, which yields approximately 5 × 10^{6} CD4^{+} splenocytes and for which splenic chimerism of an injected cohort is 20–50%. To determine if host-derived Tregs, specifically, prevent GVHD in this model, CD4^{+}CD25^{+} Tregs were isolated using CD4^{+}CD25^{+} Regulatory T cell Isolation Kit, mouse (Miltenyi Biotec Cat#130–091-041) after donor cell depletion. FoxP3^{+} expression among CD4^{+}CD25^{+} T cells purified with this kit was confirmed to be >90%.

IL-10 enzyme-linked immunosorbent assay (ELISA)

Interleukin-10 (IL-10) potentiates the generation of induced Tregs in the periphery from naïve CD4^{+} T cells[20], and elevated levels of IL-10 have been suggested to contribute to the tolerogenic fetal environment[21]. Blood was collected from 14 DPC fetuses and 20 DPC neonates by decapitation. Serum was collected after centrifugation at 10,000rpm for 10 minutes and frozen at −80°C until analysis. IL-10 was measured using Invitrogen Mouse IL-10 ELISA Kit (ThermoFisher Cat#BMS614), which has a threshold of detection of >15pg/mL (the lowest among commercially-available kits). To achieve the minimum necessary sample volume (100uL), sera from all fetuses/neonates in each litter were pooled, and therefore each individual value represents a litter. Samples were run in duplicate at a 1:1 dilution. To confirm that serum IL-10 levels of 20 DPC neonates were truly below 15pg/mL, analysis was repeated in a second cohort using undiluted serum samples run singly. Optical density was measured by absorbance at 450nm using Varioskan LUX Multimode Microplate Reader (ThermoFisher).

Statistics

Binary outcomes such as the incidence of GVHD were compared using Chi-square or Fisher's exact test. Equality of survival curves was assessed using the Log-rank (Mantel-Cox) test. Continuous, parametric outcomes such as weight/weight gain were compared using analysis of variance (ANOVA) with multiple comparison. The ordinal outcome of GVHD phenotype score was analyzed using Kruskal-Wallis. IL-10 concentration was analyzed using one-sample t test, which the assay detection threshold (15pg/uL) used as the hypothesized mean. Statistical analysis and graphing were performed using Prism, version 9 (GraphPad, La Jolla, CA). All statistical tests were two-sided with an alpha level set at 0.05 for statistical significance unless otherwise specified.

RESULTS

The fetal environment reduces the risk for GVHD following allogeneic WBM transplant

Allogeneic IUCHT at 14 DPC was performed in 83 fetuses. Fifty-eight (70%) survived to birth. Of these, 3 (5%) had no chimerism (due to a missed injection) and were excluded

from GVHD analysis. The overall incidence of postnatal GVHD was 3/55 (5%), with 2 mice (3%) dying within a week after birth (Figure 2A), and 1 mouse (2%) developing mild, non-lethal GVHD. This was not statistically higher than the incidence of GVHD among uninjected controls (0/32 (0%), P=0.3, relative risk (RR): 1.6 (0.7–7.6)). In aggregate, mean bodyweight of the cohort was significantly lower at 1 week of age compared to uninjected controls (4.2g vs. 5.1g, P<0.001). However, weight gain normalized by 3 weeks of age (Figure 2B). Phenotype scores remained below the threshold of GVHD in all but the 1 injected animal referred to above, which exhibited a mild GVHD phenotype score of 3 (Figure 2C). Mean donor cell chimerism among the survivors to 4 weeks of age was 15.8% (standard deviation (SD): 16.7%). Chimerism of the 1 mouse with mild GVHD was 5.5%.

In order to determine whether the observed 30% prenatal mortality was caused by acute GVHD mediated by T cells within the WBM donor allograft or, alternatively, by the procedure itself, allogeneic IUHCT was performed with T cell-depleted BM in a separate cohort of 14 DPC fetuses (see Supplemental Methods). Survival to birth following injection of TCD BM was 26/42 (62%), equivalent to that observed following injection of WBM (8/83 (70%), P=0.4). This suggested that the prenatal mortality following IUHCT was not caused by acute GVHD mediated by T cells within the donor allograft but was procedural mortality.

Allogenic transplantation was then performed at 20 DPC in 101 neonates. Of these, 32 (32%) had no chimerism (either due to allograft rejection or a missed injection) and were excluded. A total of 69 mice were therefore included in the GVHD analysis. The overall incidence of GVHD was 44/69 (64%), with 33 (75%) mice developing lethal GVHD (Figure 2A) and 11 mice (25%) developing non-lethal GVHD. Among the 33 mice with lethal GVHD, 27 died spontaneously and 6 were euthanized (2 for severe GVHD scores of 8 and 4 with moderate GVHD scores of 5–6 but with severe diarrhea causing dehydration/lethargy requiring euthanasia). The overall incidence of GVHD (64%) was significantly higher than that observed following injection of WBM at 14 DPC (5%, P<0.0001, RR 10.6 (3.9–31.1)). Weight gain among the cohort was significantly lower than uninjected controls at 2, 3 and 4 weeks of age (Figure 2B) associated with significantly elevated GVHD phenotype scores compared to both uninjected controls and mice injected at 14 DPC (Figure 1C) at those time points. Mean donor cell chimerism among the survivors at 4 weeks of age was 30.6% (SD: 38.9%). No correlation was observed between the magnitude of chimerism and GVHD score (R^2=0.07, P=0.12 for non-linearity, see Supplemental Figure 1). Representative gross pathology at 3 weeks of age (Figure 3A) demonstrated characteristic signs of GVHD among mice injected at 20 DPC: small size for age, hunched posture, ruffled fur, skin ulceration, and diarrhea. Gross pathology was notable for bile stasis and parenchymal necrosis of the liver. Representative microscopic histology also demonstrated characteristic features of GVHD, including periportal inflammation with multifocal necrosis in the liver, skin vacuolization at the dermal-epidermal border, and epithelial necrosis in the bowel (Figure 3B). In aggregate, these findings demonstrated a high incidence of GVHD among neonates following transplantation of T cell-replete allografts with comparative resistance to GVHD among fetal recipients of the same donor cell inoculum (RR reduction: 0.91 (0.74–0.97)).

The fetal environment is characterized by elevated IL-10

To investigate the potential mechanism of fetal resistant to GVHD, we compared serum IL-10 levels among uninjected 14 DPC fetuses and 20 DPC neonates. 14 DPC fetuses had a mean serum IL-10 concentration of 36.2 ± 4.6pg/mL (n=4 litters). In contrast, serum IL-10 levels in 20 DPC neonates were undetectable (below the assay threshold of 15pg/mL) (n=4 litters). As such, fetal serum IL-10 levels were at least 2.4 times higher than those of neonates and significantly higher than the assay threshold (P=0.0027).

Adoptive transfer of IUHCT-induced Tregs prevents neonatal GVHD

IUHCT recipients have an increased prevalence of Tregs among $CD4^+$ T cells in their bone marrow and spleens compared to uninjected controls[15], an effect that is further amplified after postnatal donor lymphocyte infusion into prior IUHCT recipients (see Supplemental Figure 2). Having now demonstrated that the fetus is resistant to GVHD compared to the neonate and that the fetal serum has elevated IL-10 that may be conducive to Treg differentiation, we hypothesized that IUHCT induces regulatory T cells that contribute to the prevention of GVHD. To test this, we assessed whether the adoptive transfer of $CD4^+$ T cells from allogeneic IUHCT recipients to neonates could prevent GVHD following allogeneic transplantation.

Ten million $B6^{GFP}$ WBM cells were injected into 20 DPC Balb/c pups in combination with 5×10^6 $CD4^+$ cells harvested from the spleens chimeric mice (Figure 1). The incidence of GVHD was compared to our cohorts of uninjected Balb/c mice (healthy control) and 20 DPC Balb/c pups injected with WBM cells only (GVHD control). Two of the 14 mice (14%) had no chimerism (either due to allograft rejection or a missed injection) and were excluded. The overall incidence of lethal or nonlethal GVHD was 0/12 (0%), significantly lower than that observed following injection of WBM only at 20 DPC (44/69 (64%), P<0.0001). Weight gain and phenotype scores were equal to uninjected Balb/c controls (Figure 2B and 2C). Mean donor cell chimerism was 6.1% (SD: 8.9%). Representative gross pathology (Figure 3A) and microscopic histology of the liver, skin, and bowel (Figure 3B) showed no features of GVHD.

In order to determine if the capacity to prevent GVHD in this model was unique to $CD4^+$ T cells from IUHCT-tolerant mice, WBM was co-injected with 5×10^6 $CD4^+$ cells harvested from the spleens of age-matched naïve B6 ($H2k^b$) or Balb/c ($H2k^d$) mice. Co-injection of $CD4^+$ splenocytes harvested from naïve B6 mice worsened GVHD, with all animals developing lethal GVHD. Co-injection of $CD4^+$ splenocytes harvested from naïve Balb/c mice resulted in allograft rejection in all recipients (Table 1).

To determine if the capacity $CD4^+$ T cells from IUHCT-tolerant mice to prevent GVHD in this model was unique to either the host- or donor-derived subpopulation, each was injected separately. Co-injection of host-derived ($H2k^{d+}$) $CD4^+$ splenocytes from chimeric mice prevented GVHD in all recipients while co-injection of donor-derived ($H2k^{b+}$) $CD4^+$ splenocytes from chimeric mice failed to prevent GVHD (Table 1). In order to definitively demonstrate that host-derived Tregs were the critical subpopulation among the host $CD4^+$ T cell milieu for preventing GVHD, we co-injected a purified subpopulation of these cells

($H2k^{d+}CD4^{+}CD25^{+}$, approximately 10% of the total) along with allogeneic B6 WBM into 20 DPC Balb/c mice. No GVHD was observed (Table 1), confirming that the resistance to GVHD conferred through adoptive transfer was indeed host Treg-mediated.

DISCUSSION

IUHCT is a potential treatment for congenital immune, metabolic, and hematologic disorders with several potential advantages over postnatal HSCT including a reduced risk of GVHD. Thymic deletion of developing host-reactive donor T cells following the HSCT partially explains the resistance to GVHD. However, it does not account for T cells that escape thymic deletion. Suppression of reactive T cells that escape thymic deletion by regulatory T cells may explain this phenomenon. In this study, we demonstrate that the murine fetus is resistant to GVHD compared to the neonate following transplantation of T cell-replete allografts. When host-derived Tregs are adoptively transferred from previous IUHCT recipients to naïve neonates, these cells reliably prevent GVHD. Taken together, these data suggest that the introduction of allogeneic donor cells into the uniquely tolerogenic fetal environment fosters the development of regulatory T cells capable of suppressing GVHD. As such, the study elucidates a mechanism by which the fetus is resistant to GVHD and is thereby an attractive candidate for hematopoietic transplantation.

Understanding the fetal resistance to GVHD is clinically important, as the ability to use T cell-containing allografts safely for clinical IUHCT has several potential benefits. Sickle cell disease and beta-thalassemia can be phenotypically corrected with lymphomyeloid chimerism levels of ~20%[1,22–25]. Through a graft-versus-hematopoietic (GVH) effect, donor T cells improve engraftment, helping to achieve this therapeutic level of chimerism[10]. Additionally, the GVH effect of donor T cells may help overcome a developing fetal immune barrier associated with IUHCT performed later in gestation, when the technical difficulty of the procedure is lower and the population of treatable fetuses is larger[15,26].

The findings of the current study have additional relevance to previous studies of allogenic IUHCT in preclinical large animal models with an eye towards clinical translation. Our canine model of IUHCT utilizes allografts with 1% $CD3^{+}$ cells to optimize engraftment and chimerism while remaining safely below the threshold $CD3^{+}$ level (≥5%) that induces postnatal GVHD[2,8]. It has been suggested that GVHD following postnatal HSCT results when donor T cell concentrations overwhelm the suppressive effect of host Tregs[27,28]. Because we were unable to differentiate between donor- and host-derived Tregs populations in our canine IUHCT studies, we could not elucidate whether the same pathophysiology applies to IUHCT. Returning to the murine model, we were able to differentiate between host- and donor-derived populations using differences in MHC class I to show that host-derived Tregs are necessary for suppressing GVHD. This shows that, while the fetal recipient is resistant to GVHD compared to postnatal recipients, this resistance still depends on the finite suppressive effects of host Tregs.

It is important to note limitations to the current study. Treg-mediated suppression is not the only mechanism of peripheral tolerance by which GVHD could be suppressed following IUHCT, and the capacity of Tregs from IUHCT recipients to prevent GVHD in the neonate

does exclude the contribution of additional mechanisms to host-specific donor cell tolerance after IUHCT. Kim, et al demonstrated exhaustion among donor reactive host T cell clones that do not undergo thymic deletion following IUHCT, showing that the reactivity of these populations to postnatal donor skin grafts could be restored with exogenous IL-2 stimulation[14]. Exhaustion among host-reactive donor T cells could similarly contribute to GVHD suppression following IUHCT. Additionally, the focus of this study is limited to Tregs and does not investigate the parallel contribution of antigen-presenting cells (APCs) to the tolerogenic fetal environment[29] following hematopoietic transplantation. A future study of the differences in APC and Treg function after fetal versus neonatal hematopoietic transplantation should be performed to more clearly delineate the unique mechanisms of fetal tolerance induction.

IUHCT remains a promising experimental treatment for an array of hematologic, metabolic and immune diseases including beta hemoglobinopathies and other congenital anemias. It has long been appreciated that a major rationale for IUHCT is to take advantage of the tolerant fetal immune system to allow for the induction of allogeneic donor-specific tolerance and alloengraftment without immunosuppressive or myeloablative conditioning. Overlooked, however, was the possibility that the tolerant fetal immune system could also influence host-reactive donor cells and the incidence of GVHD following allogeneic IUHCT. The current study, demonstrating the resistance of the fetus to GVHD and a mechanism behind this resistance, adds to the rationale for IUHCT, advancing the field closer to safe, effective clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Injection of allogeneic T cell-replete marrow induces GVHD in the neonatal mouse, whereas the fetal mouse is resistant.
- This resistance to GVHD can be transferred from the fetal IUHCT recipient to the neonate by adoptive transfer of regulatory T cells.

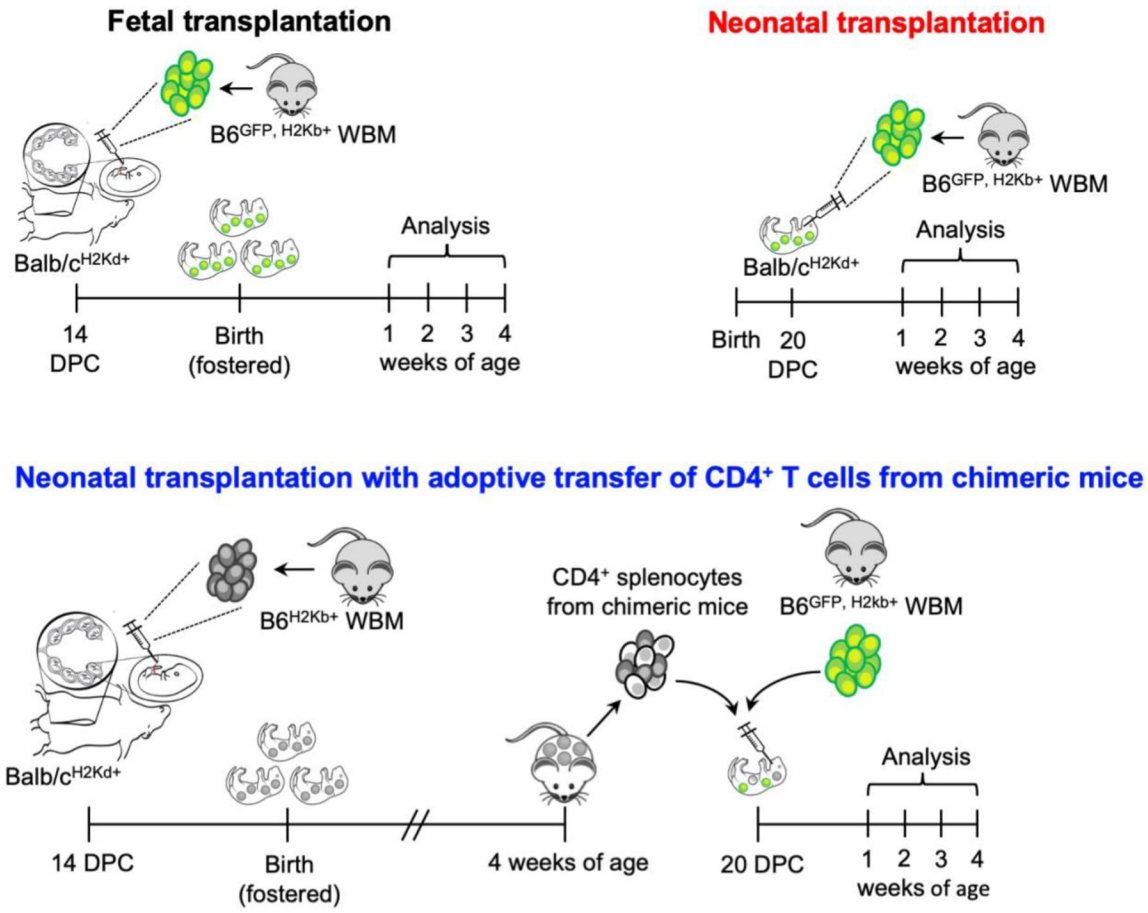

Figure 1: Experimental design.

Allogeneic WBM was injected either before birth at 14 day post-coitum (DPC) via the vitelline vein or after birth at 20 DPC via the facial vein. The capacity of IUHCT-induced Tregs to prevent GVHD was tested though adoptive transfer of $CD4^+$ T cells (and subsequently various sub-populations within that milieu) into neonates receiving concurrent allogeneic WBM transplantation. Uninjected Balb/c mice served as healthy controls.

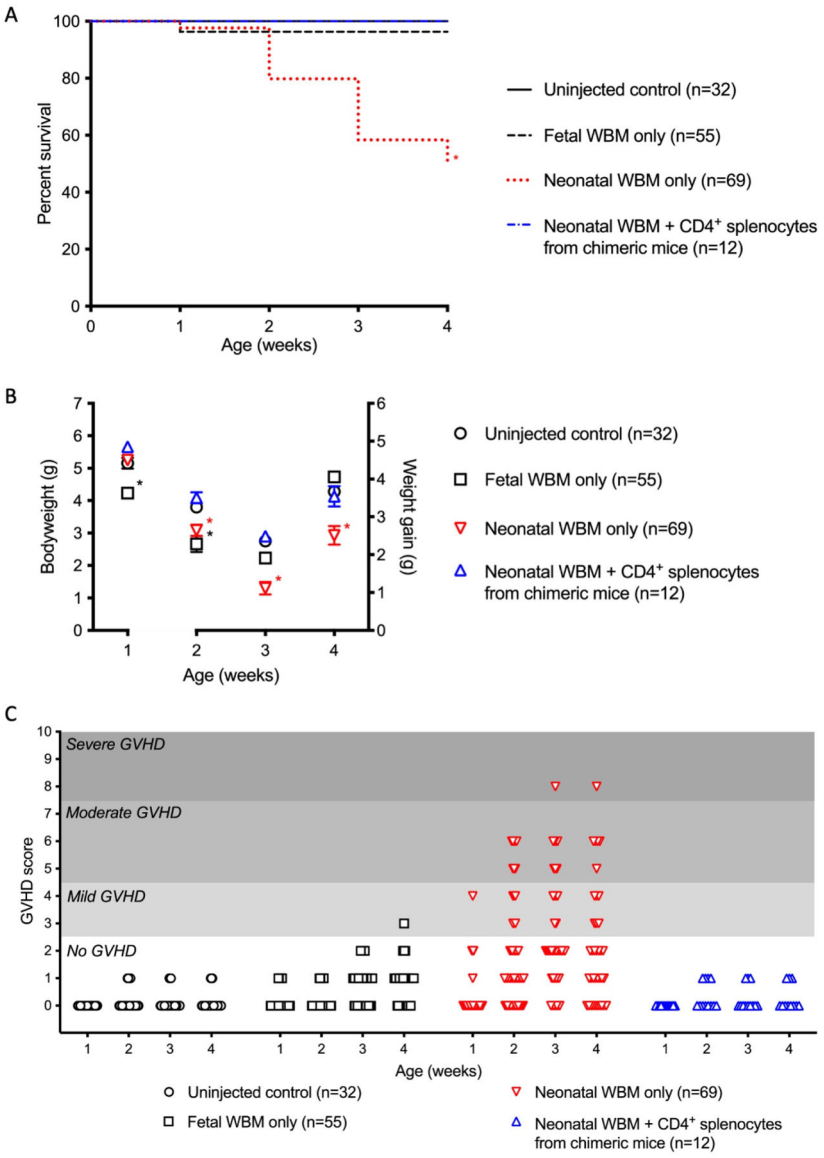

Figure 2: Clinical assessment of GVHD.

A) Kaplan-Meier curves of postnatal survival. Log-rank (Mantel-Cox) test generated P<0.0001. B) Bodyweight and weight gain. Shown is mean bodyweight ± SEM at 1 week of age and subsequent mean weight gain ± SEM at weeks 2, 3, and 4. Data were analyzed using ANOVA with statistical significance (P<0.05) compared to uninjected controls indicated by *. C) Distribution of GVHD phenotype scores in the first 4 weeks of life. Data were analyzed using Kruskal-Wallis. Mice injected with WBM only at 20 DPC showed significantly elevated scores compared to both uninjected controls and mice injected at 14 DPC with WBM at 2, 3, and 4 weeks (P<0.05 for each comparison at all time points). Mice injected with WBM + $CD4^+$ splenocytes from chimeric mice at 20 DPC did not demonstrate phenotypic signs of GHVD.

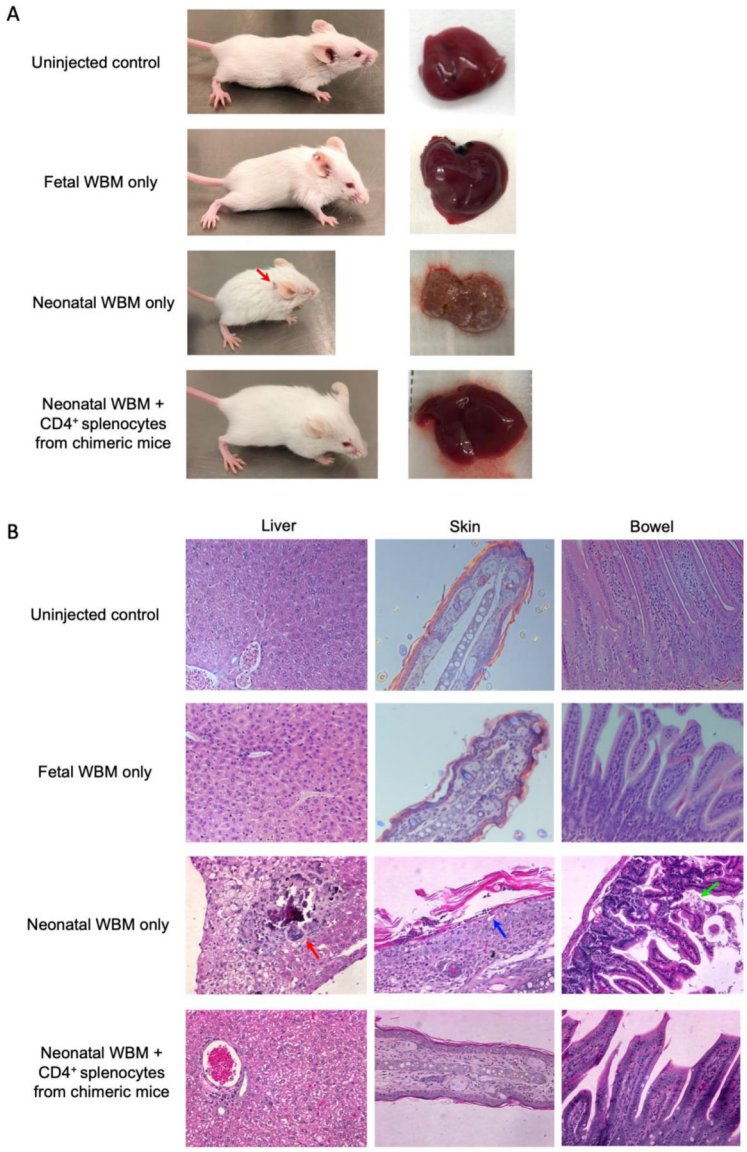

Figure 3: Histologic assessment of GVHD.

A) Mice injected with WBM only 20 DPC showed characteristic signs of GVHD, including hunched posture, diarrhea, ruffled fur, and skin lesions (red arrow). Grossly, the livers of mice injected at 20 DPC with WBM showed multifocal necrosis and bile stasis, while the livers in the other groups all appeared normal. B) Microscopic histology of the liver, skin, and bowel stained with hematoxylin and eosin and imaged at 20× magnification. Features of GVHD were visualized only among mice injected with WBM only at 20 DPC. These features included necrosis of the liver characterized by apoptotic hepatocytes with adjacent multinucleated giant cells (red arrow), skin vacuolization at dermal-epidermal border (blue arrow), and epithelial necrosis in the small bowel (green arrow). These features were not observed among uninjected controls, mice injected at 14 DPC with WBM, or mice injected at 20 DPC with WBM + $CD4^+$ splenocytes from chimeric mice, as shown.

Table 1:

Incidence of GVHD following co-injection of various cell (sub)populations with allogeneic WBM at 20 DPC

Cells co-injected with whole bone marrow	Number of cells injected (millions)	Number of mice injected	Number with no chimerism (excluded from GVHD analysis)	Incidence of GVHD
None	n/a	101	32 (32%)	44/69 (64%)
Naïve mice				
B6 ($H2k^b$) $CD4^+$	5	7	0 (0%)	7/7 (100%)
Balb/c ($H2k^d$) $CD4^+$	5	13	13 (100%)	n/a
Chimeric mice				
All ($H2k^{d+}$ and $H2k^{b+}$) $CD4^+$	5.0	14	2 (17%)	0/12 (0%)
→ Host-derived ($H2k^{d+}$)	2.5	10	2 (20%)	0/8 (0%)
→CD25$^+$ (Tregs)	**0.05**	**10**	**5 (50%)**	**0/5 (0%)**
→CD25$^-$	0.25	12	8 (67%)	2/4 (50%)
→ Donor-derived ($H2k^{b+}$)	1.0	6	0 (0%)	4/6 (66%)