REGULAR ARTICLE

Regulatory T cells promote alloengraftment in a model of late-gestation in utero hematopoietic cell transplantation

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Key Points

- Traditional and nontraditional regulatory T cells are elevated after early-gestation IUHCT.

- Allograft enrichment with regulatory T cells promotes engraftment later in development after the acquisition of T-cell immunity.

In utero hematopoietic cell transplantation (IUHCT) has the potential to cure congenital hematologic disorders including sickle cell disease. However, the window of opportunity for IUHCT closes with the acquisition of T-cell immunity, beginning at approximately 14 weeks gestation, posing significant technical challenges and excluding from treatment fetuses evaluated after the first trimester. Here we report that regulatory T cells can promote alloengraftment and preserve allograft tolerance after the acquisition of T-cell immunity in a mouse model of late-gestation IUHCT. We show that allografts enriched with regulatory T cells harvested from either IUHCT-tolerant or naive mice engraft at 20 days post coitum (DPC) with equal frequency to unenriched allografts transplanted at 14 DPC. Long-term, multilineage donor cell chimerism was achieved in the absence of graft-versus-host disease or mortality. Decreased alloreactivity among recipient T cells was observed consistent with donor-specific tolerance. These findings suggest that donor graft enrichment with regulatory T cells could be used to successfully perform IUHCT later in gestation.

Introduction

In utero hematopoietic cell transplantation (IUHCT) is a nonmyeloablative nonimmunosuppressive transplant approach that results in donor cell engraftment across immune barriers.[1,2] It has the potential to treat a number of congenital immune, metabolic, and hematologic disorders, including sickle cell disease and thalassemia.[3-6] IUHCT has been successful in preclinical studies in the murine, canine, ovine, and porcine models.[1,2,7,8] The clinical translation of IUHCT, however, has been heretofore disappointing. Among the approximately 50 reported cases of clinical IUHCT, efficacy has been limited to lineage-specific engraftment in fetuses with severe combined immunodeficiency disease and low-level, nontherapeutic engraftment in immunologically normal fetuses after early-gestation transplantation.[9-12]

The gestational age of the fetus and the predisposition of the fetal immune system toward tolerance early in gestation are key determinants of successful alloengraftment after IUHCT,[13,14] and the success of IUHCT in severe combined immunodeficiency disease suggests that the fetal T-cell response is particularly important. In the human fetus, alloreactive T cells emerge in the peripheral blood (PB) and spleen as early as 14 weeks' gestation.[15,16] Clinical experience with IUHCT suggests this to be the gestational age after which immunologically normal fetuses can reject allotransplants.[12,13,17] The impetus to perform IUHCT before this point, however, is counterbalanced by technical and practical constraints on the procedure. Intravascular injection, which optimizes engraftment,[18] is challenging at 14 weeks' gestation as a result of the small size of the target sites, namely the umbilical cord (diameter: 3.7-4.4 mm[19]) and fetal heart (internal diameter of left and right ventricle: 2.5-3 mm[20]). In addition, performing IUHCT by 14 weeks' gestation requires a series of events to occur very early in pregnancy: the mother must realize she is pregnant, she must undergo prenatal testing that confirms a treatable fetal diagnosis, she must receive multidisciplinary

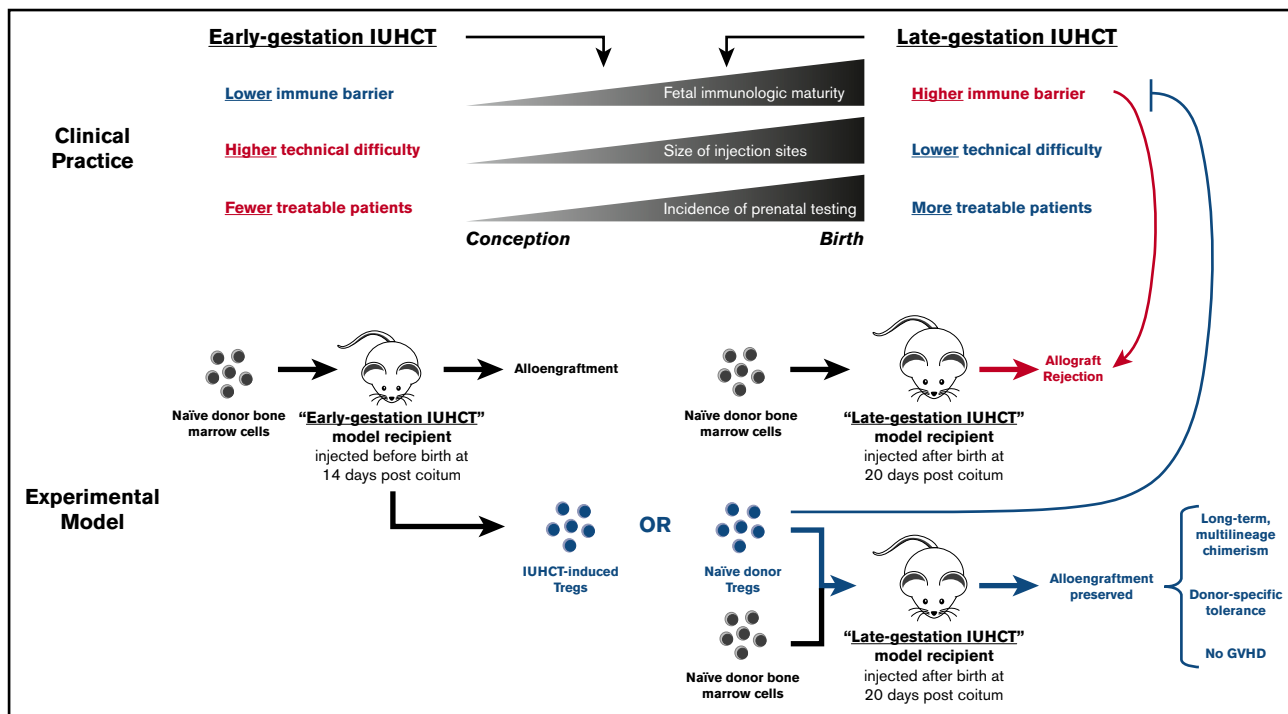The full-text version of this article contains a data supplement.

Figure 1. Study concept. In clinical practice, early-gestation IUHCT affords the lowest fetal immune barrier but is impeded by higher technical difficulty and fewer treatable patients. Late-gestation IUHCT, in contrast, affords lower technical difficulty and more treatable patients, but is impeded by a higher immune barrier leading to allograft rejection. To study this problem, we employed murine models of early-gestation IUHCT (injection of allogeneic hematopoietic cells before birth at 14 DPC) and late-gestation IUHCT (injection of allogeneic hematopoietic cells after birth at 20 DPC). First, we investigated which regulatory T-cell populations, if any, are elevated after early-gestation IUHCT and may contribute to alloengraftment and tolerance. Next, we validated our model of late-gestation IUHCT, showing it to reliably lead to allograft rejection. Next, we tested whether IUHCT-induced Tregs or naive donor Tregs (a clinically translatable approach) could prevent allograft rejection in our model of late-gestation IUHCT. Outcomes assessed included long-term, multilineage chimerism, donor-specific tolerance, and the absence of graft-versus-host disease.

counseling, donor cells must be prepared, and finally the procedure itself must be performed. For these reasons, only a minority of clinical IUHCTs have been performed by 14 weeks' gestation.[12]

An improved understanding of the tolerogenic fetal environment in the context of fetal transplantation may present opportunities to extend the window of opportunity for IUHCT to later in gestation. We know that IUHCT performed early in gestation results in clonal deletion of donor-reactive host T cells in the fetal thymus (ie, central tolerance induction).[21-23] However, we also know that clonal deletion after IUHCT is incomplete, with donor-reactive host T cells remaining long after birth without causing graft rejection.[24,25] Peripheral tolerance, including regulatory T cell–mediated suppression of donor-reactive T cells, has been suggested as an important secondary contributor to IUHCT-induced donor-specific tolerance[23,24] and may prove useful for overcoming the increased immune barrier associated with late-gestation IUHCT. In this study, we characterize donor and host regulatory T cells in the setting of allogenic IUHCT and demonstrate that regulatory T cells, either from tolerant mice after early gestation IUHCT or from naive donors, can preserve alloengraftment after the acquisition of T-cell immunity in a mouse model of late-gestation IUHCT.

Methods

Study concept

The overall study concept is summarized in Figure 1. To model IUHCT performed early and late in gestation, allogeneic hematopoietic

cell transplantation was performed at 2 different points in the mouse model. Injection performed before birth at 14 days postcoitum (DPC) was used as the murine immune-equivalent model of early-gestation human IUHCT, as previously described.[26] Injection performed after birth at 20 DPC served as the murine immune-equivalent model of late-gestation human IUHCT. The effect of IUHCT on regulatory T-cell induction was assessed after IUHCT at 14 DPC, and the ability of IUHCT-induced regulatory T cells or naive allogeneic donor regulatory T cells to promote alloengraftment in the late-gestation IUHCT model was assessed.

Mice

Balb/cJ (Balb/c, H2k^d, Jackson Cat. No. 000651), C57BL/6J (B6, H2k^b, Jackson Cat. No. 000664), C.Cg-Foxp3^{tm2Tch}/J (Balb/c^{FoxP3GFP}, H2k^d, Jackson Cat. No. 006769), B6.Cg-Foxp3^{tm2Tch}/J (B6^{FoxP3GFP}, H2k^b, Jackson Cat. No. 006772), and B6.129S6-Il10^{tm1Flv}/J (B6$^{IL-10GFP}$, H2k^b, Jackson Cat. No. 008379) mice were purchased from Jackson Laboratories. Note, Balb/c^{FoxP3GFP}, B6^{FoxP3GFP} and B6$^{IL-10GFP}$ express GFP when FoxP3 and interleukin 10 (IL-10) are expressed, respectively. B6^{FoxP3GFP} and Balb/c^{FoxP3GFP} were used as allogeneic donor and recipient strains in the early-gestation model to facilitate identification of traditional Tregs. B6$^{IL-10GFP}$ mice were also used as allogeneic donors to facilitate identification of regulatory cell populations enriched for Tr1 cells. C57BL/6TgN(act-EGFP)OsbY01 (B6^{GFP}, H2k^b) mice were provided by M. Okabe (Osaka University, 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.

Early-gestation in utero hematopoietic cell transplantation

Whole bone marrow (WBM) was harvested from 6- to 8-week-old donors, as previously described.[26] Briefly, tibias, femurs, and iliac bones were flushed with sterile phosphate-buffered saline (Mediatech, Manassas, VA). The WBM was then filtered and layered over sterile Ficoll-Paque PLUS (Ficoll, GE Healthcare Bio-Sciences, Pittsburgh, PA) to isolate the low-density mononuclear cell layer. Fetuses of time-dated pregnant mice were injected at 14 DPC, as previously described.[18] Briefly, a midline laparotomy was performed under isoflurane anesthesia (3%) to expose the uterine horns. The vitelline vein was injected using a programmable microinjector (IM-300 Microinjector; MicroData Instrument Inc, S. Plainfield, NJ), a 100-μm beveled glass micropipette, and a dissecting microscope. All pups in a litter were injected. To account for an effect on engraftment of maternal antibodies transferred via the breastmilk to newborn pups,[27] all litters transplanted before birth were fostered immediately after birth with naive Balb/c dams. Analysis of regulatory T-cell populations after early-gestation IUHCT is described in supplemental Methods.

Mouse model of late-gestation IUHCT in immunocompetent recipients

Allogeneic murine IUHCT performed at 14 DPC achieves donor-specific tolerance and long-term engraftment.[28,29] The upper limit of the immunologic window for allogeneic IUHCT in mice has not been defined, although the capacity for tolerance induction until at least the immediate postnatal period in mice has been described in other transplant models.[30,31] In humans, immunocompetence with respect to allograft rejection coincides with the emergence of mature fetal T cells in the PB, which begins at approximately 14 weeks' gestation.[12,13,15,16] To determine whether a similar marker exists in the mouse, large litters of Balb/c pups born at 19 DPC were selected, and ~3 pups were euthanized every 12 hours for 48 hours. PB was harvested by decapitation, and red blood cells were lysed using BD Pharm Lyse (BD Biosciences Cat. No. 555899). Cells were stained with CD3-PerCP/Cy5.5 (Biolegend Cat. No. 100218), CD4-PE (BD Biosciences Cat. No. 553653), and CD8-APC (Biolegend Cat. No. 100712) at a dilution of 1:100 at 4°C for 25 minutes. The frequency of CD3$^+$ T cells and the expression of CD4 and CD8 among them were assessed by flow cytometry.

Next, multiple litters of Balb/c pups were injected with 9.7×10^6 B6^{GFP} T cell–depleted (TCD) BM cells via the facial vein at 19 to 21 DPC. We included all litters, not just those born at 19 DPC. As maternal sensitization only occurs with prenatal injection,[27] pups injected postnatally were not fostered. The rate of facial vein injection failure (which led to the exclusion of the animal from the study) was approximately 1%. The only observed complication was a small, self-resolving hematoma at the injection site, occurring in 10% of recipients. No procedural mortality was observed. CD3 depletion was performed by magnetic activated cell sorting, using CD3ε Microbead Kit, mouse (Miltenyi Biotec Cat. No. 130-094-973). CD3$^-$ purity was confirmed to be higher than 99.9%. One to

3 pups were taken from each litter immediately before injection, and PB was assessed for T-cell prevalence and CD4/CD8 expression. The correlation between the frequency of T-cell subtypes in the PB of these pups and the frequency of macroengraftment among the injected littermates was assessed. To determine the kinetics of graft rejection in this model, multiple litters were injected with TCD BM at 20 DPC and bled weekly via the facial vein for 4 weeks.

Isolation of regulatory T cells from tolerant mice

To determine whether regulatory T cells harvested from tolerant mice can prevent allograft rejection, spleens were harvested from 4-week-old male and female B6→Balb/c macroengrafted mice. CD4-positive selection was performed by magnetic activated cell sorting using CD4 (L3T4) Microbeads, mouse (Miltenyi Biotec Cat. No. 130-042-401). Postselection CD4$^+$ purity higher than 90% was confirmed using CD4-APC (eBioscience Ref. No. 17-0042-82) at a dilution of 1:100 for 25 minutes at 4°C. Equal numbers of CD4$^+$ splenocytes isolated from age-matched naive B6 and Balb/c mice served as nontolerant controls. Cells were co-injected with TCD BM cells into newborn pups at 20 DPC at a dose of 5×10^6 CD4$^+$ cells/pup in a total volume of 20 μL. Separation into subpopulations is described in supplemental Methods.

Isolation of naive donor Tregs

To determine whether alloengraftment could be achieved at 20 DPC using a clinically translatable protocol, 9.7×10^6 B6^{GFP} TCD BM cells were co-injected with 0.5×10^6 (5%) CD4$^+$CD25$^+$ cells harvested from the spleens of 6- to 8-week-old B6 mice. This represents a 100-fold enrichment for traditional Tregs, as the normal frequency of CD4$^+$CD25$^+$ cells in B6^{GFP} WBM is 0.05%, or approximately 5000 cells per 10×10^6. Cells were isolated using CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit, mouse (Miltenyi Biotec Cat. No. 130-091-041). CD4$^+$ cells were confirmed to be at least 98% CD25$^+$, and 94% of CD4$^+$CD25$^+$ cells were found to express FoxP3.

Assessment of chimerism and macroengraftment

Mice were bled at 4, 8, 12, and 24 weeks of life by retroorbital venipuncture, using Heparinized Micro-hematocrit Capillary Tubes (Kimble Chase Cat. No. 40C505) under isoflurane anesthesia. Cells were stained with CD45-PE (Biolegend Cat. No. 103106), H2k^b-APC (eBioscience Ref. No. 17-5958-82), and H2k^d-PerCP/Cy5.5 (Biolegend Cat. No. 116618) at 1:100 for 25 minutes at 4°C. Allograft-derived donor cell chimerism was assessed among CD45$^+$ cells and was calculated as (H2k^{b+}GFP$^+$/(H2k^{b+}GFP$^+$ + H2k^{d+}) × 100. Macroengraftment was defined as donor cell chimerism above 1% at 4 weeks of age.

Multilineage analysis

To determine whether engraftment constituted all hematopoietic lineages, PB was obtained at 6 months of age. Cells were stained with CD45.2-PerCP/Cy5.5 (eBioscience Ref. No. 45-0454-82, 1:66), CD3-APC (eBioscience Ref. No. 17-0032-82, 1:50), B220-APC (Biolegend Cat. No. 103212, 1:66), CD11b-PE-eFluor610 (eBioscience Ref. No. 61-0112-82, 1:66), and Ly-6G(Gr1)-PE (eBioscience Ref. No. 12-5931-82, 1:666) for 25 minutes at 4°C. The frequency of lineage-positive cells was determined among donor cells by gating on the CD45$^+$GFP$^+$ population. Age-matched uninjected B6^{GFP} male and female mice served as controls.
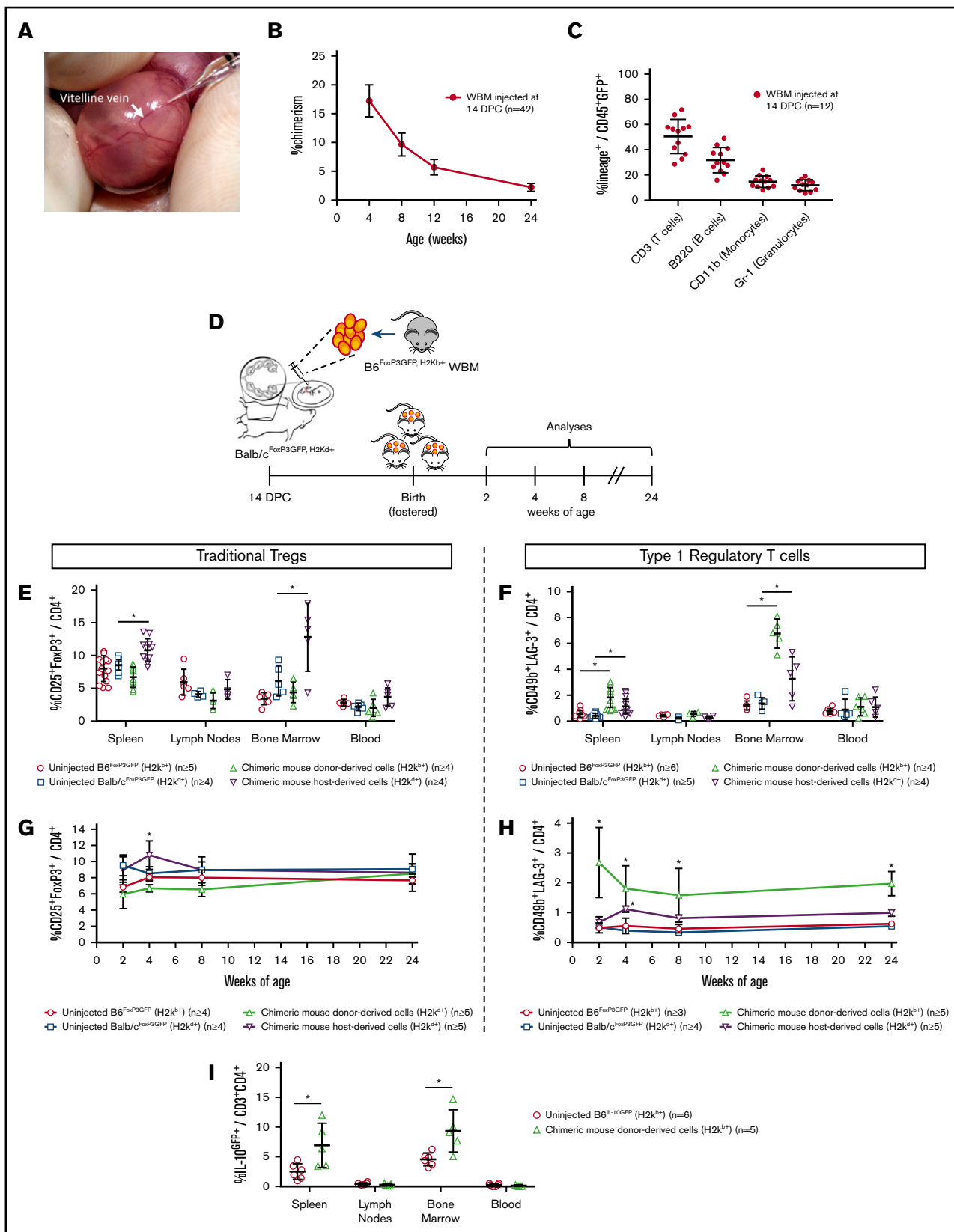
Figure 2. Frequency of regulatory T cells after early-gestation IUHCT. Allogeneic transplantation was performed at 14 DPC by injection of WBM cells into the vitelline vein (A), which results in long-term (B), multilineage (C) donor cell chimerism. All fetuses from a litter were injected. To account for the effect of maternal milk-borne antibodies on engraftment, all litters were fostered immediately after birth with naive Balb/c dams. Of the 69 fetuses injected, 48 (70%) survived to birth and 46 (67%) survived to

In vivo mixed lymphocyte reaction

To assess long-term donor-specific tolerance, an in vivo MLR was performed at 6 months of age. Lymph nodes (LNs) and spleens were harvested together, and low-density mononuclear cells were isolated. Cells were stained with Cell Proliferation Dye eFluor 670 (eBioscience Cat. No. 65-0840-85, fluorescence equivalent to APC) at a dilution of 1:250 in 1 mL cRPMI 1640 medium (Gibco Cat. No. 11875093) for 40 minutes at 37°C. Cells were washed and resuspended in 200 μL phosphate-buffered saline for injection via the tail vein into 8-week-old B6 × Balb/c F1 offspring (H2k^{b}H2k^d). After 72 hours, the LNs and spleens were isolated separately. Low-density mononuclear cells were stained with CD3-PE (BD Biosciences Cat. No. 555275) and H2kb-PECy7 (eBioscience Cat. No. 25-5958-82) at a dilution of 1:100 for 30 minutes at 4°C. The percentage proliferating was determined by flow cytometry for host T cells as the percentage of H2k^{b-} GFP$^-$CD3$^+$ cells with APC fluorescence intensity of 50% or less of undivided cells.

Assessment of graft-versus-host disease

To evaluate for graft-versus-host disease (GVHD), mice were assessed for body weight, physical signs of disease, death, and histologic evidence of organ injury. Assessment was performed at 1, 2, 3, 4, 8, 12, and 24 weeks of age, with a focus on the first 4 weeks after transplantation. A GVHD phenotype score ranging from 0 to 10 was calculated on the basis of presence of hunched posture, ruffled fur, fur loss, desquamation, and diarrhea, as previously described.[32] Histology was performed at 3 weeks of age. Liver, skin, small bowel, and lungs were harvested, fixed in formalin, and stained with hematoxylin and eosin.

Statistics

The frequency of macroengraftment was compared using Fischer's exact test. Continuous, parametric outcomes were compared between 2 groups using Student t test, assuming unequal variance. Comparison of continuous, parametric outcomes among 3 or more groups was performed using analysis of variance (ANOVA) with Bonferroni multiple comparison. Nonparametric continuous and ordinal outcomes were compared among 2 groups using Mann-Whitney U and among 3 groups using Kruskal-Wallis. Statistical analysis and graphing were performed using Stata/1C, version 14.2 (StataCorp, College Station, TX) and Prism, version 7.0 (GraphPad, La Jolla, CA). All statistical tests were 2-sided with an α level set at 0.05 for statistical significance.

Results

Traditional and nontraditional regulatory T cells are elevated after early-gestation IUHCT

IUHCT performed at 14 DPC (before birth; Figure 2A) resulted in long-term, multilineage donor cell chimerism (Figure 2B-C) in agreement with our previous studies.[28,29] Survival to birth and weaning was 70% and 67%, respectively, with successful macroengraftment observed in 91% of survivors. To evaluate a potential role for regulatory T cells after early-gestation IUHCT, we first determined the frequency of traditional CD25$^+$FoxP3$^+$ Tregs and nontraditional CD49b$^+$LAG-3$^+$ Tr1 cells in B6^{FoxP3GFP}→Balb/c^{FoxP3GFP} chimeric mice (Figure 2D). Host-derived traditional Tregs, donor-derived Tr1 cells, and host-derived Tr1 cells were elevated in the spleen and BM at 4 weeks of age (Figure 2E-F). Donor-derived Tr1 cells remained elevated in the spleen, which was enlarged in chimeric mice (supplemental Figure 1) at all times assessed up to 6 months of age (Figure 2G-H). To confirm that donor-derived CD4$^+$CD49b$^+$LAG-3$^+$ cells displayed the characteristic IL-10$^+$ Tr1 cell phenotype,[31] we next performed IUHCT at 14 DPC, using the B6$^{IL-10GFP}$→Balb/c strain combination. Splenic and BM donor-derived CD4$^+$ T cells were significantly enriched for IL-10$^+$ cells (Figure 1I), and the majority of donor-derived CD4$^+$CD49b$^+$LAG-3$^+$ cells in the spleen (53%) and the BM (55%) were confirmed to be IL-10$^+$.

To determine whether donor-derived Tr1 cells arise from CD4$^+$ cells present in the allograft or CD4$^-$ progenitors, we next injected CD4-depleted B6 BM into Balb/c fetuses at 14 DPC. Although the frequency of donor-derived Tr1 cells was lower after injection of CD4-depleted BM, it nevertheless remained elevated above uninjected controls, suggesting that both induction of naive CD4$^+$ T cells present in the allograft and education of progenitor T cells contribute to long-term elevation of donor-derived Tr1 cells (supplemental Figure 2).

The emergence of CD4$^+$CD8$^-$ T cells in the PB correlates with allograft rejection

The enrichment of traditional and nontraditional regulatory T cells after early-gestation IUHCT suggested that they may contribute to allograft tolerance. We next sought to determine whether increasing the regulatory T-cell content of the donor allograft could promote alloengraftment later in development, when the recipient is no longer receptive to allotransplantation. To achieve this, we developed a mouse model of late-gestation IUHCT that reliably rejects the allograft and is defined by developmental T-cell populations. Analysis of the PB after birth at 19 to 21 DPC identified a steady rise in CD3$^+$ T cells predominantly resulting from the emergence of CD4$^+$CD8$^-$ T cells associated with the disappearance of CD4$^-$CD8$^-$ T cells (Figure 3A-B). After postnatal injection via the facial vein (Figure 3C), correlation of macroengraftment with PB CD4$^+$CD8$^-$ T cells and DPC (Figure 3D) demonstrated that a frequency of CD4$^+$CD8$^-$ cells of at least 10% and at least 20 DPC were consistently associated with the absence of macroengraftment after transplantation of allogeneic TCD BM (Figure 3E-F). In contrast, macroengraftment frequency ranged from 17% to 80% in litters in which the frequency of CD4$^+$CD8$^-$ cells was less than 5%, with macroengrafted animals demonstrating

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Figure 2. (continued) weaning at 4 weeks of age. Among these survivors, 42 (91%) macroengrafted. (D) To evaluate the possible contribution of regulatory T cells to tolerance and engraftment, B6^{FoxP3GFP} WBM cells were injected into Balb/c^{FoxP3GFP} fetuses at 14 DPC, and the resulting chimeric mice were analyzed at multiple times after birth. Donor- and host-derived traditional Tregs (E) and type 1 regulatory T cells (Tr1 cells) (F) were first measured as a percentage of CD4$^+$ cells in the spleen, LNs, BM, and PB at 4 weeks of age and compared with uninjected, age-matched B6^{FoxP3GFP} and Balb/c^{FoxP3GFP} controls. The frequency of traditional Tregs (G) and Tr1 cells (H) was then assessed in the spleen at additional times. Early-gestation IUHCT was next performed using the B6$^{IL-10GFP}$→Balb/c strain combination. (I) The percentage of donor-derived CD4$^+$ cells expressing IL-10 was measured in the spleen, BM, LN, and PB at 4 weeks of age. Mean and standard deviation are included in addition to individual data points. Data were analyzed using ANOVA with multiple comparisons with statistically significant differences indicated by * ($P <$ .05).
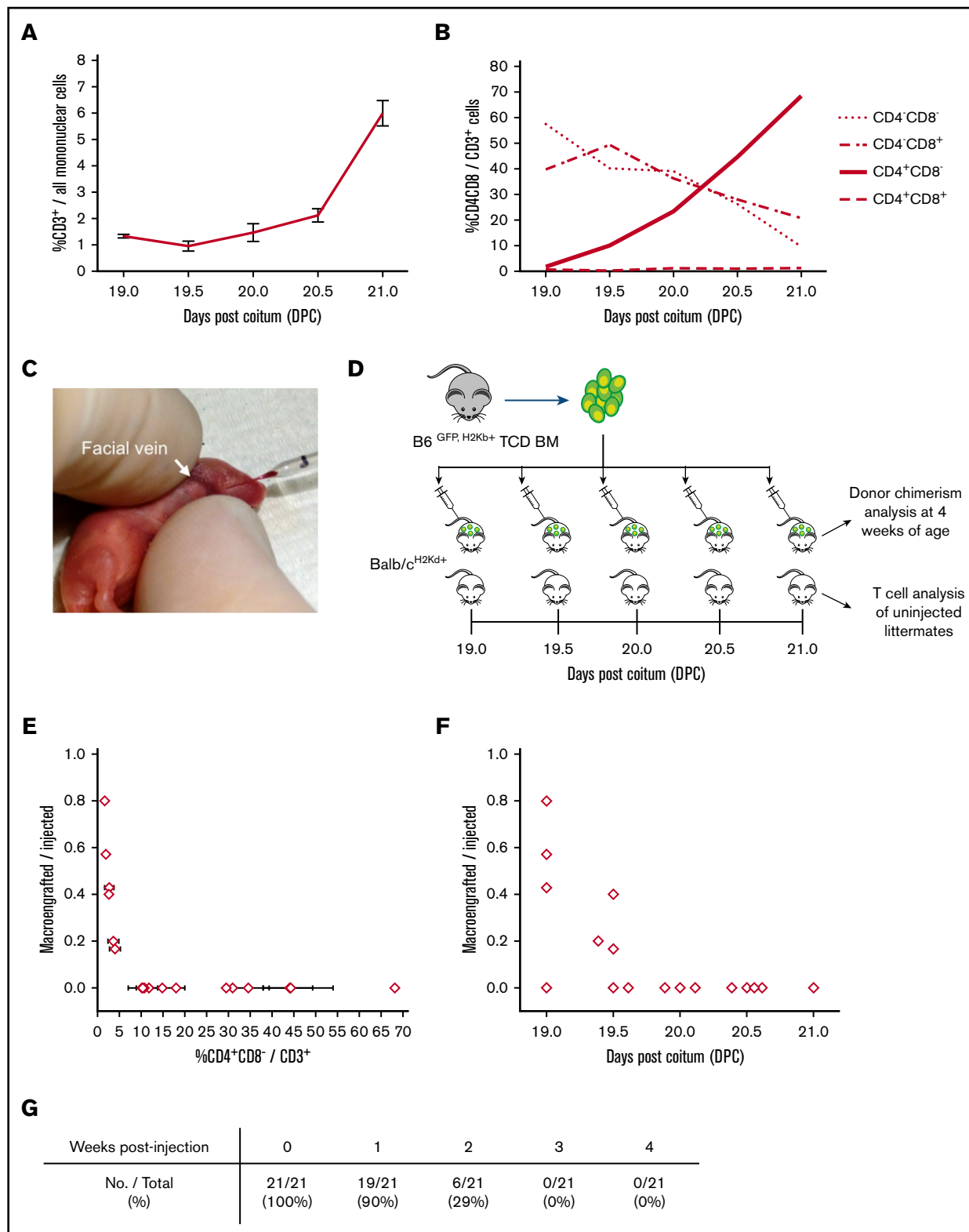
Figure 3. The emergence of CD4$^+$CD8$^-$ T cells in the PB of the developing mouse correlates with allograft rejection. (A) T cells were measured as a percentage of all PB mononuclear cells after birth at 19 to 21 DPC. (B) CD4 and CD8 expression among PB T cells was then measured at 19 to 21 DPC. Allogeneic transplantation was performed by injecting litters of newborn pups via the facial vein (C) with TCD BM cells at 19 to 21 DPC, with 1 to 3 uninjected pups from each litter euthanized for T-cell analysis (D). As maternal sensitization only occurs with prenatal injection, pups injected postnatally were not fostered. The frequency of macroengraftment (PB chimerism > 1% at 4 weeks of age) among injected animals was correlated with the frequency of CD4$^+$CD8$^-$ cells among PB T cells in uninjected littermates (E) and the developmental

significantly lower T-cell proliferation by in vivo MLR compared with their nonengrafted littermates, consistent with the induction of donor-specific tolerance (supplemental Figure 3). In nonengrafted animals injected at 20 DPC (which is after birth), donor cell rejection occurred between 1 and 3 weeks after injection (Figure 3G). These findings suggested that the window of opportunity for allograft tolerance and macroengraftment in mice closes in the perinatal period with the emergence of mature CD4$^+$CD8$^-$ T cells. As such, 20 DPC was chosen for our mouse model of late-gestation IUHCT, and the frequency of CD4$^+$CD8$^-$ PB T cells in uninjected littermates was used as a confirmatory measure of immunocompetence in this model.

Co-injection of CD4$^+$ splenocytes from chimeric mice prevents allograft rejection by immunocompetent recipients

To evaluate the ability of regulatory T cells from IUHCT-tolerant mice to prevent allograft rejection in our model of late-gestation IUHCT, B6^{GFP} TCD BM cells were injected into Balb/c pups at 20 DPC alone or in combination with CD4$^+$ cells harvested from the spleens of 4-week-old B6→Balb/c chimeric mice (Figure 4A). Co-injection of CD4$^+$ chimeric splenocytes significantly improved the frequency of macroengraftment compared with TCD BM alone (100% vs 4%; $P < .0001$). The frequency of macroengraftment and mean chimerism in recipients of TCD BM + CD4$^+$ chimeric splenocytes at 20 DPC were comparable with those observed after injection of 9.7 × 10^6 B6^{GFP} TCD BM at 14 DPC (Figure 4B-C). In both groups, donor chimerism decreased initially with stabilization by 6 months of age (Figure 4C), at which point engraftment was multilineage (Figure 4D) supporting engraftment at the level of the stem or progenitor cell. An in vivo MLR was performed to confirm the presence of donor-specific tolerance. T cells harvested from mice injected at 20 DPC with TCD BM + CD4$^+$ chimeric splenocytes demonstrated significantly less proliferation than those from mice injected at 20 DPC with TCD BM only (Figure 4E). Notably, there was no significant difference in T-cell proliferation between mice injected at 20 DPC with TCD BM + CD4$^+$ chimeric splenocytes and mice injected with TCD BM at 14 DPC (LNs: 2.7% vs 3.2% [$P = 1.0$]; spleen: 9.9% vs 6.0% [$P = .3$]), confirming that long-term, donor-specific tolerance was induced despite injection later in development.

Host-derived traditional Tregs are sufficient to promote alloengraftment

To determine which cell subpopulation or subpopulations was crucial for preventing allograft rejection in our model, donor allografts were enriched with subpopulations of CD4$^+$ splenocytes from 4-week-old chimeric mice. Host-derived CD4$^+$ cells were most effective in preventing graft rejection, preserving engraftment in all recipients (Table 1). Removal of traditional Tregs by CD25 depletion significantly impaired the efficacy of host-derived CD4$^+$ cells to prevent graft rejection, reducing the incidence of macroengraftment from 100% to 40% ($P < .0005$). Injection of purified host-derived traditional Tregs mice restored engraftment to 86%

(vs 100% in mice injected with all host-derived CD4$^+$ cells; $P = .26$), demonstrating this subpopulation to be sufficient to prevent allograft rejection in our model.

Allograft enrichment with naive donor traditional Tregs promotes engraftment

These studies suggest that Tregs may play an important role in maintaining tolerance/engraftment after early-gestation IUHCT; that this property can be transferred to a model of late-gestation, nontolerant IUHCT; and that host-derived Tregs have the most robust effect. Given the inability to use host-derived, IUHCT-tolerant cells for clinical IUHCT, we next tested whether naive donor-derived Tregs can similarly promote engraftment in the model of late-gestation IUHCT (Figure 4A). Co-injection of B6^{GFP} TCD BM cells with naive B6 CD4$^+$CD25$^+$ Tregs at 20 DPC allowed for macroengraftment in 21 (84%) of 25 recipients with a mean chimerism at 4 weeks of age of 7.1%, statistically equivalent to that achieved by injecting TCD BM at 14 DPC and TCD BM + CD4$^+$ splenocytes from chimeric mice at 20 DPC (Figure 4B-C). Long-term, multilineage engraftment was achieved with a frequency of lineages equivalent to that of age-matched, uninjected B6^{GFP} controls (Figure 4D). Engrafted mice also demonstrated decreased proliferation on in vivo MLR, consistent with long-term donor-specific tolerance (Figure 4E). Notably, robust engraftment was achieved in litters in which the frequency of CD4$^+$CD8$^-$ cells among PB T cells far exceeded the predetermined threshold of immunocompetence ($\geq$10%), as high as 47% (Figure 5).

Allograft enrichment with regulatory T cells achieves macroengraftment in the absence of GVHD

GVHD is a concern with allogeneic BM transplants, especially when the allograft is enriched for T-cell populations. IUHCT recipients were serially weighed and monitored for signs of GVHD after birth. Recipients of TCD BM as well as those in which the allograft was enriched with either CD4$^+$ splenocytes from tolerant mice or Tregs from naive B6 mice were healthy, demonstrating normal weight gain, no clinical signs or histologic evidence of GVHD, and 100% survival (Figure 6).

Discussion

IUHCT is a potential treatment of congenital hemoglobinopathies with established efficacy in several animal models but with heretofore disappointing clinical results. The early acquisition of T-cell immunity in the human fetus (supplemental Figure 4) and the practical challenges of performing IUHCT before that time contribute to the lack of clinical success. It is therefore necessary to develop strategies to extend the window of opportunity for IUHCT to later in gestation. In this study, we demonstrated that Tregs harvested from either IUHCT-tolerant or naive mice promote multilineage alloengraftment associated with donor-specific tolerance in a mouse model of immunocompetent late-gestation IUHCT. These findings suggest that Tregs could be used clinically to perform IUHCT later in gestation.

Figure 3. (continued) age of the litter (F). (G) Finally, to determine the kinetics of graft rejection in this model, several litters were injected at 20 DPC, and the frequency of mice with chimerism higher than 1% was measured weekly for 4 weeks, demonstrating that the majority of animals reject the allograft between 1 and 3 weeks posttransplant consistent with an adaptive immune response.
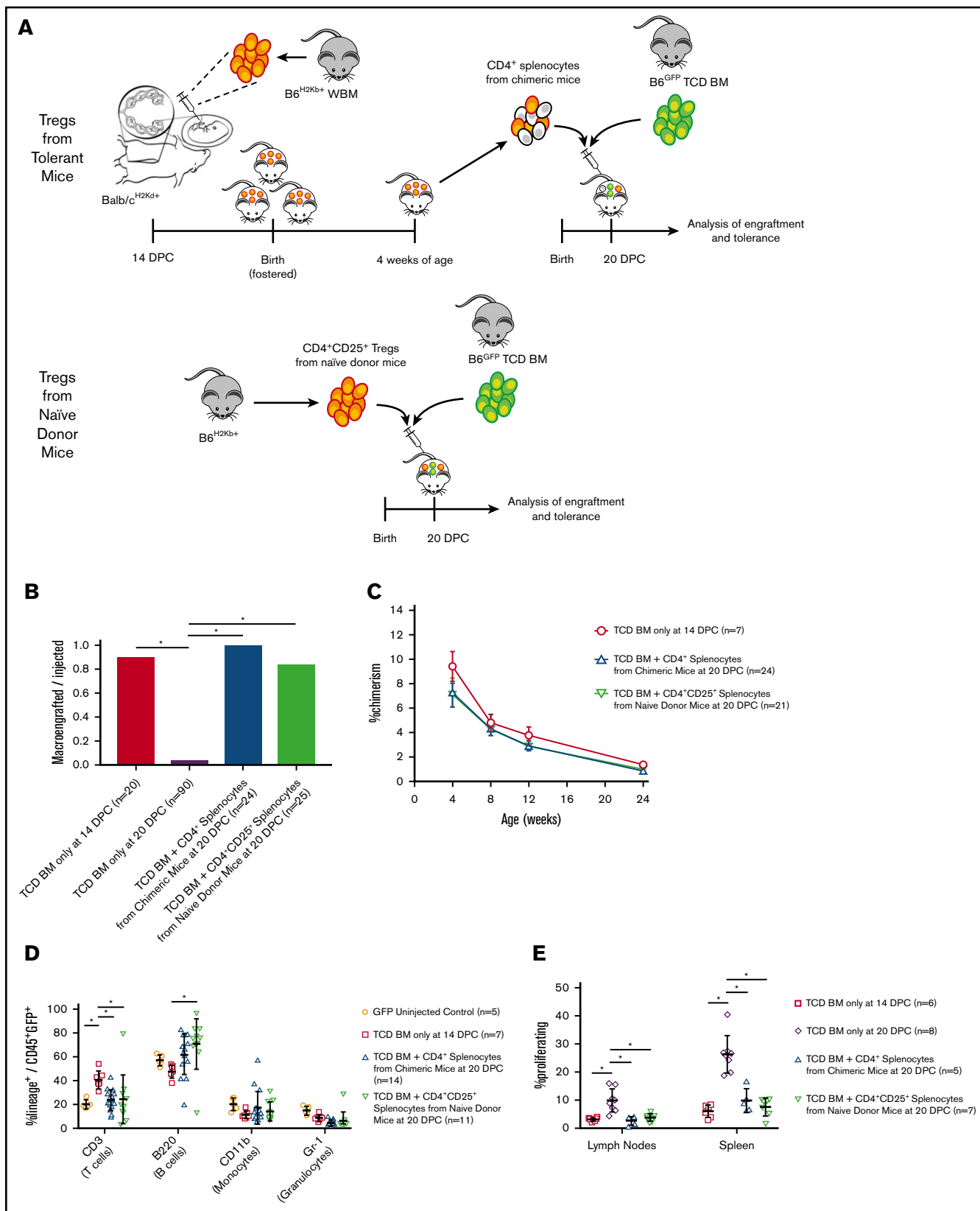
Figure 4. Regulatory T cells preserve alloengraftment in model of late-gestation IUHCT in immunocompetent recipients. (A) Newborn Balb/c pups were injected at 20 DPC with TCD BM harvested from B6GFP mice in combination with regulatory T cells harvested from either chimeric or naive donor mice, as displayed. As maternal sensitization only occurs with prenatal injection, pups injected postnatally were not fostered. (B) Frequency of macroengraftment defined as the number of pups with PB

Table 1. GVHD-free survival, frequency of macroengraftment, and PB chimerism after co-injection of various subpopulations with TCD BM into newborn pups at 20 DPC

Cells co-injected with TCD BM	Number of cells injected (millions)	Number of mice injected	GVHD-free survival, n (%)	Frequency of macroengraftment among survivors, n (%)	Mean chimerism at 4 weeks among survivors, %
None	0	90	90 (100)	4 (4)	0.2
Naive mice					
B6 (H2k^b) CD4$^+$	5.0	11	4 (36)	0 (0)	0
Balb/c (H2k^d) CD4$^+$	5.0	8	8 (100)	0 (0)	0
Chimeric mice					
All (H2k^{d+} and H2k^{b+}) CD4$^+$	5.0	24	24 (100)	24 (100)	7.3
Host-derived (H2k^{d+})	2.5	10	10 (100)	10 (100)	5.6
CD25$^-$	2.25	15	15 (100)	6 (40)	2.0
CD25$^+$	0.25	7	7 (100)	6 (86)	5.5
Donor-derived (H2k^{b+})	1.0	9	9 (100)	6 (67)	5.4
CD25$^-$	0.95	8	8 (100)	6 (75)	2.6
IL-10$^+$	0.04	7	7 (100)	1 (14)	0.3

The importance of regulatory T cells to donor-specific tolerance after IUHCT is controversial. Nijagal et al elegantly showed that the increase in peripheral Treg prevalence after IUHCT is not absolute but, rather, a result of disproportionate deletion of donor-specific effector T cells. Furthermore, given no observed difference in Treg proliferation between chimeric and nonchimeric mice after in vivo MLR experiments, the authors concluded that Tregs have a minimal role, if any, in maintaining tolerance after IUHCT.[21] Although the increases in Treg frequency among CD4$^+$ T cells are relative, our results challenge the assertion that these Treg populations are functionally insignificant. We demonstrated that the adoptive transfer of host-derived CD4$^+$ T cells from IUHCT-tolerant mice restores engraftment at 20 DPC, a point in development at which the recipient consistently rejects allografts. This observation cannot be explained by the deletion of donor-specific effector T cells alone, suggesting that the changes in the host regulatory T-cell milieu induced by IUHCT contribute to the donor-cell tolerogenic state.
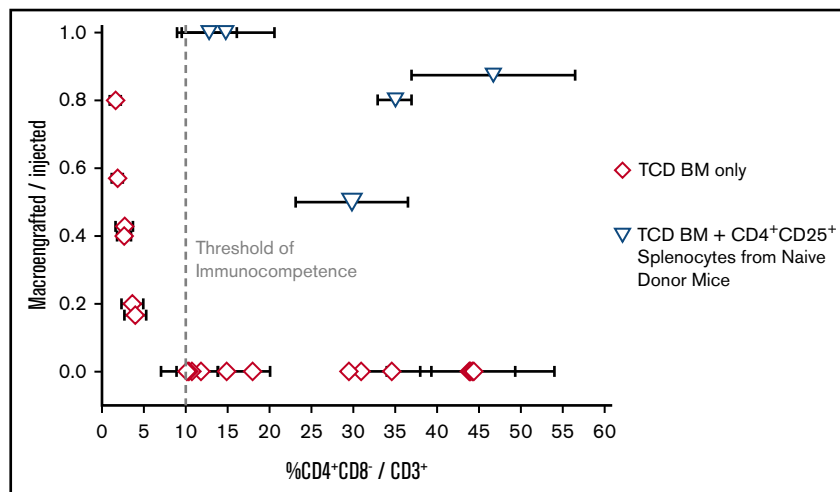
In addition to traditional Tregs, we observed increased Tr1 cell prevalence in the spleen and BM after early-gestation IUHCT at 14 DPC. Tr1 cells have been shown to contribute to immune homeostasis in patients with persistent mixed chimerism after postnatal hematopoietic cell transplantation[33,34] as well as reciprocal tolerance at the maternal-fetal interface.[35] Tr1 cells can be induced in the periphery when naive CD4$^+$ T cells encounter antigen in the presence of IL-10.[36,37] Given that IL-10 is elevated during pregnancy in humans and mice,[38] Tr1 cells may be induced after IUHCT when naive donor CD4$^+$ T cells are exposed to host antigen and vice versa. The elevation of Tr1 cells in the BM and spleen is not surprising given the role of these organs as hematopoietic stem cell reservoirs,[39] the tendency for murine Tr1 cells to reside in the spleen,[40,41] and the role of the spleen in the induction of peripheral tolerance.[42,43] Although early-gestation IUHCT-induced H2k^{b+}CD4$^+$IL-10$^+$ cells, which are enriched for Tr1 cells, did not reliably prevent allograft rejection when co-injected in our late-gestation IUHCT model (and therefore naive donor CD4$^+$CD49b$^+$LAG-3$^+$ Tr1 cells, which are difficult to purify in adequate quantities for in vivo studies, were not subsequently tested), Tr1 cells may contribute to reciprocal tolerance after IUHCT in other ways beyond the scope of this study, including the prevention of GVHD and/or fetal demise.

The ability to preserve alloengraftment later in development at 20 DPC using Tregs not only from IUHCT-tolerant mice but also from naive donors supports the potential feasibility of this approach for clinical translation. The ability of donor-strain Tregs to suppress the antidonor host T-cell response is consistent with studies supporting the ability of Tregs to suppress effector T cells expressing unmatched major histocompatibility complex class I.[44] Our naive donor Tregs were polyclonal, not enriched for T-cell receptor clones specific for self-antigen beyond that which occurs during natural Treg induction.[45-47] However, Tregs have several contact-independent mechanisms, including inhibitory cytokines, cytolysis, and metabolic disruption of effector T cells, that contribute to the suppressive effects of polyclonal Treg populations.[48,49] As such, antigen-specific Tregs have been shown to be only marginally more effective than polyclonal Tregs for adoptive immunotherapy in postnatal HSCT.[50-52] Similarly, Graca et al demonstrated that CD4$^+$CD25$^+$ T cells from naive mice suppress mismatched skin graft rejection equally as well as those from tolerant mice.[53] Thus, we propose that the high dose of naive polyclonal donor Tregs used in our study transiently suppressed the developing recipient immune response adequately to allow central tolerance induction to occur. This is supported by the persistently depressed alloreactivity of

Figure 4. (continued) chimerism higher than 1% at 4 weeks of age divided by the total number of injected pups in that group. Data were analyzed using Fischer's exact test. (C) Mean PB chimerism among macroengrafters over time. Shown are the mean ± standard error of the mean. Data were analyzed using ANOVA. (D) Lineage frequency among donor-derived (GFP$^+$) cells in the PB at 6 months of age. Mean and standard deviation are included in addition to individual data points. Data were analyzed using ANOVA. (E) In vivo mixed lymphocyte reaction performed at 6 months of age. Percentage proliferating was defined as the percentage of H2k^{b-}GFP$^-$CD3$^+$ cells with APC fluorescence intensity of 50% or less of undivided cells. Mean and standard deviation are included in addition to individual data points. Data were analyzed using ANOVA. In all panels, statistically significant differences between groups are indicated by * ($P < .05$).

Figure 5. Allograft enrichment with naive donor Tregs promotes macroengraftment beyond the threshold of immunocompetence. The frequency of macroengraftment is plotted against the frequency of CD4$^+$CD8$^-$ cells among CD3$^+$ T cells in the PB of uninjected littermates with the previously determined threshold of immunocompetence ($\geq$10%) delineated by the dashed line.

host-derived T cells we observed at 6 months of age by MLR as well as by studies showing that thymic deletion is an ongoing process continuing into the neonatal period.[54-56]

We used the co-expression of CD4 and CD25 to purify traditional Tregs. Although these markers have high fidelity for FoxP3 expression in mice, confirmed to be 94% in our study, these specific markers have less fidelity for Tregs in humans.[49,57] Nonetheless, clinical

studies with CD4$^+$CD25$^+$ Tregs have been performed and demonstrate that imperfect purity does not preclude either safety or efficacy.[50] In addition, the use of CD4$^+$CD25$^+$ to purify donor Tregs carries the possibility that we are injecting not only regulatory T cells but also some effector T cells, as CD25 can also be expressed in this population after activation.[58] This raises the possibility that a graft-versus-hematopoietic effect, in addition to a suppressive effect, is contributing to the macroengraftment we observed in the
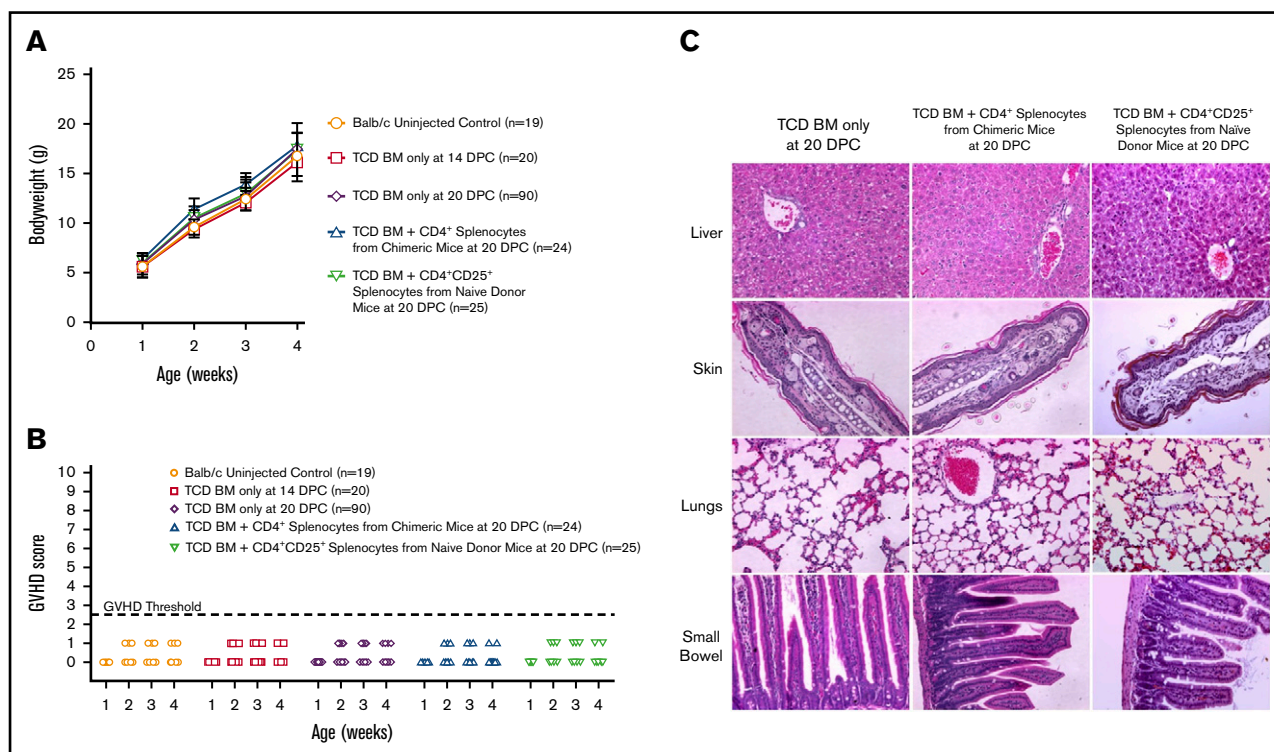

Figure 6. Assessment of GVHD. (A) Mean bodyweight in the first 4 weeks of life. Shown is the mean ± standard deviation over time. No experimental group was found by ANOVA to have a lower mean bodyweight than uninjected Balb/c controls at any point. (B) A GVHD phenotype score ranging from 0 to 10 was calculated on the basis of the presence of hunched posture, ruffled fur, fur loss, desquamation, and diarrhea with scores lower than 3 consistent with no GVHD. No animals developed GVHD. (C) Histology of the liver, skin, lungs, and small bowel at 3 weeks of age. Slides were stained with hematoxylin and eosin. Images were collected at 20× magnification. No features of GVHD were detected on histology.

late-gestation model. Even if this were the case, however, the normal survival, weight gain, phenotype, and histology of these animals suggests that any graft-versus-hematopoietic effect was not harmful.

The model of late-gestation IUHCT used in our study has limitations. As we and others have demonstrated, the maternal and fetal immune systems interact in important ways during normal pregnancy and after fetal transplantation, some conducive to tolerance[38,59] and some detrimental.[27,60-62] Mouse studies have demonstrated the elicitation of donor-specific maternal antibodies after allogeneic IUHCT secondary to spillage of donor cells in the maternal abdomen and resorption of injected, aborted fetuses, and these antibodies can be transferred postnatally in the breast milk leading to postnatal allograft rejection.[27] Subsequent mouse studies have demonstrated that preexisting donor-specific maternal antibodies can cause prenatal allograft rejection, thus making the mother the preferred donor for clinical IUHCT.[62] As a result of differences between humans and mice in the timing of immunologic maturation relative to birth, our mouse model of late-gestation IUHCT required postnatal injection and, as a result, did not capture these interactions. It is therefore important to confirm our results in an animal model in which the fetus becomes immunocompetent while still in utero.[2,63,64] In addition, although this study shows an associated between the emergence of $CD4^+CD8^-$ in the PB and the capacity for allograft rejection, we do not show direct causation by reversing the effect with either transgenic mice or the use of antibodies. This represents not only an opportunity to more firmly demonstrate mechanism but also an alternative strategy for transient, targeted immunosuppression worth testing in future studies.

In summary, IUHCT is a promising experimental treatment of congenital hematologic disorders including α- and β-hemoglobinopathies, but the need to perform IUHCT very early in gestation remains a significant barrier to its successful clinical translation. Our study demonstrates that regulatory T cells can be used to achieve tolerance and engraftment beyond the acquisition of T-cell immunity in mice, extending the window of opportunity for IUHCT to a later point in development. This work will serve as a useful foundation for subsequent preclinical and clinical studies, helping to fulfill the promise of IUHCT as a treatment of inherited blood disorders.

Authorship

Contribution: J.S.R. designed the research study, conducted the experiments, acquired the data, analyzed the data, and wrote the manuscript; L.E.M. designed the research study, conducted the experiments, and acquired the data; J.D.S., B.E.C., and N.J.A conducted the experiments; H.L. acquired the data and analyzed the data; S.P.L, C.G.F., and A.I.B.S.D. designed the research study and conducted the experiments; A.W.F. analyzed the data; and W.H.P. designed the research study, analyzed the data, and wrote the manuscript.

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