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Regulatory T Cell Amelioration of Graft-versus-Host Disease following Allogeneic/Xenogeneic Hematopoietic Stem Cell Transplantation Using Mobilized Mouse and Human Peripheral Blood Donors.

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Journal

Biology of Blood and Marrow Transplantation, 29(5)

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Publication Date

2023-05-01

DOI

10.1016/j.jtct.2023.02.015

Peer reviewed

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Author manuscript

Transplant Cell Ther. Author manuscript; available in PMC 2024 May 01.

Published in final edited form as:

Transplant Cell Ther. 2023 May ; 29(5): 341.e1–341.e9. doi:10.1016/j.jtct.2023.02.015.

Treg amelioration of GVHD following allo/xeno-HSCT using mobilized mouse and human peripheral blood donors

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INTRODUCTION

The use of $CD4^+FoxP3^+$ regulatory T cells (Tregs) to inhibit graft vs host disease (GVHD) following allogeneic hematopoietic stem cell transplantation (aHSCT) has been explored for more than a decade [1–10]. Both donor and host Tregs have been demonstrated to possess regulatory potential to ameliorate experimental GVHD [11–12]. Clinical trials infusing donor and cord blood derived Tregs in aHSCT patients have reported

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Conflict of interest statement: RBL is a compensated consultant/advisory board member for and equity holder in NightHawk Biosciences, Inc. (formally Heat Biologics, Inc.). KVK is an ad hoc consultant for Janssen, Novartis, Genentech/Roche, Kite, Takeda, Iovance, United Healthcare, Avacta Therapeutics and Kiadis. None are directly relevant to the efforts of this study. MMS, RRJ and JW are, or were former, employees of NightHawk Biosciences, Heat Biologics and/or Pelican Therapeutics during the collection of such data that constituted this research report. GRH has consulted for Generon Corporation, NapaJen Pharma, iTeos Therapeutics, Neoleukin Therapeutics, Commonwealth Serum Laboratories, Cynata Therapeutics and has received research funding from Compass Therapeutics, Syndax Pharmaceuticals, Applied Molecular Transport, Serplus Technology, NightHawk Biosciences, Inc. (formally Heat Biologics, Inc.), Laevoroc Oncology and iTeos Therapeutics. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. TRM no conflict. RJ no conflict.

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their safe application and results from prior and ongoing studies have shown promise [13–15, NCT00529035/NCT01937468/NCT03912064/NCT02991898/NCT01660607 and NCT04013685). Challenges nonetheless remain for their widespread usage as an effective strategy for regulating clinical HSCT [16]. Patient applications necessitate that sufficient Treg numbers (donor or host) will be available at the time of an HSCT. Historically, a similar requirement involving availability and access to sufficient numbers of donor stem / progenitor cells was resolved through development of mobilization procedures in the donor prior to transplant [17–21]. Peripheral blood (PB) from donors for allogeneic as well as autologous HSCT could be obtained following stem / progenitor cell mobilization regimens and successfully transplanted resulting in hematopoietic engraftment and graft versus leukemia (GVL) activity [21,22]. These regimens typically consist of the infusion of filgrastim (G-CSF) and plerixafor, as some individuals respond inadequately to filgrastim failing to mobilize sufficient $CD34^+$ HSC into the peripheral blood to enable harvest of sufficient numbers (~5×10^6 HSC / kg) for transplant [19, 21, 23].

Notably, with regard to the present studies, G-CSF and plerixafor can also effectively mobilize hematopoietic progenitors in mice [19,24]. Here, we report an experimental aHSCT protocol in which donor mice were prepared using G-CSF alone or with plerixafor regimens to mobilize stem/progenitor cells while enabling concomitant expansion of their peripheral $CD4^+FoxP3^+$ Treg compartment. Mobilized animals were administered TNFRSF25 agonists together with low dose IL-2 (rhIL-2_{LD}) to rapidly and markedly expand the $CD4^+FoxP3^+$ Treg compartment [5,6,25]. Utilizing multiple donor / recipient strain combinations involving MHC-matched and MHC-mismatched genetic disparities, the findings demonstrated efficient and simultaneous donor HSC / progenitor cell mobilization and Treg expansion. Transplant of PB from such donors resulted in diminished GVHD as assessed by clinical score, histopathology and immune parameters. We conclude HSC/PC mobilization was effective in the presence of Treg expansion and the donor HSC/PC populations exposed to TNFRSF25 agonists and rhIL-2_{LD} were functional post-transplant in recipients. Additionally, donor Tregs could be efficiently expanded in the presence of G-CSF + plerixafor and subsequently were functionally suppressive in recipients. Lastly, using MLL-AF9 leukemia cells, graft vs. leukemia responses (GVL) remained intact in animals transplanted using this “dual” donor stem/progenitor cell and Treg expansion protocol, consistent with prior observations that Treg amelioration of GVHD does not abolish anti-tumor activity [5,9]. In total, these findings support the notion that during the donor stem / progenitor cell mobilization process, targeting and activating Tregs using a two receptor, i.e. TNFRSF25 + CD25 pathway strategy can result in a potential translational approach using PB for allo-HSCT with reduced GVHD severity while maintaining GVL.

MATERIALS AND METHODS

Animals:

C57BL/6J (B6; stock: 000664), B6-CD45.1 breeder (stock: 002014) ($H2^b$), LP/J ($H2^b$), B10.D2 ($H2^d$) and C3H.SW ($H2^b$) mice were purchased from The Jackson laboratory and maintained in University of Miami animal facilities. The FoxP3 reporter mice on a C57BL/6 background (B6-$FoxP3^{RFP}$= B6-Fir) were originally provided by R. Flavell (Yale

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University, New Haven, CT). Wild-type BALB/c ($H2^d$) mice were purchased from Taconic Biosciences or The Jackson Laboratory. BALB/c-FoxP3.DTR were obtained from the Fred Hutchinson Cancer Center. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were obtained from the University of Miami animal core. All mice were used at 6–12 weeks of age and were maintained in pathogen-free conditions at the University of Miami (UM) animal facilities. All animal procedures used were performed under protocols approved by the UM IACUC.

Flow cytometry:

Commercial antibodies for use in flow cytometry were purchased from BD Biosciences (San Jose, CA), Biolegend (San Jose, CA), or eBioscience / ThermoFisher (Waltham, MA). All antibodies used in this study are included in Supplemental Table I. Single-cell suspensions were prepared from different organs (spleen, peripheral lymph nodes [pLN]), bone marrow (BM). Peripheral blood (PB) was collected in heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll density gradient centrifugation. Next, 10^6 cells were pre-blocked with anti-mouse CD16/CD32 and stained with different antibody combinations. Intracellular staining was performed according to standard procedures. The following mAbs to the indicated molecules and their fluorescent labels were used in this study: CD4, CD8, CD19, CD25, CD44, CD62L, KLRG1, CD39, CD73, I-COS, Nrp-1, PD-1, CTLA-4, Ly-6C, Ki-67, Annexin V, H2Kb, H2Kd, CD45.1 and CD45.2.

Stem/Progenitor cell mobilization:

Donor mice were treated with recombinant murine G-CSF (Biolegend, San Jose, CA) or Neupogen (Filgrastim, AMGEN, Thousand Oaks, CA) for 4 days (2.5 ug/mouse sc) sometimes followed by Plerixafor (AMD3100, (Sigma-Aldrich, St. Louis, MO) (5mg/kg sc) on Days −1 and Day 0. PB was collected and PBMC obtained by density centrifugation (see below) followed by staining with monoclonal antibodies to c-kit (CD117), Ly6A/E (Sca-1), CD11b, Ly6G, CD4, CD8, CD19 and FoxP3 (see Suppl Table I).

Staining was assessed via flow cytometry (see above) and phenotypic analysis to identify populations in the peripheral blood of un-mobilized and mobilized mice for Hematopoietic Stem and Progenitor Cells (HSPCs) including: HSCs (Hematopoietic Stem Cells $Lin^-Sca\text{-}1^+c\text{-}kit^+$ = (LSK which contain MPP: multipotent progenitors) $CD150^+$ $CD48^-$ cells), CLPs (Common Lymphoid Progenitors Lin^- IL-$7R^+$ Sca-$1l^{low}$ c-kit^{low}), GMPs (Granulocyte – Macrophage Progenitors Lin^- Sca-1^- c-kit^+ $CD16^+CD32^+$ $CD34^+$), MEPs (Megakaryocyte – Erythroid Progenitors Lin^- Sca-1^- c-kit^+ $CD16^-CD32^-CD34^-$) and CMPs (Common Myeloid Progenitors Lin^- Sca-1^- c-kit^+ $CD16^-CD32^-$ $CD34^+$) that reside within Lin-Sca-1^-c-Kit^+ (LK) population.

Treg cell expansion:

TNFRSF25 agonists - (fusion protein: TL1A-Ig, mAbs: 4C12, mPTX-35 (Heat Biologics / Pelican Therapeutics) were administered intraperitoneally. TL1A-Ig+rIL-2 (recombinant IL-2): *in vivo* treatment with TL1A-Ig (on days 1 to 4) and human rIL-2 (10,000 units / injection) on Days 4,5 and 6. Recombinant mouse IL-2 and α-IL-2 monoclonal antibody,

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clone JES6–5H4, were purchased from ThermoFisher Scientific, (Waltham, MA.). TL1A-Ig was generated in our laboratory as described previously[14].

Xenogeneic human to mouse transplantation with ex-vivo expanded human Tregs

PBMC were isolated from human mobilized (Filgrastim) peripheral blood by ficoll separation and viable T cells counted (all human cells were obtained from consented donors according to IRB approved (20160363). NSG mice were irradiated (2 Gy, total body irradiation) and transplanted the following day with 6×10^6 PBMC which included 3.6×10^6 T cells and ~2×10^4 $CD34^+$ cells from the same PBMC donor.

Cryopreserved PBMC from healthy donors mobilized with Neupogen were thawed and phenotyped and Tregs (defined as $CD4^+CD25^{hi}CD127^{lo}$) were obtained by cell sorting (>98.0% $Foxp3^+$). Sorted Tregs (5×10^5/well/ml) were cultured in 24-well plates with anti-CD3/CD28 Dynabeads (4:1 ratio of Dynabeads to cells, ThermoFisher Scientific) and human IL2 (500 unit/mL, Novartis) in OpTmizer CTS™ T-cell expansion medium (designated as SFM) (Life Technologies) on day 0, and then subcultured in SFM with human IL2 (500 unit/mL) for 7–8 days. Post-culture analysis assessed by $CD4^+FoxP3^+CD127^{lo}$ expression indicated significant Treg expansion with maintenance of high FoxP3 expression (>98%). Tregs were counted and added to the PBMC at a 1:1 ratio. Mice were monitored 3x per week for GVHD clinical score (as above), weight loss, and survival until 6 weeks post-transplantation.

Hematopoietic stem cell transplantation: Models for HSCTs used were: 1) a major MHC-mismatched model (B6→BALB/c). Female BALB/c mice were conditioned with 7.5–8.0 Gy total body irradiation 1 day prior to transplantation, and 2) an MHC matched minor antigen mismatched model (LP/J or C3H.SW→B6). B6 female mice were conditioned with 10.0 Gy TBI on the day of transplantation. Peripheral blood (see above) cells were obtained from the appropriate donor animals for each experiment. Donor cells were stained for T cells (anti-CD4, clone RM4–5; anti CD8, clone 53-6-7) and adjusted to 0.5×10^6 to 1.0×10^6 T cells per mouse. In some experiments, tumor cells (B6-MLL-$AF9^{GFP}$) previously generated by our laboratory were employed [26]. B6 H2b tumor cells (6.0×10^3) were added to the PB population prior to infusion into recipients. Recipient mice underwent transplantation (day 0) via i.v. infusion using a 0.2 mL volume via tail vein injection. GVHD was assessed by monitoring recipients for changes in total body weight, clinical signs, and overall survival. Clinical scores for GVHD were recorded for individual mice. Recipients were scored on a scale from 0 to 2 for 5 clinical parameters: weight loss, diarrhea, fur texture, posture, and alopecia according to our previous published studies using a modification of the scoring system previously reported [5, 6,27].

DT depletion of Tregs in vitro.

BALB/c-FoxP3-diptheria toxin knock-in mice were either mobilized only, mobilized + Treg expanded and mobilized + expanded then given (1μg) diphtheria toxin. Cells from PB were then obtained at 24 hrs. after DT was given and plated in round bottom 96 well plate (100,000 cells/well). Anti-CD3 (2C11) hybridoma protein G 1μg/ml was added to the wells. Three and four days later wells were manually counted.

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Histologic Analysis

Briefly, GI tissues from animals 5–7 weeks after aHSCT were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) and images were acquired using the Keyence BZ-X700 microscope. Tissue samples were scored following a modified system described by Kaplan D, *et al* using the multiple parameters hyperplasia, inflammation, submucosal edema and necrosis [28].

Statistical analyses: Numbers of animals per group are described in the figure legends. All Figure panels include data sets obtained from individual animals. All graphing and statistical analysis were performed using GraphPad Prism 9 (La Jolla, CA). Significance of differences between two experimental groups were determined using two-tailed unpaired *t* test. For experiments comparing more than two groups, data was analyzed using a one-way ANOVA with a post-hoc Tukey's multiple comparisons test. For survival analyses, a Kaplan Meyer (Wilcoxin) test was performed. Statistical tests performed are indicated in the figure legends. Significance indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns=non-significant. Data shown are means ± SEM.

RESULTS and DISCUSSION:

Development of a regimen to induce concomitant stem / progenitor cell mobilization and highly elevated Treg levels in mouse peripheral blood.

To test a potential strategy whereby Tregs present in transplanted donor blood could be assessed for their ability to ameliorate GVHD, a protocol to induce mobilization of stem / progenitor cells (HSC/PC) together with elevated levels of circulating Tregs was developed. Mice were initially examined for mobilization following the administration of G-CSF alone or together with plerixafor (Fig. S1A).

Neupogen (Filgrastim: rG-CSF) was administered to B6 mice ($H2^b$) over 4 consecutive days and PB analyzed for c-kit expression and several hematopoietic cell markers including CD11b and Ly6G. Significant increases in the overall levels of c-kit^+ cells as well as $CD11b^+$ and $Ly6G^+$ populations were noted compared to untreated peripheral blood (Fig. S1B).

In vivo mobilization with G-CSF together with plerixafor concomitantly (simultaneously) resulted in multifold increase of HSCs and MEP, CLP and GMP lineage committed progenitors numbers in the PB of mobilized B6 mice (Fig. 1A–E). Frequencies of HSC and lineage committed progenitors including MEPS, CLPs, GMPs and LSKs were elevated in these mobilized B6 mice (Fig. 1A–E). Additionally, the numbers of progenitor cell populations were increased in peripheral blood from mobilized B6 animals (Supplemental Table 2). Mobilization was confirmed in an independent mouse (LP/J) strain following administration of Filgrastim + plerixafor where elevated levels of c-kit^+ populations as well as granulocytes and monocyte cells were readily apparent in mobilized vs untreated mice (Fig. 1F–H). To manipulate the Treg compartment concomitantly with mobilization, some animals also received a fusion protein (TL1A-Ig FP) specific for TNFRSF25 and $rhIL\text{-}2_{LD}$ which induces proliferation of $CD4^+FoxP3^+$ Tregs [5,29]. Treated animals demonstrating

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mobilization also exhibited highly elevated Treg (>30% $CD4^+FoxP3^+$ / $CD4^+$) levels as assessed by frequency and numbers (Fig. 1I **legend**).

To verify the ability to target Tregs via TNFRSF25 in mice undergoing HSC/PC mobilization, a third strain (B10.D2, $H2^d$) was administered Filgrastim + plerixafor and either TL1A-Ig + $IL\text{-}2_{LD}$ or an anti-TNFRSF25 specific agonistic mAb (4C12) plus $rhIL\text{-}2_{LD}$ (Fig. 1J–M) [5]. Heightened levels of c-Kit^+, myeloid cells and monocytes were detected in mobilized animals receiving either TNFRSF25 agonistic reagent (TL1A-Ig FP or 4C12mAb) (Fig. 1J,K,L). Levels of Tregs were markedly increased in all mobilized animals who also were treated with anti-TNFRSF25 agonists as evidenced by frequency and numbers (Fig. 1M **legend**).

Phenotypic and functional assessment of Tregs in blood following mobilization with or without TNFRSF25 and CD25 stimulation

Following *in vivo* stimulation with TLIA-Ig FP+ $rhIL\text{-}2_{LD}$, PB Tregs were analyzed to assess the impact of 2-pathway TNFRS25 and CD25 stimulation on their phenotype. Increased levels of Tregs following administration of the expansion protocol, characterized by diminished frequencies of central Tregs Ly-$6C^+$ and significantly elevated levels of effector Tregs in the 2-pathway stimulated animals compared to unmanipulated PB were detected (Fig. 2A, B). Mobilization alone tended to affect the Treg populations in a similar although less impactive manner. Additionally, Tregs from mobilized and Treg expanded animals compared to animals undergoing mobilization alone expressed elevated levels of ICOS-1 and Nrp-1 (Fig. S1C). Notably, prior findings demonstrated that TL1A-Ig + $rhIL\text{-}2_{LD}$ *in vivo* expanded Tregs exhibited higher suppressive function evidenced by a lower Treg:Teff ratio required to ameliorate GVHD [6].

pSTAT5 expression was examined in PB from untreated, mobilized and mobilized + Treg expanded animals. Following the final $IL\text{-}2_{LD}$ injection, pSTAT5 levels were markedly elevated on PB Tregs (but not Tconv) only from the mobilized and Treg expanded animals (Fig. 2C, S1D) demonstrating that the downstream IL-2R signaling pathway was functional in these $CD4^+FoxP3^+$ cells. To directly assess suppressive capacity, PBMC were obtained from untreated, mobilized and mobilized plus Treg expanded animals and cultured with an anti-TcR (CD3) mAb (Fig. 2D). Stimulation by the anti-TcR mAb in cultures from animals mobilized and concomitantly Treg expanded resulted in substantially reduced cell numbers compared to cultures established from mobilized only or untreated animals (Fig. 2D). Following mobilization and Treg expansion in FoxP3-diptheria toxin knock-in mice, Tregs were depleted *in vivo* with DT prior to *in vitro* PBMC culture. DT depletion of Tregs abolished the suppression of the anti-CD3 mAb T cell response (Fig. S2A). These findings illustrate a correlation between the diminished *in vitro* responses by PB T cells in samples and the presence of elevated levels of Treg cells in PB.

Allogeneic transplantation and analysis of GVHD using peripheral blood from non-mobilized, mobilized or mobilized and Treg expanded donors

Experiments were next performed to compare the GVHD capacity of donor blood from $FoxP3^{RFP}$ knock-in donor B6 mice undergoing either mobilization only or mobilization

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together with concurrent Treg expansion (Fig. S1A). Based on the levels of $CD4^+$ and $CD8^+$ T cells in the donor PB, cell numbers were adjusted, and all recipient BALB/c mice received 1×10^6 total T cells. A third group of BALB/c recipients received spleen cells also recovered from mobilized only B6-FIR donors (1×10^6 total T cells). Recipients receiving either blood or spleen cells from mobilized only donors generated significant GVHD clinical scores (Fig. 2E). In contrast, recipients receiving donor PB from mice concomitantly mobilized and Treg expanded exhibited significantly lower GVHD clinical scores (Fig. 2E) and within the first month post-HSCT contained higher frequency of Tregs in the PB (Fig. S2B). Additionally, 4/5 vs 1/5 mice survived to one month if the mobilized donor PB had been Treg expanded using TL1A-Ig+ IL-2_{LD} (data not shown). Treg expanded donors were alive at 1 month, only 1/5 Transplants were independently performed utilizing MHC-matched minor histocompatibility antigen mismatched LP/J ($H2^b$) donors and B6 ($H2^b$) recipients. Recipients of concurrently mobilized and Treg expanded donor PB (Fig. 2F) and exhibited significantly reduced GVHD clinical scores compared to recipients of PB from either untreated or mobilized only (Fig. 2G).

Our prior studies found that individual treatment with rhIL-2_{LD} alone or TL1A-Ig (or anti-TNFRSF25mAb) alone resulted in the TNFRSF25 agonists inducing a greater frequency of PB (spleen and lymph nodes) Tregs within the CD4 compartment although combining stimulation of both CD25 and TNFRSF25 induced the highest levels in these compartments [5,6,25]. Transplants using mobilized and rhIL-2_{LD} treated donors compared to transplants using mobilized and rhIL-2_{LD} plus TL1A-Ig Treg expanded donors did not show any statistically significant difference in survival benefit (Fig. S2C **left panel**). However, the recipients of mobilized and rhIL-2_{LD} Treg expanded donors demonstrated little improvement in clinical GVHD scores compared to recipients of mobilized donors receiving rhIL-2_{LD} plus TL1A-Ig to expand Tregs (Fig. S2C **right panel**). Notably, mobilized donors receiving only the TNFRSF25 agonist, TL1A-Ig also did not result in improved survival compared to recipients of control (mobilized only) donors (Fig. S2D **left panel**). Again, there was no statistically significant difference in survival benefit between groups transplanted with mobilized donors who were either Treg expanded with one reagent (TL1A-Ig) or the combination of TL1A-Ig+IL-2 (Fig. S2D **left panel**). However, in contrast to recipients of mobilized and rhIL-2_{LD} treated donors (Fig. S2C, **right panel**), these mobilized and TL1A-Ig only treated Treg expanded donors exhibited diminished GVHD clinical scores (Fig. S2D **right panel**). Again, the strongest amelioration of GVHD score were detected in recipients of mobilized together with combination TL1A-Ig+IL-2_{LD} Treg expanded donors (Fig. S2D **right panel**). Pathology evaluation of GI tissue indicated significantly reduced damage in recipients of mobilized and concomitantly Treg expanded donors compared to recipients of mobilized only or untreated donors (Fig. S2E).

To begin assessing potential clinical application of this strategy, LP/J mice were concomitantly mobilized and treated with an agonistic anti-TNFRSF25 mAb provided to our laboratory by Pelican Therapeutics. This specific mAb is an anti-human TNFRSF25 containing several amino acid differences from the original hamster anti-mouse reagent (4C12mAb) in the complementarity determining regions [30,31]. Treg levels in PB were >30% of $CD4^+$ T cells in stem cell mobilized and mPTX35+IL-2_{LD} treated donor animals

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(Fig. S3A). PB assessed ~ two weeks post-HSCT indicated that Treg levels were elevated in recipients of mobilized and Treg expanded compared to mobilized only donors (Fig. S3B). Overall weight (not shown) and clinical GVHD scores were decreased in B6 recipients of mobilized and mPTX35 Treg expanded donors compared to recipients of mobilized only (Fig. 2H).

Recipients of mobilized allogeneic peripheral blood containing elevated levels of Tregs mediated GVL against leukemia cells

To assess if animals receiving mobilized PB from donors who were concomitantly Treg expanded could affect graft vs. leukemia (GVL) responses, MLL-AF9 B6 tumor cells were administered at the time of C3H.SW⟶B6 HSCT. Some animals underwent an HSCT using mobilized syngeneic donor PB (B6->B6) and also received MLL-AF9 cells. As anticipated, GVHD was reduced in recipients of tumor + mobilized + Treg expanded donors compared to tumor + mobilized recipients (Fig. 3A). Tumor cells were readily identified in the spleen and marrow of recipients of syngeneic, mobilized PB donors ~1 month post-HSCT, (Fig. 3B, **left panels**). As morbidity ensued, blood was collected shortly prior to anticipated death. In contrast, recipients of mobilized allogeneic PB (Fig. 3B, **middle panels)** had very low levels of detectable tumor cell and mobilized allogeneic PB containing elevated levels of expanded Tregs (Fig. 3B, **right panels)** did not contain detectable tumor at early time points examined (Fig. 3B). At later time-points post-HSCT, low tumor levels could again be detected in some of the allo-HSCT recipients (Fig. 3C). In total, while no apparent difference in the GVL response was observed assessing recipients of mobilized vs. mobilized + Treg expanded donors, the former demonstrated significantly higher levels of GVHD (Fig. 3A). Earlier studies examining the use of Treg cells to ameliorate experimental GVHD in mice did not find ablation of GVL activity (9). Notably within this context, several clinical trials have reported that donor Treg infusion does not increase leukemia relapse in patients (32–36).

Ex-vivo expanded human donor Tregs inhibit xenogeneic GVHD development in animals receiving transplants of mobilized human peripheral blood

The ability of donor human Tregs to inhibit GVHD induced by mobilized human PB cells was also examined. PBMC from healthy donors mobilized with Neupogen were aliquoted and cryopreserved prior to use. Subsequently, a sample was phenotyped for T conventional ($CD4^+FoxP3^-$), CD8 ($CD8^+CD4^-FoxP3^-$) (not shown) and Tregs ($CD4^+CD8^-CD25^+FoxP3^+CD127^{lo}$) (Fig. S3C). $CD4^+CD25^+CD127^{lo}$ T cells (Treg) were obtained by cell sorting and cultured with anti-CD3/CD28 Dynabeads and IL-2 (Methods) for 7–8 days (Fig. S3C). Post-culture analysis indicated significant Treg expansion (~30x, Fig. S3D) with yields ranging from 0.4 to 1.2×10^8 (Fig. 3D **left panel**) and maintenance of high FoxP3 levels (>98%) (Fig. 3D **right panel**). Expanded donor Tregs were then mixed with PBMC from a freshly thawed aliquot of the same donor and transplanted (1:1) into NSG mice. Recipients receiving PBMC from the mobilized donor without Tregs exhibited severe GVHD and did not survive >21 days post-transplant (Fig. 3E). In contrast, recipients of PBMC containing *ex-vivo* expanded Tregs had significantly diminished GVHD clinical scores and 100% survival through 5 weeks post-HSCT (Fig. 3E+F) as well as higher levels of hu $CD4^+FoxP3^+$ Tregs compared to recipients of mobilized PB without ex-vivo expanded

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Tregs (Fig 3G, **left panel**). Human CD45$^+$ CD4 and CD8 T cells were present 1–2 weeks post-transplant in all mice, however, the frequency of CD4 and CD8 T cells was much higher in non-Treg treated recipients (Fig 3G, **middle and right panel**). Cell proliferation was assessed two weeks post-transplant and Tconv cells (CD4$^+$FoxP3$^-$) in animals receiving PBMC without added *in vitro* expanded donor Tregs exhibited elevated Ki67 expression compared to Tconv cells in Treg treated recipients which expressed barely detectable Ki67 (Fig. 3H). In total, recipients of allogeneic mobilized human PB without *ex-vivo* expanded donor Tregs contained higher levels of donor CD4 Tconv and CD8 T cells with increased Ki67 expression compared with recipients of human Tregs.

Concluding remarks—GVHD remains the major immunological complication preventing more wide-spread application of aHSCT. According to the CIBMTR, among adult recipients of matched related donor transplants as well as among adult recipients of unrelated donor transplants, mobilized PB cells is the most common graft type accounting for ~80% of all transplants through 2019 https://www.cibmtr.org/ReferenceCenter/SlidesReports/SummarySlides/pages/index.aspx). A number of labs including our own have been exploring potential applications of CD4$^+$FoxP3$^+$ T cells (Tregs) to ameliorate GVHD [1–15,25]. The ability to concomitantly mobilize stem cell donors and effectively augment the peripheral Treg compartment could represent a clinically useful advance. We have previously reported a two pathway Treg expansion strategy developed by our lab to amplify the suppressive effect of the donor inoculum and diminish GVHD in experimental transplant recipients [5,6]. The present studies established a procedure to concurrently induce stem/progenitor cell mobilization and Treg expansion in murine HSCT donors by infusion of TNFRSF25 agonistic fusion protein and IL-2$_{LD}$ into donors receiving G-CSF+/−plerixafor. The findings demonstrated that both the expanded Tregs and progenitor cells were functional as evidenced by survival and suppression of GVHD. It should be noted that at present, an FDA approved human anti-TNFRSF25mab is not available. Nonetheless addition of *in vitro* expanded human Tregs to mobilized human PB - as did mobilized mouse PB together with *in vivo* expanded mouse Tregs - suppress GVHD. Importantly, concomitant donor mouse treatment *in vivo* with rIL-2 and G-CSF did not provide GVHD protection nearly as effective as donors administered anti-TNFRSF25+CD25 agonists. Therefore, the use of IL-2 infusion alone while mobilizing human donors is not likely to be as effective to suppress GVHD. Accordingly, we posit that for translational purposes, co-administration of an FDA approved anti-TNFRSF25 together with a CD25 (IL-2) agonist would be required for donor treatment to produce PB that would optimally ameliorate GVHD. Nonetheless, the xenogeneic GVHD data also supported another potential translational strategy i.e., using *ex-vivo* expanded donor Tregs (anti-CD3 beads + hulL 2) added to mobilized human PB. Since prior studies did not identify any lingering phenotypic or pathologic changes in blood or tissues following the transient two pathway Treg cell expansion protocol employed here [5], we posit there is potential for administering anti-TNFRSF25 and anti-CD25 reagents *in vivo* to manipulate the donor – and potentially the host - CD4$^+$FoxP3$^+$ compartment as a novel approach for GVHD prophylaxis [37,38].

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Supplementary Material

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ACKNOWLEDGMENTS:

The authors would like to acknowledge the support of the SCCC Flow Cytometry Core and the Flow Cytometry Core Facility at Diabetes Research Institute (Dr. Oliver Umland). We also thank the staff at the Division of Veterinary Resources at the University of Miami Miller School of Medicine for their outstanding animal maintenance and care of the mice used in these studies, we would thank Drs Stephen Nimer and Na Man for providing the construct to induce MLL-AF9 tumor cells in B6 mice used in our GVL experiments. The authors also thank Brandon Kale, MD and Clarisel Lozano for help with injection of the animals in some of these experiments.

Financial disclosure:

This work was supported by funds from the National Institutes of Health (R01 EY024484-06, R01 EY030283-01, R41 AI149916-01, to M.S and R.B.L.; R01 AI31648 to T.R.M.; and R01 CA208634 to R.J. K00CA245728 to C.S.B. and SC is the recipient of a ASTCT New Investigator Award. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funding bodies were not involved in the study design; collection, analysis, and interpretation of data; manuscript preparation; or the decision to submit the manuscript for publication.

Data availability:

Data reported in this article will be shared under the terms of a Data Use Agreement and may be used only for approved proposals. Requests may be made to hbarreras@med.miami.edu

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Highlights

- Donor peripheral blood in mice undergoing stem/progenitor cell mobilization can be concurrently treated *in vivo* with reagents targeting and expanding their Treg compartment.
- Use of this PB for MHC-matched and in MHC mismatched HSCT transplants ameliorated GVHD while maintaining GVT.
- Addback of *ex-vivo* expanded donor human Tregs from mobilized PB to the same mobilized PB donor resulted in marked reduction of xenogeneic GVHD.

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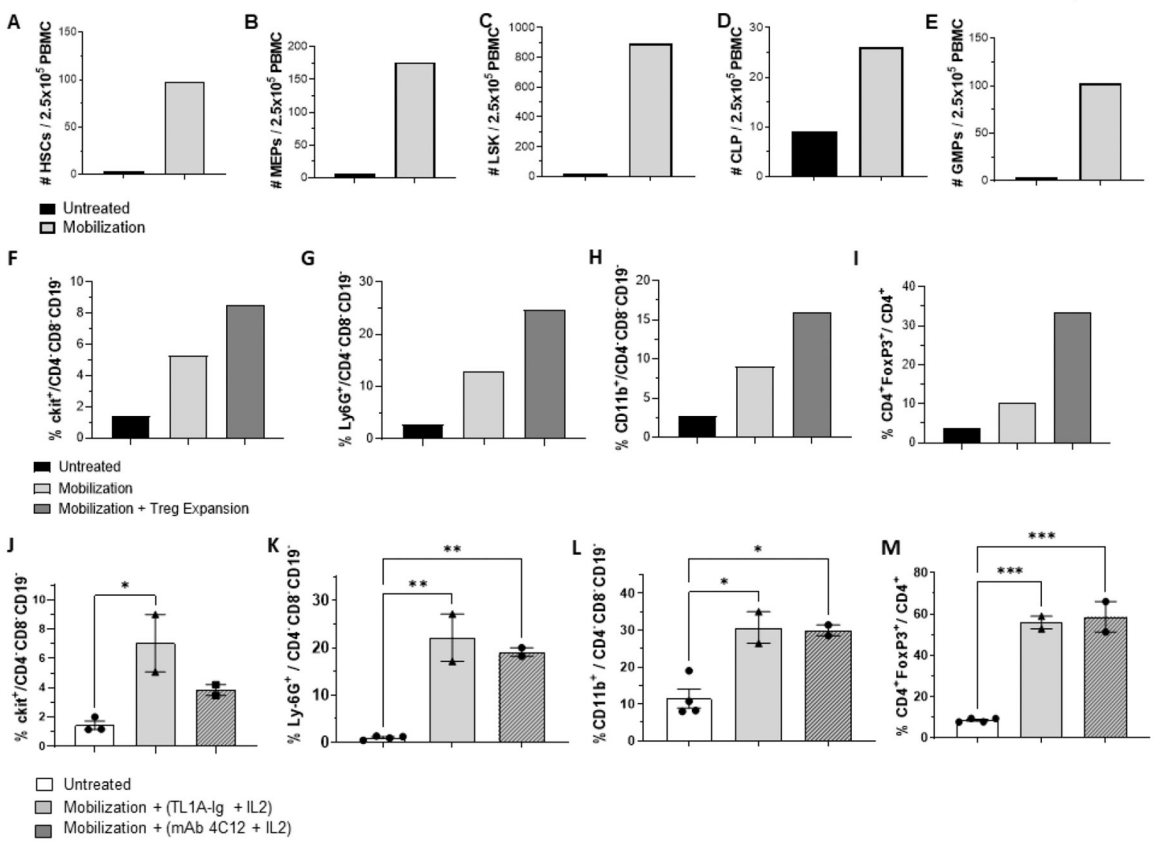

Fig. 1. Tregs can be expanded concomitantly with HSC and progenitor cell increases in peripheral blood following mobilization with Filgrastim and plerixafor infusion.

(A-E) B6-Fir,H2^b mice were administered rGCSF (2.5ugs / injection) on Days 1–4 daily and plerixafor (5.0mgs/kg) on Days 4 and 5. (Fig. S1). Peripheral blood was collected in the morning of Day 5, 1 hr. following plerixafor injection and PBMC isolated (Methods). Following staining with selected mAbs and analysis via flow cytometry, **(A-E)** mobilization treatment resulted in 24-, 29-, and 60-fold increases of HSC **(A)** MEP **(B)** and LSK **(C)** populations respectively. CLP (3 fold) **(D)** and GMP (34 fold) **(E)** were also elevated compared to control (peripheral blood from non-injected normal mice). Data represents results of pooled peripheral blood from 2 mobilized B6-Fir male mice. **(F-I)** Mice (LP/J, H2)^b were mobilized as above, and administered TL1A-Ig fusion protein and rhIL-2LD. Increased levels of c-kit$^+$ and WBC fractions (**F-H** populations calculated within the non-lymphoid fraction) together with elevated Treg frequency following mobilization and treatment with TL1a-Ig and rhIL-2LD. Data represents pooled peripheral blood from 5 individual mice in each group (n=5 mice / group). **(I)** FoxP3$^+$ Tregs within the CD4$^+$ population. Numbers of Tregs were calculated for each group indicated (1.0ml peripheral was collected from each mouse, PBMC collected and pooled (n=5/group). Total PBMC were counted and Treg numbers calculated based on the frequency of CD4$^+$FoxP3$^+$/CD4$^+$ cells. Untreated: 7,502; Mobilized: 102,538; Mobilized plus Treg expansion: 914,514. **(J-M)** Targeting TNFRS25 with a second agonist (mAb 4C12) also expands Tregs in mobilized peripheral blood. B10.D2 (H2d) mice were mobilized and Treg expanded with either TL1A-Ig (50ug) or mAb 4C12 (100ug) plus rhIL-2LD. **(J-L)** Heightened levels of c-kit$^+$,

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myeloid cells and monocytes were detected in mobilized animals receiving eitherTNFRSF25 agonistic reagent. **(M)** Levels of Tregs were increased in all mobilized B10.D2 animals treated with either agonist (n=2 for mAb 4C12+IL-2LD), n=2 for TL1A-Ig+IL-2LD) vs non-mobilized B10.D2 animals (n=4): Cells per 200ul of peripheral blood = Non-mobilized, 818–984; Mobilized via 4C12, 1640, 2476; mobilized via TL1A-Ig, 1485,3320. **(J-M)** Data were collected from individual mice and are expressed as mean ± SD and were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

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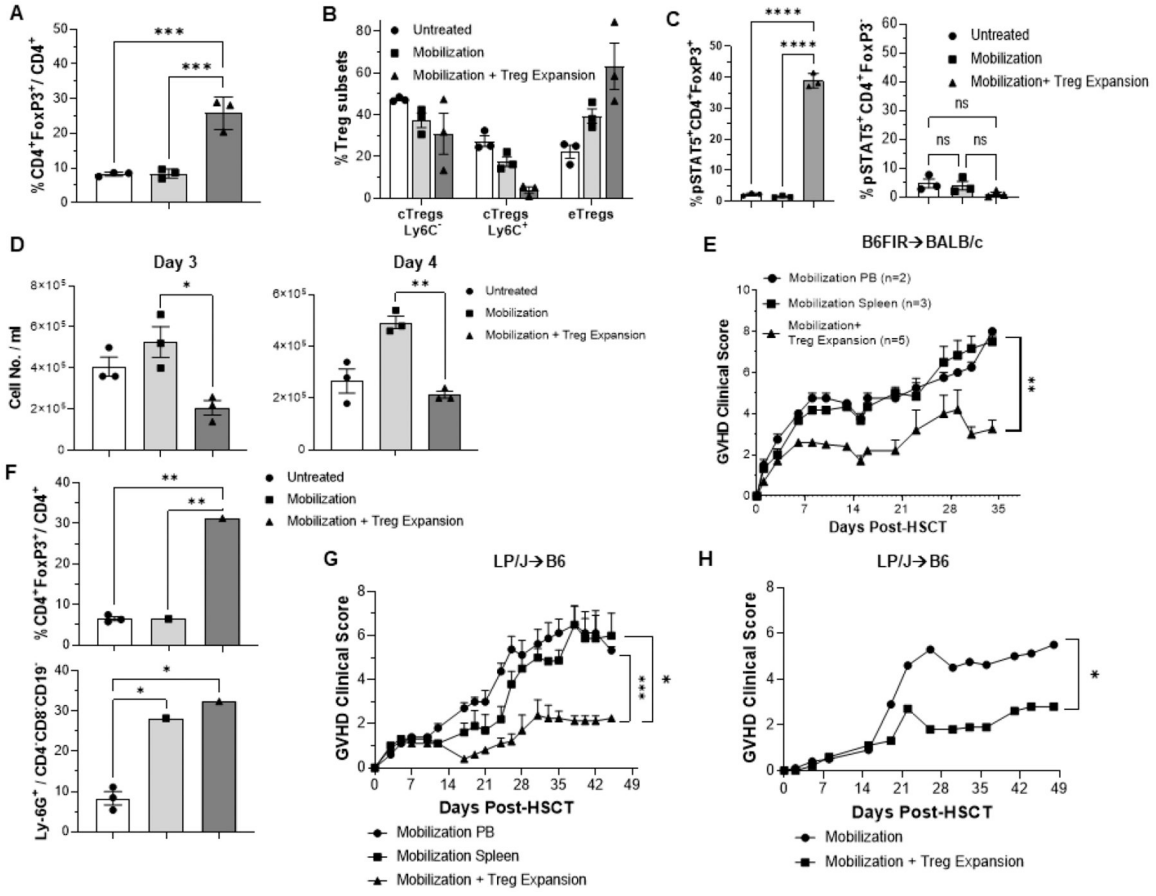

Fig 2. Tregs Expanded with TL1a-Ig and IL-2(LD) in mobilized donor peripheral blood (PB) exhibited suppressive activity and ameliorated GVHD.

Mice were injected i.p. with TL1A-Ig (50ug) (days 1–4) and IL-2 LD (days 4 to 6). Mice administered TL1A-Ig+IL-2_{LD} showed an increase in overall Treg ($CD4^+$ $FoxP3^+$) frequency in mobilized (rGCSF + Plerixafor) plus Treg expanded PB versus mobilized or untreated mice **(A,F). (B)** Diminished frequencies of central Tregs and significantly elevated levels of effector Tregs were present in mobilized plus Treg expansion compared to mobilized or untreated mice. **(C)** One hour after the last IL-2 injection (combined TL1A-Ig+IL-2_{LD}), pSTAT5 staining showed heightened activation of PB Tregs compared to Tcon CD4 T cells. **(D)** Decreased T cell proliferation in mobilized peripheral blood in animals concomitantly Treg expanded. PBMC were activated with anti-CD3 mab and assessed for proliferation after 72 and 96 h **(E)** GVHD was diminished in animals receiving mobilized and Treg expanded donor cells. MHC-mismatched HSCT (B6→BALB/c) using T cells from donor B6-Fir mice PB either mobilized or mobilized plus Treg expanded (TL1A-Ig+IL-2LD) adjusted to contain 1.0×10^6 total T cells. Clinical scoring is presented. (**F-H**) GVHD was diminished in animals after an MHC-matched minor histocompatibility antigen mismatched HSCT (LP/J -> B6) using T cells from donor PB mobilized (rGCSF + Plerixafor) plus Treg expanded (TL1A-Ig fusion protein + IL-2_{LD}) versus mobilized only. **(G)** and mPTX mab+IL-2LD **(H)** adjusted to contain1.0×10^6 total T cells. **(F)** Treg and $Ly6G^+$ granulocyte levels in PB donors for the transplant results in panel **(G)** are shown. Values are means ± SEM and were analyzed by multiple variable analysis using

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ANOVA. A P-value < 0.05 was considered significant. **(E,G)** a 2-tailed unpaired t-test. was used for comparisons between 2 experimental groups **(H)** *P < .05; **P < .01; ***P < .001.Significance indicated by * p < 0.05, ** p < 0.01, *** p < 0.001, ns=not significant.

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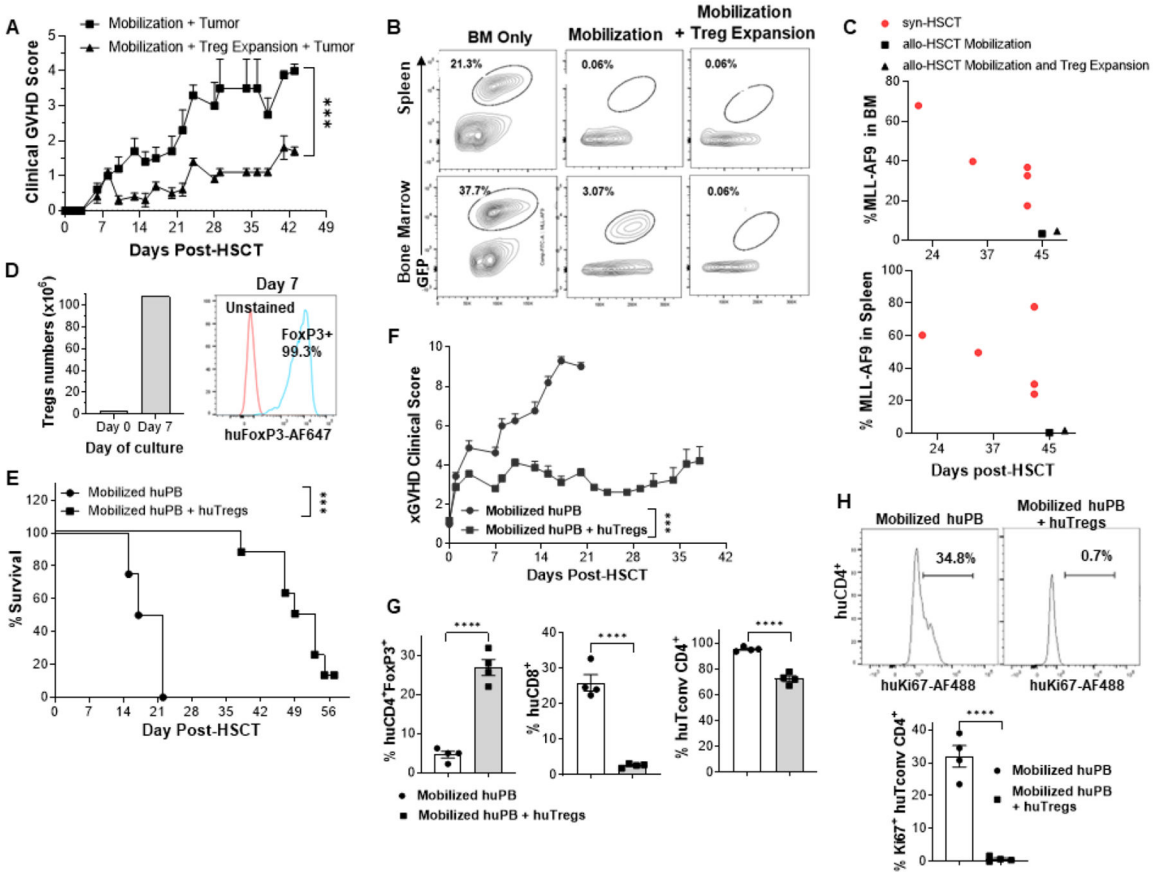

Fig 3. Maintaining mouse GVL and suppressing xenogeneic GVHD : Transplants with mobilized mouse and mobilized human peripheral blood.

(A-C) An MHC-matched transplant C3H.SW⟶B6 HSCT was performed. Mice received PB T cells from either mobilized only (rGCSF) or mobilized (rGCSF) plus expanded Treg donor C3H.SW mice. MLL-AF9 B6 tumor cells were administered to all mice at the time of transplant. **(A)** GVHD was reduced in recipients of MLL-AF9+mobilized + Treg expanded donors compared to MLL-AF9 + mobilized only recipients**. (B)** Representative flow contour plots of spleen and bone marrow cells 28–30 days post-HSCT from individual recipients of BM only (syngeneic, C3H.SW), mobilization only (allogeneic, B6), and mobilization (allogeneic, B6) + Treg expansion (TL1A-Ig + IL-2 LD). **(C)** GVL is maintained in animals with reduced GVHD. Allo-HSCT recipient groups were examined for MLL-AF9 presence post-HSCT. Tumor cell frequency was always greater in the recipients of syngeneic mobilized PB donors compared to levels in recipients who received allogeneic mobilized PB without or with expanded Tregs. **(D-H)** Addition of *ex-vivo* expanded human Tregs to mobilized human peripheral blood suppresses xeno GVHD. Sorted Tregs isolated from huPB were cultured and expanded for 7 days using aCD3/aCD28 beads (Fig. S3C). **(D)** Treg numbers and FoxP3 expression at Day 7 of culture are shown prior to use in transplant. NSG mice were irradiated on day −1 and injected with mobilized huPBMCs with or without these expanded huTregs on day 0 (n=8 mice/group). **(E,F)** Mice treated with huTregs exhibited significantly less lethality and better clinical GVHD scores. **(G)** Mice receiving huTregs with huPB showed persistence of elevated Treg levels in PB, less huCD8^{+} and conventional

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$CD4^+$ T cell levels in the blood 13 days post-transplant compared to recipients of huPB without Tregs. (**E**) Representative histogram and graph of individual mice illustrating $huCD4^+$ Tconv proliferation in blood 13 days post-transplant. *** $p<0.001$; **** $p<0.0001$.