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Third Party Umbilical Cord Blood-Derived Regulatory T cells Prevent Xenogenic Graft-versus-Host Disease

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Abstract

Naturally occurring regulatory T cells (Treg) are emerging as a promising approach for prevention of graft-versus-host Disease (GvHD), which remains an obstacle to the successful outcome of allogeneic hematopoietic stem cell transplantation. However, Treg only constitute 1-5% of total nucleated cells in cord blood (CB) ($<3\times10^6$ cells) and therefore novel methods of Treg expansion to generate clinically-relevant numbers are needed. Several methodologies are currently being utilized for ex vivo Treg expansion. Here, we report a new approach to expand Treg from CB and demonstrate their efficacy in vitro by blunting allogeneic mixed lymphocyte reactions and in vivo by preventing GvHD using a xenogenic GvHD mouse model. Using magnetic cell sorting, naturally-occurring Treg were isolated from CB by the positive selection of $CD25^+$ cells. These were expanded to clinically-relevant numbers using CD3/28 co-expressing Dynabeads and interleukin (IL)-2. Ex vivo-expanded Treg were $CD4^+25^+FOXP3^+127^{lo}$ and expressed a polyclonal T-cell receptor Vβ repertoire. When compared to conventional T-lymphocytes ($CD4^+25^-$ cells), Treg consistently showed demethylation of the FOXP3 TSDR promoter region and suppression of allogeneic proliferation responses in vitro. In our NOD-SCID IL-2Rγ^{null} (NSG) xenogeneic model of GvHD, prophylactic injection of 3rd party CB-derived, ex vivo-expanded Treg led to the prevention of GvHD that translated into improved GvHD score, decreased circulating inflammatory cytokines and significantly superior overall survival. This

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model of xenogenic GvHD can be used to study the mechanism of action of CB Treg as well as other therapeutic interventions.

Introduction

Graft-versus-host disease (GvHD) remains one of the major challenges to the successful outcome of allogeneic stem cell transplantation. Although ongoing research for over a decade has been able to identify several potential therapeutic targets, only a few are proving to be successful in clinical practice. To date steroids remain the cornerstone of GvHD treatment, although the specter of steroid-refractory GvHD remains a considerable concern, as do the side-effects associated with long-term steroid administration. More recent advances in our understanding of GvHD immunobiology have identified a preventive role for a subset of T-cells ($CD4^+CD25^+FOXP3^+CD127^{lo}$), referred to as a regulatory T-cells (Treg)[1]. Murine studies have demonstrated that the infusion of donor grafts enriched in Treg, reduces the incidence of lethal GvHD, and may even facilitate allogeneic transplantation across HLA barriers[2,3]. The use of cord blood (CB)-derived, ex vivo-expanded Treg is currently being evaluated as one strategy to prevent GvHD and their adoptive transfer has been associated with improved survival in mice[4] Furthermore, in a clinical setting, cellular therapy in the form of ex vivo-expanded adult donor[5] and/or CB derived Treg[6] is emerging as a potential prophylactic intervention for GvHD.

However, several challenges need to be overcome before the clinical potential of Treg can be realized. These include (i) large scale ex vivo expansion to yield clinically-applicable doses and (ii) the identification of an appropriate GvHD model to demonstrate in vivo efficacy in pre-clinical studies. While, elegant models exist for the study of GvHD in mice[7,8], more studies are needed to validate the translational potential of possible therapeutic interventions. The goal of our study is to demonstrate the efficacy of third party, ex vivo expanded CB-derived Treg in preventing GvHD and develop xenogeneic GvHD mouse model that will allow continued refinement of current approaches.

Methods

Treg isolation and ex vivo expansion

Cryopreserved CB units were provided under University of Texas M. D. Anderson Cancer Center (MDACC) Institutional Review Board (IRB)-approved protocols. Cryopreserved human CB units were thawed and washed in CliniMACS buffer (Miltenyi Biotec, Bergish Gladbach, Germany) containing 0.5% HSA (Baxter Healthcare, Westlake Village, CA) to yield CB mononuclear cells (MNC). CB MNC were then subjected to $CD25^+$ cell enrichment using magnetic activated cell sorting (MACS) according to manufacturer's instructions (Miltenyi Biotec, Bergish Gladbach, Germany). Positively selected cells were co-cultured with CD3/28 co-expressing Dynabeads® (ClinExVivo™ CD3/CD28, Invitrogen Dynal AS, Oslo, Norway) in a 1 cell: 3 bead ratio[9] and re-suspended at 1×10^6 cells/ml in X-VIVO 15 medium (Cambrex BioScience, Walkersville, MD) supplemented with 10% human AB serum (Gemini Bio-Products, Sacramento, CA), 2 mM L-glutamine (Sigma, St.

Louis, MO), 1% Penicillin-Streptomycin (Gibco/Invitrogen, Grand Island, NY)] [9] and 200 IU/ml interleukin (IL)-2 (CHIRON Corporation, Emeryville, CA).

Ex vivo co-culture of the $CD25^+$ cells and beads was performed in tissue culture flasks at 37°C in a 5% CO_2-in-air atmosphere (as shown in Figure 1A). The CB-derived $CD25^+$ enriched T-cells were maintained at 1×10^6 cells/ml by the addition of fresh medium and IL-2 (maintaining 200 IU/ml) every 48-72 hours [2, 9]

Flow cytometric analysis

Phenotypic analysis of cells was performed by analysis of surface or intracellular staining with anti-human specific antibodies including: CD4, CD8, CD25, CD127 and CD45 (BD Biosciences, San Jose, CA). Anti-mouse CD45 antibody (BD Biosciences, San Jose, CA) was used as negative control in the xenogeneic mouse model. Events were acquired using a FACSCalibur flow cytometer (BD Biosciences) and data analysis was performed using CellQuest™ Pro software (BD Biosciences).

Spectratyping assay

Total RNA was extracted from the Treg using a commercial kit (Tel-Test, Friendswood, TX) and cDNA was prepared using reverse transcription (Applied Biosystems, Foster City, CA). The CDR3 regions were then amplified for 23 TCR Vβ subsets by polymerase chain reaction (PCR). The resulting PCR products were subjected to capilary electrophoresis and quantitative densitometry to assess the diversity of fragment length within each of the TCR Vβ families, as previously described [10].

DNA bisulfite treatment and FOXP3 methylation analysis

DNA was extracted using standard phenol-chloroform methods and DNA was modified with sodium bisulfite[11]. Briefly, 2 μg of DNA was treated with bisulfite, and DNA was denatured in 0.2N NaOH at 37°C for 10 min and incubated with 3M sodium bisulfite at 50°C for 16 h, purified with the Wizard cleanup system (Promega, Madison, WI) and desulfonated with 0.3N NaOH at 25°C for 5 min. DNA was then precipitated with ammonium acetate and ethanol, washed with 70% ethanol, dried, and re-suspended in H_2O. Bisulfite pyro-sequencing revealed DNA methylation. For pyro-sequencing, PCR was performed using primers shown in supplementary Table 1[11]. DNA methylation was calculated using PSQ HS 96A 1.2 software (Biotage AB, Uppsala, Sweden). Several amplicons were analyzed, including amplicons 7, 9, 10 and 11[11].

Mixed Lymphocyte Reaction (MLR)

Two way, allogeneic mixed lymphocyte reactions were performed using peripheral blood mononuclear cells (PBMCs) isolated from two fresh, unrelated donor samples[12]. Assays were performed using 96-well round-bottom plates with each condition performed in triplicate. Donor cells were mixed at a ratio of 1:1 for a total of 1×10^5 cells/well. Treg ($CD4^+25^+$ cells) or Tcon ($CD4^+25^-$ cells) were added to a total volume of 200 μl of culture media. Ex vivo culture was performed at 37°C in a 5% CO_2-in-air atmosphere. Tritiated (^3H)-thymidine (PerkinElmer, Waltham, MA) was added on day 5 and culture continued for an additional 18 to 24 hours. The suppression of MLR-associated proliferation was assessed

over serially diluted Treg (or Tcon) and each condition was repeated in triplicate. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine. Cells were harvested (PerkinElmer, Waltham, MA) and tritium incorporation determined by liquid scintillation (Packard Meriden, Prospect, CT). Results were expressed in counts per minute (cpm).

Since downstream signaling pathways including p38MAP kinase have been implicated in mediating Treg suppression [13], its' pharmacologic inhibitor-SB203580 (Cell Signaling Technology, Beverly, MA) was added to the allogeneic MLR to study their effect on Treg-mediated suppression with appropriate controls employed.

Xenogeneic murine GvHD model

All animal work was performed under University of Texas MDACC Institutional Animal Care and Use Committee (IACUC)-approved protocols. Two different xenogeneic GvHD models were explored (as shown in Figure 1B). In one model, mice (NOD/SCID IL-2Rγ^{null} (NSG), Jackson Laboratory, Bar Harbor, ME) received sub lethal whole body irradiation (300 cGy from a ^{137}Cs source delivered over 1 minute by a J. L. Shepherd and Associates Mark I-25 Irradiator, San Fernando, CA) 1 day prior to intravenous infusion of human PBMCs (day -1), and in the other model they did not receive irradiation prior to PBMC injection. On day 0, mice received PBMCs at a dose of 1×10^{7} (Figure 1B). In order to study the effect of CB Treg in the xenogenic GvHD model, mice received an additional injection of 10^{7} ex vivo expanded CB-derived Treg shortly after irradiation on day -1. Mice were evaluated daily using a scoring system developed by Ferrara et al. [14], where GvHD 'scores' were assigned based on: (i) weight, (ii) posture, (iii) activity level, (iv) skin integrity and (v) fur texture. Although several mice were followed past 30 days in a survival study, the majority of mice were euthanized between 14 to 21 days post-transplant. Flow cytometric analysis was performed on RBC-lysed PB samples (Pharm Lyse, BD Pharmingen) for human CD4, CD8 and CD45 and mouse CD45-positive cells. Tissues including femoral bones and bone marrow, liver, spleen, lungs, skin and gastrointestinal tract were also harvested and fixed in formalin for subsequent histopathology analysis by veterinary and clinical pathologists.

Serum cytokine analysis

Approximately 100μl of peripheral blood was collected from mice into EDTA-containing blood collection tubes and blood centrifuged at 400 x g for 10min, serum collected and stored at -80°C until analyzed. Cytokine concentrations were estimated using the Bio-Plex® Pro human Cytokine 27-Plex Panel kit and analyzed usingthe Bio-Plex® system according to manufacturer's instructions (BioRad, Hercules, CA). Concentrations were interpolated from contemporaneously acquired standard curves using Bio-Plex® software. Samples were assayed in duplicate and data (mean ± standard deviation) compared.

Bioluminescence imaging

Production and imaging of labeled Treg—Treg were labeled using a retroviral vector that expressed Firefly luciferase and eGFP (eGFP-FFLuc). This vector was developed by the Center for Cell and Gene Therapy (Baylor College of Medicine, Houston, TX). To produce a retroviral supernatant for transduction, HEK 293T cells were co-transfected with the

retroviral vector eGFP-FFLuc and Peg-Pam-e (containing the sequence for the MoMLV gag-pol) and RDF plasmids (encoding for the RD114 envelop) (kindly provided by Dr. GianPietro Dotti, Baylor College of Medicine, Houston, Texas). Retroviral supernatant was collected at 48 and 72 hours after transfection, filtered (0.45μm) and stored at -80°C until required. Prior to transfection, $CD25^+$ cells were enriched from CB by MACS and activated by culture at 1×10^6 cells/ml in X-Vivo medium containing 200 IU/ml IL-2 and CD3/28 co-expressing Dynal® beads at a 3 bead to 1 cell ratio. Culture at 37°C was performed for 3 days. For transduction, Treg were plated at a concentration of 5×10^5 cells/ml in thawed retroviral supernatant in Retronectin-coated 24-well tissue culture plates and continually cultured for 11 days by using the same procedure as described in "Treg isolation and ex vivo expansion". Treg were then harvested by centrifugation, washed, resuspended in saline and injected intravenously into sublethally-irradiated (300 cGy) NSG mice at 10^7 PBMC per mouse. Non-invasive, bioluminescent imaging was performed using a Xenogen IVIS imaging system (Caliper Life Sciences, Hopkinton, MA). At specified time-points, mice were anesthetized with 2% isoflurane gas and the presence of Firefly luciferase-expressing Treg revealed by the subcutaneous administration of 100μl, 20mg/ml D-luciferin (Gold Biotechnology). Mice were imaged within 10 minutes of injection using a 5 minute exposure to acquire the image. The minimum and maximum photons/second/cm^2 for each figure was indicated by a rainbow bar scale.

Statistics

Where appropriate, statistical analyses were performed using the Student's T-test (Excel, Microsoft Corp., Redmond, VA) with significance assumed at $P\leq0.05$. The Kaplan Meier method was used to generate survival curves and Log Rank test was used to compare the two groups.

Results

Isolation and expansion of Treg from CB

Naturally occurring $CD25^+$ cells routinely comprised 1 -5% of CB MNC[15] and following $CD25^+$ MACS selection, the purity of the 'positive' fraction determined by the co-expression of CD4 / CD25 / FoxP3 was > 90% ($0.5\text{-}3.0\times10^6$ cells). The 'negative' fraction containing $CD25^-$ cells was defined as Tcon'. For the purpose of expansion, the whole of the positive fraction (Treg) was used. For Tcon, where appropriate, only a part (5×10^6 cells) of the negative fraction was cultured. Ex vivo culture was initiated with $CD25^+$ cells at a concentration of 1×10^6 cells/ml in medium supplemented (as previously described) with IL-2 at 200 IU/ml, in the continued presence of CD3/28 beads with an initial ratio of 3 Dynabeads: 1 cell. The cell numbers were assessed on an every-other-day basis and maintained at a concentration of 1×10^6 cell/ml. At the end of the 14 days of ex vivo culture, the Treg were expanded to 300×10^6 cells (range: 250-900 $\times$ 10^6 cells, n=12).

Confirmation of the Treg phenotype of the ex vivo expanded product

In the representative data, >90% of the final Treg product at 14 days of ex vivo culture were $CD4^+25^+FOXP3^+127^{lo}$ (Figure 2A). By comparison, <50% of the expanded Tcon were $CD4^+25^+$. Since CD25 and FOXP3 expression can be upregulated in response to activation

by exposure to IL-2, we analyzed the methylation status of the FOXP3 TSDR region. This is positively correlated with Treg phenotype and function[11]. When compared to Tcon, significant levels of FOXP3 TSDR demethylation were demonstrated in the ex vivo expanded CB Treg (Figure 2B). Further, ex vivo expanded CB Treg maintained the polyclonality of their TCR Vβ repertoire (Figure 2C) showing that the process of ex vivo expansion did not skew their TCR Vβ repertoire.

In vitro efficacy of ex vivo expanded CB Treg

Two-way MLR were performed, as described, between 2 random donor s PB MCs (D1 + D2).. As expected, significant proliferation was observed when D1 and D2 were mixed together indicating a strong allogeneic response (Figure 2D). This proliferation also occurred following the addition of ex vivo expanded Tcon (D1 + D2 + Tcon) (Figure 2D). However, the allogeneic proliferation response was significantly blunted by the addition of ex vivo expanded CB Treg at a 1:1 ratio (D1 + D2 + Treg) (Figure 2D). These data therefore provide evidence that ex vivo generated Treg suppress in vitro allogeneic proliferation generated during MLR. Further correlation was established between the Treg phenotype, FOXP3 demethylation and suppression of lymphocyte proliferation (supplemental figure 1)

In previous studies, the p38MAP kinase[13] pathway have been shown to mediate the suppressive effect of Treg. This was assessed in our model. Addition of pharmacologic inhibitors of p38MAP kinase (SB203580: 1 μM) to the allogeneic-MLR were suggestive of possible additive effect of the blockade of these pathways on the CB Treg mediated inhibition of the allogeneic proliferation (Figure 2E).

Xenogenic GvHD Model

Two xenogeneic GvHD models were investigated: (i) mice receiving sublethal irradiation (300 cGy) and (ii) no sublethal irradiation, prior to the injection of PBMC (Figure 1B). In the model without irradiation, the average time to hair loss was 42±5 days and weight loss was 50±5 days. On average, the mice were moribund at a median of 90 days (range: 65-120 days). In the model with sub lethal irradiation, the mice showed signs of GvHD including loss of fur, hunched posture, loss of weight and decreased activity at as early as day 12 and the majority of the mice were moribund by day 28. Histopathologic analysis revealed typical features of GvHD in both cases. These included the diagnostic presence of apoptotic bodies, lymphocytic infiltration, interstitial edema and tissue destruction (supplemental figure 2). The GvHD scoring system provided objective evaluation of the health of the mice and the average GvHD score in the PBMC recipients was ‘4’ at the time of euthanasia, indicating that the initial radiation had no impact on the severity of the GvHD. However, in the irradiated mice, more rapid development of a higher GvHD score (by an average time of 30 days) was observed and therefore suggested the use of the sub lethally-irradiated model for the study of Treg-based strategies for the prevention of GvHD.

Ex vivo expanded CB Treg prevent xenogenic GvHD

Flow cytometry analysis of representative peripheral blood samples from mice receiving PBMC with or without Treg were compared (Figure 3A). Gating on human (CD45) cells revealed that the majority (approaching 100%) of circulating human cells were T-

lymphocytes and that there was no significant difference between the pattern of circulating CD4 or CD8 cells irrespective of whether the mice received CB Treg. Despite this similar pattern of circulating human T-lymphocytes in the peripheral blood , prophylactic injection of ex vivo expanded CB Tregs on day -1 (prior to the PBMC injection) led to the prevention of the gross sequelæ of GvHD leading to the preservation of fur and condition of the skin (Figure 3B). In addition, analysis of changes in body weight revealed the significant loss of weight in the PBMC recipients as compared to the preservation of weight in the Treg + PBMC recipients (Figure 3C). These differences were also reflected by a higher overall GvHD score in the PBMC only mice (Figure 3D). In addition, intravenous injection of Treg alone in sub lethally-irradiated NSG recipients did not induce GvHD.

Microscopic examination revealed that the administration of CB Treg was associated with the preservation of normal tissue architecture. Histopathologic analysis by veterinary and clinical pathologists concurred with this observation and reported diagnostic indicators (including tissue destruction, lymphocytic infiltration, aplasia and the presence of apoptotic bodies) of GvHD present in the small intestine, liver, lung and skin of the recipients of PBMC alone that were absent in the same tissues from recipients of Treg + PBMC (Figure 3E). Generally, tissue structures were better preserved in the Treg + PBMC recipients than in the recipients of PBMC alone. Significant differences were also seen in terms of bone marrow aplasia and lymphocytic infiltration of the spleen in PBMC only recipients.

Ex vivo expanded CB Treg Prevent Xenogenic GvHD by Suppressing the production of Inflammatory Cytokines

Mouse serum was analyzed for the presence of circulating human inflammatory cytokines including: interleukin-6 (IL-6)[16], interferon γ (IFNγ) [17], interleukin-5 (IL-5) [18], monocyte chemotactic protein-1 (MCP-1) [19], macrophage inflammatory protein (MIP)-1β [20], interferon inducible protein-10 (IP-10) [21] and tumor necrosis factor α (TNFα) [22] to determine whether the administration of Treg had any impact on the in situ production of these factors. In all cases, levels of circulating inflammatory cytokines were decreased if CB Treg were introduced before administration of PBMC. These data suggest a Treg-associated decrease in inflammatory cytokines is correlated with the prevention of xenogenic GvHD (Figure 3F).

Ex vivo expanded CB Treg injection improved Overall Survival in the Xenogenic GVHD mouse model

Overall, the administration of CB Treg and the benefit it conferred in terms of prevention of GvHD was associated with improved survival of the recipient mice (Figure 3G). In a limited number of NSG mice received identically ex vivo expanded Treg or Tcon from the same CB unit prior to (day -1) the administration of PBMC (day 0), to confirm that the prevention of GvHD was due to Treg and not simply an artifact associated with the administration of an ex vivo expanded T-cell product. While the administration of Treg prevented GvHD, no benefit was associated with the administration of Tcon and symptoms in these mice (Tcon + PBMC) were as aggressive as those seen in recipients of PBMC alone. These data confirm that prevention of GvHD in the xenogenic model was due to the prophylactic injection of Treg. In other limited experiments, it was shown that while Treg can prevent GvHD when

administered prior to PBMC, they cannot treat or ameliorate pre-existing sequelæ of established GvHD or 'rescue' the mice (data not shown). This shows that our prophylactic strategy is an appropriate means of utilizing Treg as a cellular therapy to prevent GvHD.

In vivo homing and distribution

In order to better understand the distribution of ex vivo expanded CB Treg and their interaction with PBMC in the mouse model, we labeled Treg with Firefly Luciferase (Figure 4A). Following their intravenous injection on day -1, evidence of trapping in the lung microvasculature was observed, followed by signal detection in the lungs, liver, spleen, skin and long bones at day 0 (Figure 4B – left panel). Such signal was no longer detectable at 3 days post injection. Whether this was a consequence of CB Treg dilution throughout the blood stream and body of the mice, with no foci of cells sufficient for the signal to reach threshold for detection and/or the lack of proliferation stimuli for Treg in the absence of sites of GvHD is unclear. However, when the intravenous injection of labeled ex vivo expanded CB Tregs (injected day -1) was followed by the intravenous injection of unlabeled PBMC (administered day 0), (Figure 4B – right panel), the CB Treg associated signal persisted beyond day 10, suggesting a CB Treg response to PBMC-induced GvHD with homing and proliferation. Signal associated with the CB Treg was lost by day 14 and no gross GvHD sequelæ were observed in these recipients, suggesting that there was a correlation between the activity of the labeled CB Treg and the absence of otherwise aggressive PBMC-induced xenogeneic GvHD. Confirming the absence of CB Treg persistence following their initial role in preventing GvHD, neither the intravenous administration of additional PBMC (as a further stimulus to Treg proliferation) nor the intra-peritoneal administration of IL-2 yield to any further bioluminescence associated with CB Treg activity (data not shown).

Discussion

Using $CD25^+$ MACS enrichment and an ex vivo expansion technique, which includes the use of the CD3/28 co-expressing Dynabeads, 200 IU/ml IL-2 and maintenance of cultures at $\leq 1\times10^6$ cells/ml, we have been able to successfully demonstrate the generation of clinically-relevant numbers of functional, third party Treg from the limit numbers initially found in CB units and to demonstrate their efficacy in preventing GvHD. Since this methodology uses Good Manufacturing Practice (GMP) grade materials, including the CD3/28 co-expressing Dynabeads, it is therefore an approach that can be readily translated to the clinic for use in clinical trials with the goal of preventing GvHD especially in high-risk hematopoietic transplant patients. Using well described phenotypic markers[23] including intracellular FOXP3, we were able to confirm the Treg phenotype of the ex vivo expanded CB product and specifically differentiate them from similarly ex vivo expanded CB Tcon, as a control. Although, the expression of FOXP3 is used as an important phenotypic marker of Treg, it has also been shown to be transiently and non-specifically increased in activated T cells[11]. Indeed, when Tcon and Treg were prepared in a similar manner, up to 50% of Tcon were found to express FOXP3. To address this concern, the demethylation status of the TSDR region of the FOXP3 gene in Treg and Tcon were compared. These data confirmed that while Treg showed marked demethylation of the TSDR region of the FOXP3 gene, Tcon did not. In a limited number of experiments, demethylation of the TSDR region (indicative of a

Treg 'phenotype') was shown to be absolutely correlated with the suppression of allogeneic proliferation in vitro in MLR (supplemental Figure 1). This is important in the clinical setting since the FOXP3 demethylation status of the cells generated following ex vivo expansion can be used to confirm their identity as Treg and as an easy 'surrogate' marker of Treg function for clinical studies. Furthermore, data obtained here suggest that it may be possible to augment the suppressive function of ex vivo expanded CB Treg through the use of pharmacologic agents that modify the p38 MAP kinase pathway. Such inhibitors are available clinically[24] and can be utilized for possible improvements in the GVHD prevention.

In order to strengthen our pre-clinical work, we have established a xenogenic GvHD model that can act as a platform to explore experimental approaches for development of cell therapy-based preventive strategies against GvHD. Similar models have been shown to be reliable in allowing the investigation of GvHD mechanisms[25-28]. Previously, Cao et al., showed that when ex vivo expanded Treg were co-transferred with peripheral blood lymphocytes into the spleens of NOD/SCID mice, GvHD was prevented and survival significantly enhanced [29]. This model has also been used to study the GvHD-ameliorating effects of mesenchymal stem cells[30]. Similar to our findings, Hippen et al.[4], have previously shown that ex vivo expanded CB derived Tregs, expanded using both CD3/28 beads or cell based techniques, are able to inhibit xenogenic GvHD in a NK-deficient C57BL/6 $Rag^{-/-}$, $\gamma c^{-/-}$ mouse model[4].

We also reconfirmed the critical importance of the timing of the administration of CB Treg in the prevention of GvHD. In our studies, CB Tregs were unable to ameliorate pre-existing GvHD and/or 'rescue' mice already showing symptoms of GvHD. As captured by the bioluminescence signaling, the homing pattern of the ex vivo expanded CB Treg in our xenogeneic model showed that without constant engagement and stimulation by an allogeneic source, the CB Tregs did not persist beyond day 3. This provides an insight into how the ex vivo expanded CB Treg distribute in vivo and provides a platform with which to study the impact of possible manipulations of these therapeutic cells. Establishing a xenogenic murine GvHD model in our laboratory has enabled us to specifically examine the impact of ex vivo expanded CB Treg alone as GvHD prophylaxis and to study the possible mediators of suppressive effects in vivo.

Brunstein et al.,[31] have recently demonstrated the safety and clinical efficacy of 3rd party CB Treg administered after the primary CB transplant. We currently propose a phase I trial of the prophylactic infusion of 3rd party CB Treg to prevent GvHD in a double cord blood transplantation setting. It is proposed to begin at a starting dose level of 1×10^6 ex vivo expanded Treg/kg. For safety purposes, we will initially match the CB Treg at a minimum of HLA 4/6 loci. Our data suggest that the generation of CB Treg using the ex vivo expansion strategy has the potential to provide a readily available, clinically-relevant, "off-the-shelf", cellular therapy to prevent GvHD and improve transplant outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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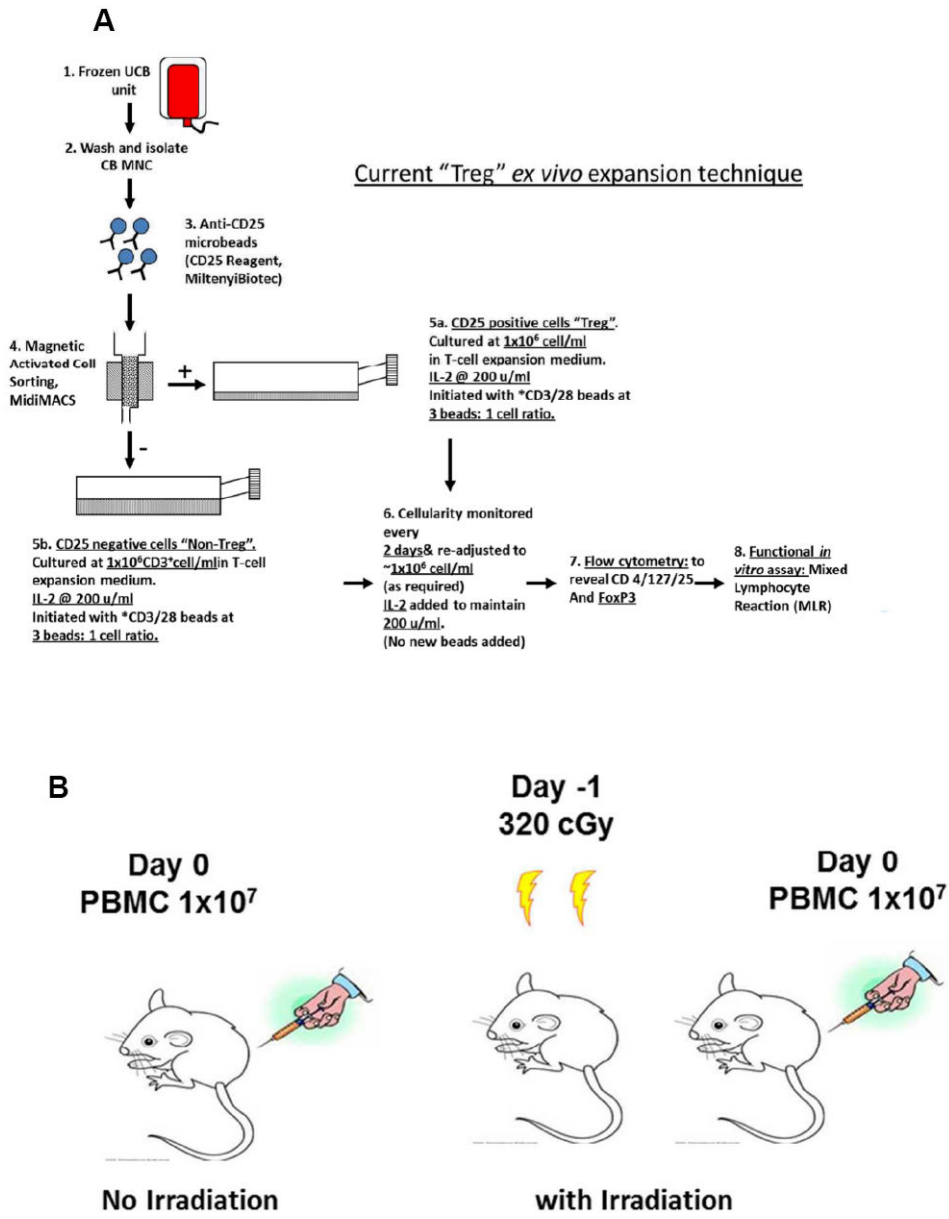

FIGURE 1.

CB Treg Expansion and Xenogenic GvHD model. A, CD25 selection. Method of enrichment of $CD25^{+}$ CB Tregs using MACS and ex vivo CB Treg expansion using IL-2 and CD3/28 beads. Treg ($CD25^{+}$) and Tcon ($CD25^{neg}$) prepared identically. B, Xenogenic GvHD model with/without sublethal irradiation (320 cGy) in NSG mice followed with/without injection of 1×10^{7} Treg/mouse prior to injection of 1×10^{7} PBMC/mouse.

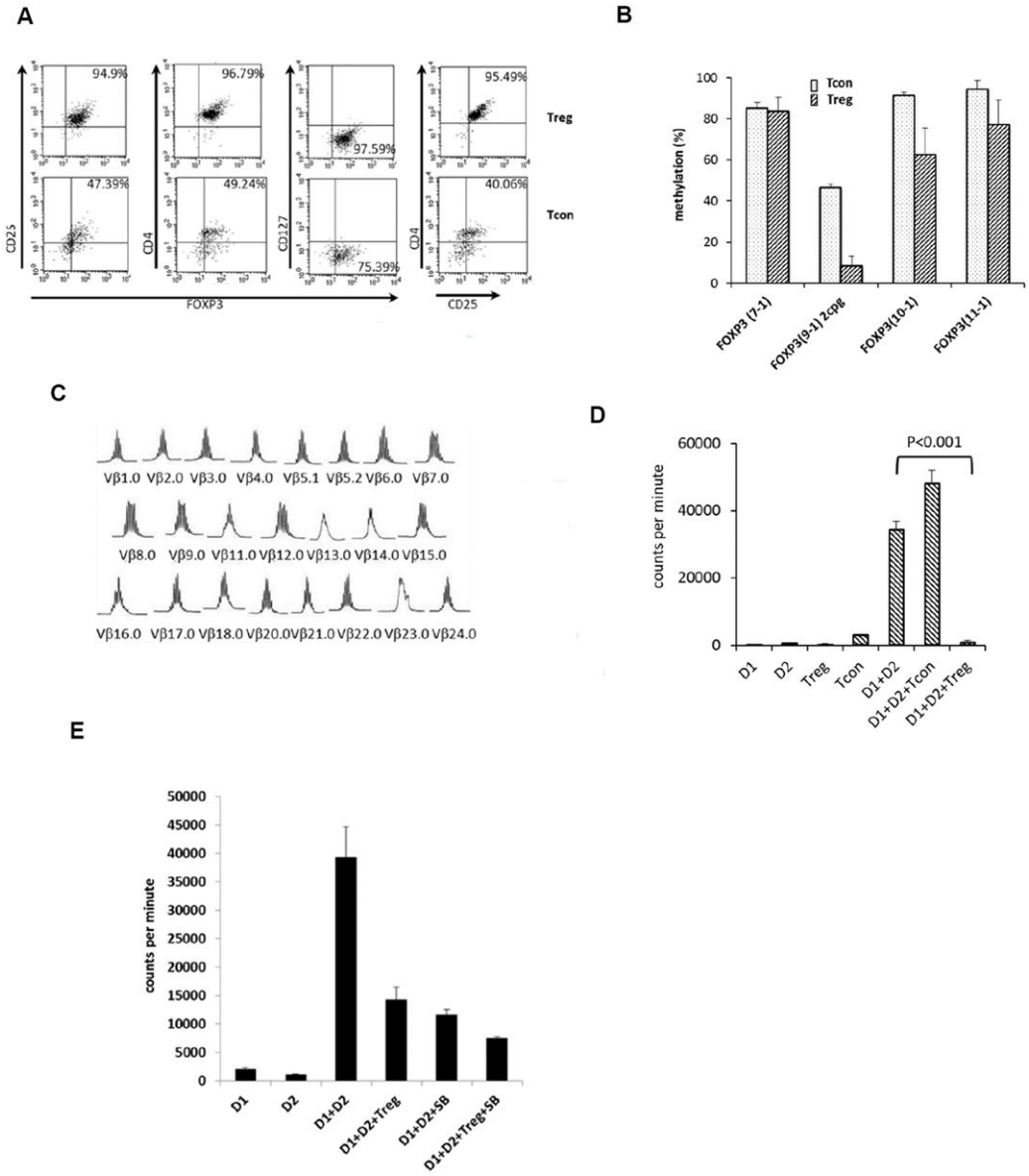

FIGURE 2.

Expanded CB Tregs exert suppressive function. A, Treg phenotype. Ex vivo expanded CB cells after 14 days of culture confirmed as $CD4^{+}25^{+}FOXP3^{+}127^{lo}$. B, Expanded Tregs show FOXP3 demethylation. Bisulfite sequencing in ex vivo expanded UCB-derived-Treg showed significant DNA demethylation of the FOXP3 gene locus at amplicons 7, 9, 10 and 11 [11] when compared to the Tcon. X axis denotes the CpG island locus and Y axis denotes percent methylation. (n=10). C. Distribution of TCR Repertoire is preserved in ex vivo expanded Tregs: TCR repertoire remains preserved post-expansion. Representative TCR repertoire distribution at day 14 of ex vivo expansion is shown. D, Ex vivo expanded CB-derived-Tregs significantly suppresses immune response in allogeneic mixed lymphocyte reaction. PBMCs from two donors, were cultured together to generate MLR (D1+D2). Addition of Treg to the donor mixture (D1+D2) at a ratio of 1:1, significantly suppressed MLR. Y-axis denotes counts per minute (Mean±SEM, n=.10). E, Additive effect of

p38MAPK inhibition. Pharmacologic inhibition of p38 MAP kinase (SB203580, 1uM) led to additive effect on CB Treg mediated MLR suppression (Mean±SEM, n= 2)

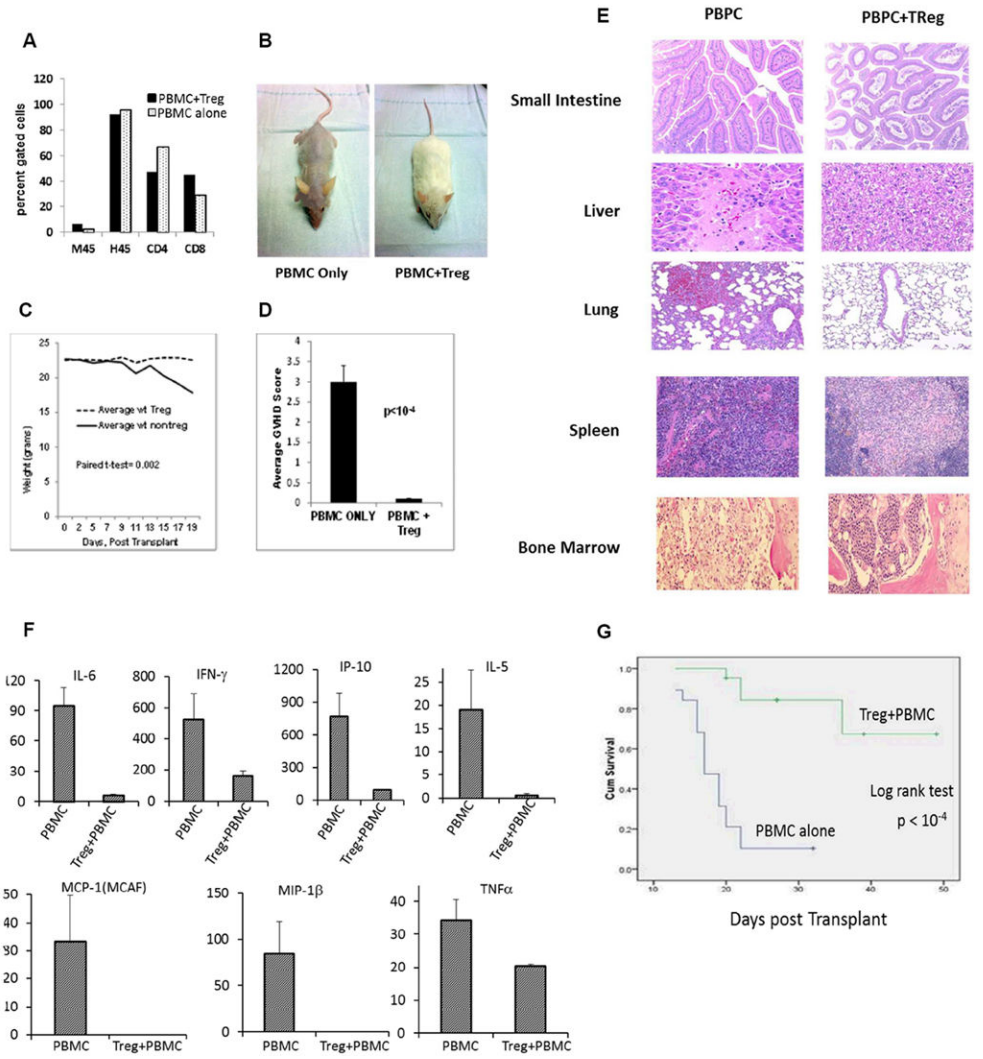

FIGURE 3.

Treg infusion prevents xenogeneic GVHD. A, Circulating Human lymphocytes. No effect of addition of CB Treg on day -1 was demonstrated on the circulating human lymphocytes in the PB of xenogenic mouse model. B, Condition of coat. While PBMC recipients (left) show extensive loss of hair and skin erythema, recipients of Treg and PBMC (right) show an intact coat of fur. C, Weight loss. Significantly greater weight loss was detected in the xenogeneic GVHD model in PBMC recipients after as little as 14 days post-transplant when compared to recipients of Treg and PBMC (P=0.002; t-test; n=20 mice in each arm). D, GVHD score. Recipients of prophylactic Tregs (10^7) demonstrated consistently lower GVHD scores based on the Ferrara GVHD scale (P<0.001; T-test). An assessment of GVHD scoring was performed every 48-72 hours. (n=20 mice/gp). E, Histopathologic analysis. Formalin-fixed tissues were embedded in paraffin and sections stained with H&E. Microscopic sections of lung from recipients of PBMC alone (left panel) show areas of evidence of GvHD in form of apoptotic bodies, lymphocytic infiltration and loss of architecture in small intestine, liver and lung. Bone marrow aplasia and lymphocytic infiltration into the spleen was present. In contrast, microscopic evaluation of small

intestine, liver and lung tissue from recipients of Treg and PBMC revealed preserved tissue histology. Preserved lymphoid follicles in spleen and hematopoiesis in bone marrow was observed. F, Tregs suppress levels of inflammatory cytokines in the xenogeneic GVHD mouse model. Measurements of circulating serum cytokines at day 14 showed a consistent decrease in the pro-inflammatory cytokine levels including IL-6, IFN- γ, IP-10, IL-5, MIP-1β, TNF α in the Treg and PBMC recipients as compared to the PBMC only recipients (n=5 mice/group; mean ± SEM.). The unit of y-axis is pg/ml. G, Overall survival. At a median follow up of 30 days after PBMC infusion, the overall survival of PBMC recipients that also received Treg was >80% as compared to <20% for mice receiving PBMC alone ($P<0.001$; log rank test; n=22 mice/group).

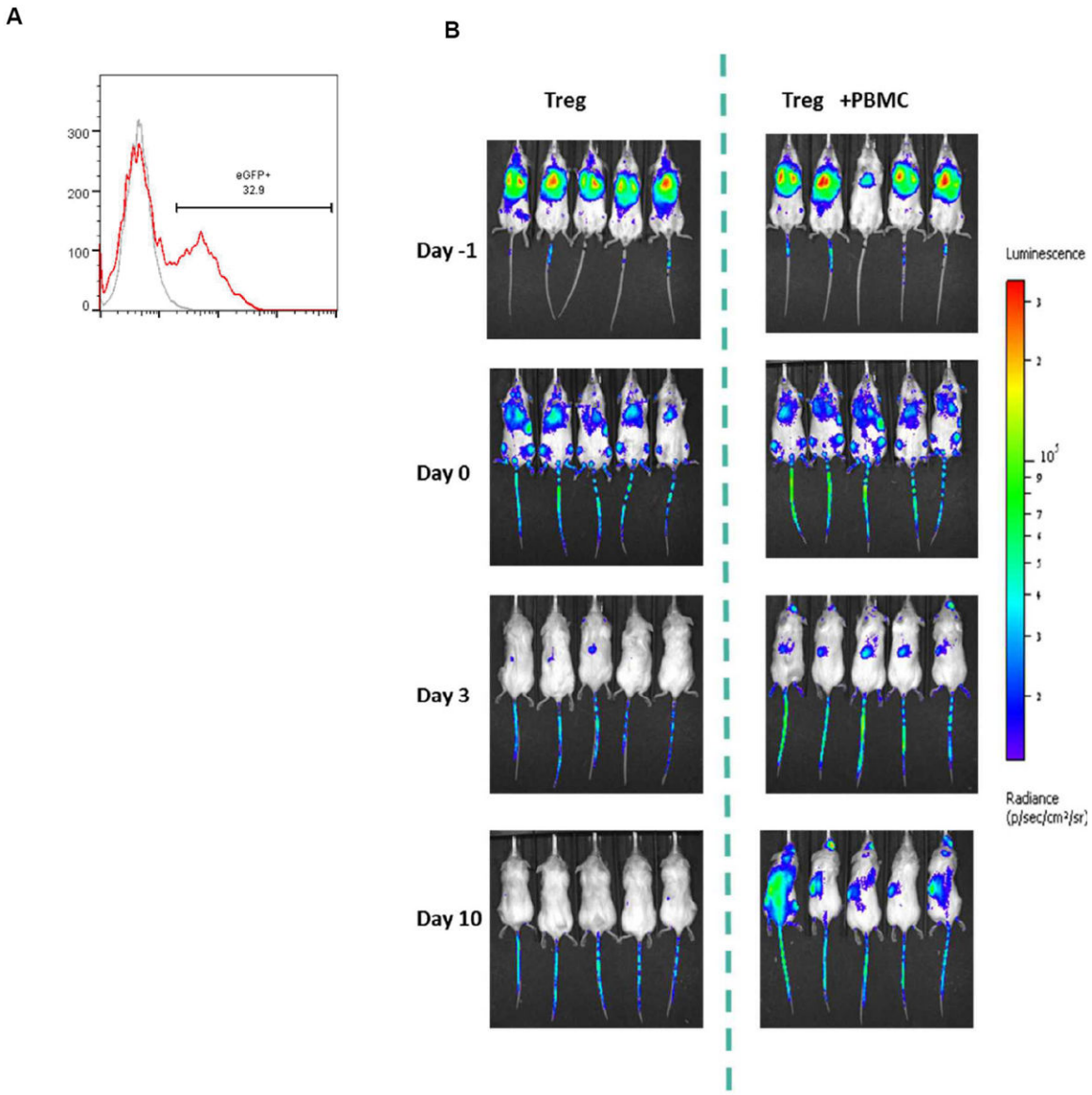

FIGURE 4.

Non-Invasive Bioluminescence. A, Treg transfection efficiency. The left panel showed negative control (non- transfected cell), the right panel showed the population of eGFP positive cell was 33% of gated lymphocyte population. B. In vivo distribution of CB Tregs. Firefly luciferase labeled CB Treg become undetectable by day 10, whereas in presence of PBMC, the CB Treg continue to proliferate and their distribution pattern is consistent with the GvHD target organs.