Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4-1BB expressed on artificial antigen-presenting cells

Keli L. Hippen,[1] Paul Harker-Murray,[1] Stephen B. Porter,[1] Sarah C. Merkel,[1] Aryel Londer,[1] Dawn K. Taylor,[1] Megan Bina,[1] Angela Panoskaltsis-Mortari,[1] Pablo Rubinstein,[2] Nico Van Rooijen,[3] Tatiana N. Golovina,[4] Megan M. Suhoski,[4] Jeffrey S. Miller,[5] John E. Wagner,[1] Carl H. June,[4] James L. Riley,[4] and Bruce R. Blazar[1]

[1]University of Minnesota Cancer Center and Department of Pediatrics, Division of Bone, Blood and Marrow Transplantation, Minneapolis; [2]National Cord Blood Program, The New York Blood Center, New York; [3]Department of Molecular Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands; [4]Abramson Family Cancer Center Research Institute, University of Pennsylvania Cancer Center, Philadelphia; and [5]University of Minnesota Cancer Center and Department of Medicine, Division of Bone Marrow Transplantation, Minneapolis

Previously, we showed that human umbilical cord blood (UCB) regulatory T cells (Tregs) could be expanded approximately 100-fold using anti-CD3/28 monoclonal antibody (mAb)–coated beads to provide T-cell receptor and costimulatory signals. Because Treg numbers from a single UCB unit are limited, we explored the use of cell-based artificial antigen-presenting cells (aAPCs) preloaded with anti-CD3/28 mAbs to achieve higher levels of Treg expansion. Compared with beads, aAPCs had similar expansion properties while significantly increasing transforming growth factor β (TGF-β) secretion and the potency of Treg suppressor function. aAPCs modified to coexpress OX40L or 4-1BBL expanded UCB Tregs to a significantly greater extent than bead- or non-modified aAPC cultures, reaching mean expansion levels exceeding 1250-fold. Despite the high expansion and in contrast to studies using other Treg sources, neither OX40 nor 4-1BB signaling of UCB Tregs reduced in vitro suppression. UCB Tregs expanded with 4-1BBL expressing aAPCs had decreased levels of proapoptotic *bim*. UCB Tregs expanded with non-modified or modified aAPCs versus beads resulted in higher survival associated with increased Treg persistence in a xenogeneic graft-versus-host disease lethality model. These data offer a novel approach for UCB Treg expansion using aAPCs, including those coexpressing OX40L or 4-1BBL. (Blood. 2008;112:2847-2857)

Introduction

Acute graft-versus-host disease (GVHD) is a significant cause of morbidity and mortality after allogeneic bone marrow transplantation (BMT) and occurs as a result of the activation, proliferation, and effector cell function of donor T cells that, along with the release of proinflammatory cytokines, result in tissue damage.[1] Recently, a subset of CD4$^+$ T cells with potent suppressor activity, CD4$^+$25$^+$ regulatory T cells (Tregs),[2] have been shown to inhibit alloreactive T-cell activation and effector cell function[3,4] and GVHD lethality in murine models.[5-7] Thus, Tregs are attractive therapeutic tools for preventing GVHD in humans.[8-11] When administered at the time of BMT, typically Tregs must be given at approximately the same number of donor T cells (1:1 ratio) to be highly effective at preventing GVHD.[5-7] This poses a technical problem since Tregs are present in low frequency in the peripheral blood ($\sim$ 1% are CD4$^+$CD25^{br} cells)[12] and contaminating CD4$^+$25$^-$ T cells out-compete Tregs for expansion and result in loss of Treg suppressor cell function.[11,13] Therefore, many laboratories have been dedicated to developing new approaches for the isolation and expansion of human Tregs from adult peripheral blood without loss of suppressor cell function.

Tregs express multiple tumor necrosis factor receptor (TNFR) family members on their cell surface, including glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40 (CD134), and 4-1BB (CD137).[14,15] While it is generally accepted that TNFR expressed on naive T cell functions contributes to T-cell survival (reviewed in Watts[16]), the exact role of TNFR signaling in Treg generation, expansion, and suppression is unclear. Shimizu and coworkers have shown that stimulation of GITR abrogates Treg suppressor function,[17] although in other studies, McHugh et al showed that loss of suppression depends on whether or not TCR triggering and IL-2 are present before GITR engagement.[15] We have shown that blockade of CD40L signaling on Tregs increases their suppressive function and tolerogenic capacity.[18-20]

Since signaling via CD40L and GITR can reduce suppressor cell function, neither would seem advantageous for human Treg expansion cultures, so we focused on OX40 and 4-1BB. While OX40 signals do not appear to increase in vitro anti-CD3 mAb/APC–driven Treg proliferation,[21-23] a recent report indicates that OX40 signals can increase endogenous Treg survival in vivo,[24] although other studies indicated that OX40 signals provided during ex vivo Treg expansion inhibited rodent Treg suppressor function and foxP3 expression.[21,22,24] In humans, OX40L-mediated signaling has been reported to inhibit the generation of IL-10–producing T-regulatory type 1 cells from naive and memory CD4$^+$ T cells and to shut down both IL-10 production and suppressor cell function.[25] 4-1BB also is expressed on resting Tregs and up-regulated within 2 days after in vitro anti-CD3 mAb plus IL-2 stimulation.[26] In vitro anti-CD3 mAb or antigen-induced rodent Treg proliferation and in

vivo Treg survival was increased via 4-1BB signaling without altering suppressor cell function.[26,27] In other studies, agonistic anti–4-1BB mAb infusion lead to an increase in splenic $CD4^+25^+$ T cells and a reduction in chemical-induced colitis.[28]

Because rodent data indicated that OX40 and 4-1BB signaling of Tregs may be advantageous for increasing Treg expansion or survival, studies were performed using UCB Tregs and a cell-based universal artificial antigen-presenting cell (aAPC) system to provide OX40 OR 4-1BB signals for Treg expansion and survival. K562 erythromyeloid leukemia cells were engineered to stably express CD32 (low-affinity FcγRII) and the human costimulatory ligands OX40L or 4-1BBL.[29,30] By adding anti-CD3/28 mAbs that bind to CD32 expressed on aAPCs, TCR, CD28 and TNFR costimulatory signals were provided to freshly isolated UCB Tregs. Using this approach, we now report that UCB Treg expansion and survival is significantly augmented by provision of OX40 or 4-1BB signals without adversely affecting in vitro or in vivo Treg-mediated suppression. In contrast to typical Treg expansion protocols using non–cell sorter isolated adult Tregs, rapamycin is not needed to maintain a high level of suppression even during prolonged ex vivo expansion cultures. These studies suggest a novel approach to expand UCB Tregs for clinical trials and indicate that neither OX40 nor 4-1BB signals provided during the early Treg expansion phase reduce UCB Treg suppressor cell function.

Methods

Treg isolation and culture

Tregs were purified from frozen UCB units (National Placental Blood Program, New York Blood Center) by positive selection using directly conjugated anti-CD25 magnetic microbeads and either manual columns as previously reported[31] or with AutoMACS or CliniMACS (Posseld2 program). $CD4CD25^+$ cells (average 64% $CD4^+$) were cultured with anti-CD3/CD28 mAb-coated Dynabeads[32] at a 3:1 bead-total cell ratio or with K562 cell lines lentiviral transduced with CD32 alone (KT32) or coexpressing 4.1BBL (KT32/4.1BBL) or OX40L (KT32/OX40L), at a Treg to KT cell ratio of 2:1.[30] KT lines were irradiated with 10 000 cGy and incubated with anti-CD3 (OKT3) and anti-CD28 (CD28.2; both from BD Pharmingen, San Diego, CA) at 1 μg/mL for 10 minutes. $CD25^+$ cells were cultured in X-Vivo-15 (BioWhittaker, Walkersville, MD) or RPMI 1640 (Invitrogen, Carlsbad, CA) media supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA), L-glutamine (Invitrogen), and N-acetylcysteine (American Regent, Shirley, NY). Recombinant IL-2 (300 IU/mL; Chiron, Emeryville, CA) was added on day 3 and maintained for culture duration. Where indicated, rapamycin (Rapammune; Wyeth-Ayerst, Princeton, NJ) at 109 nM was added on day 0 and with refeeding. Cells were cultured for 18 to 21 days and split every 2 to 3 days.

Flow cytometry and antibodies

All human-specific antibodies used for flow cytometry were purchased from BD Pharmingen except Foxp3 (eBioscience, San Diego, CA). Acquisition was performed using a FACScalibur or LSRII (BD Bioscience) and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Suppression assays

Mixed lymphocyte reaction (MLR)–based suppression assays were carried out using responder T cells ($CD4^+CD25^-$) and in vitro matured CD14 plus MoDCs purified from adult peripheral blood as previously reported.[30] Suppression was assessed with serial dilutions of Tregs from 1:1 to 1:64. Wells were pulsed on days 4 to 6 with ^3H-thymidine (1 μCi; 3.7×10^4 becquerels) for 16 to 18 hours. Each point had 6 replicates. Data were collected with a direct beta counter (no liquid scintillation).

The proliferative capacity of expanded Tregs was also tested using a 5-carboxyfluorescein diacetate succinimide ester (CFSE) inhibition assay. Tregs ($8\text{-}10 \times 10^6$) were CFSE labeled using a kit as per the manufacturer's instructions (Invitrogen). For peripheral blood mononuclear cell (PBMNC) stimulation, anti-CD3 mAb–coated beads (Dynal, Oslo, Norway) were added at a bead-to-cell ratio of 1:1. Unstained PBMNCs were plated at 10^5 per well in 96-well U-bottom plates with graded titrations of stained, cultured Tregs (1:2 to 1:32 Treg/PBMNCs) in 200 μL culture media. On day 4, cells were stained with antibodies to CD4, CD8, and/or HLA-A2. Acquired data were analyzed using the proliferation platform in FlowJo. Relative number of cells per well was determined using PKH-labeled reference beads (Sigma-Aldrich, St Louis, MO).

Analysis of cytokine secretion

Supernatants from 3 independent UCB Treg cultures were sampled on day 6 and day 17 and cytokine concentrations determined fluorimetrically using Luminex beads (IL-4, IL-5, IL-10, and IFN-γ) or by enzyme-linked immunosorbent assay (ELISA; TGF-β).

Anti- and proapoptotic gene expression analysis

RNA was extracted from cell pellets for 5 independent UCB Treg cultures (75%–90% $CD127^-$, $Foxp3^+$) using RNeasy Mini Kit with on-column DNase digestion (Qiagen, Valencia, CA). cDNA synthesis was performed as described in the Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Bcl-2, bcl-xL, and bim expression was analyzed on an Applied Biosystems 7500 Real-Time PCR System using Taqman Universal PCR Master Mix #4304437 and Assay on Demand primer/probe kits (Applied Biosystems, Foster City, CA).

Xenogeneic GVHD model

A published xenogeneic GVHD model was used.[33] Briefly, T-, B- and NK-deficient C57BL/6 $Rag^{-/-}$, $\gamma_c^{-/-}$ mice (Taconic Farms, Germantown, NY), housed in a pathogen-free facility in micro-isolator cages, were depleted of macrophages by injection of clodronate-containing liposomes at 100 μL/10 g body weight[34] on day −1. On day 0, mice were irradiated with 400 cGy (137 cesium source). Human PBMNCs (30×10^6) were injected with or without expanded Tregs (30×10^6). Mice were assessed for signs of GVHD daily, weighed thrice weekly, and evaluated for human cells in blood by flow cytometry weekly until day 80 or until they were killed. Before the animals being killed or at the conclusion of the experiment, hematocrits were determined in heparinized capillary tubes as percentage packed red blood cell volume. All animal protocols were approved by IACUC at the University of Minnesota.

Histologic and immunohistochemistry assessment

Histopathology was performed on tissues (liver, lung, small intestine, colon, skin, spleen, and bone marrow) from all mice to evaluate GVHD. Cryosections (6 mm) were acetone-fixed and stained by hematoxylin and eosin. Coded tissues were assessed for GVHD using a 0 to 4+ scale.[35] After fixation, cryosections were immunoperoxidase-stained using biotinylated mAbs to CD4 and CD8 (Ancell Immunology Research Products, Bayport, MN) or mAbs to HLA-A2 (BD Pharmingen) followed by biotinylated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Detection of biotinylated antibody was accomplished with streptavidin-HRPO from an anti-Ig HRP detection kit (BD Biosciences).

Statistical analysis

Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox summary of chi-square. Other data were analyzed by analysis of variance (ANOVA) or Student t test. Probability (P) values less than or equal to .05 were considered statistically significant.

Figure 1. Treg lines expanded with cell-based aAPCs have equivalent purity and expansion, with increased suppressive function. (A) Representative example (i) and summary (ii) of the CD4, CD25 versus Foxp3 (CD4-gated), and CD127 versus Foxp3 (CD4-gated) profiles for purified cord blood cells expanded 21 days in vitro with bead- or cell-based aAPCs. (B) Representative example (i) and summary (ii) of in vitro expansion of total cells or Tregs (CD4$^+$, CD127$^-$, Foxp3$^+$). Representative example (C) and summary (D) demonstrating that cord blood Tregs expanded with aAPCs potently suppress an in vitro MLR assay (average values for Treg-to-Tresponder ratio of 1:4), and cell-based aAPCs are even more effective than bead-based aAPCs. For each summary, the data are presented as the mean plus or minus the standard error of the mean (SEM), with n = 7. *$P \leq .05$.
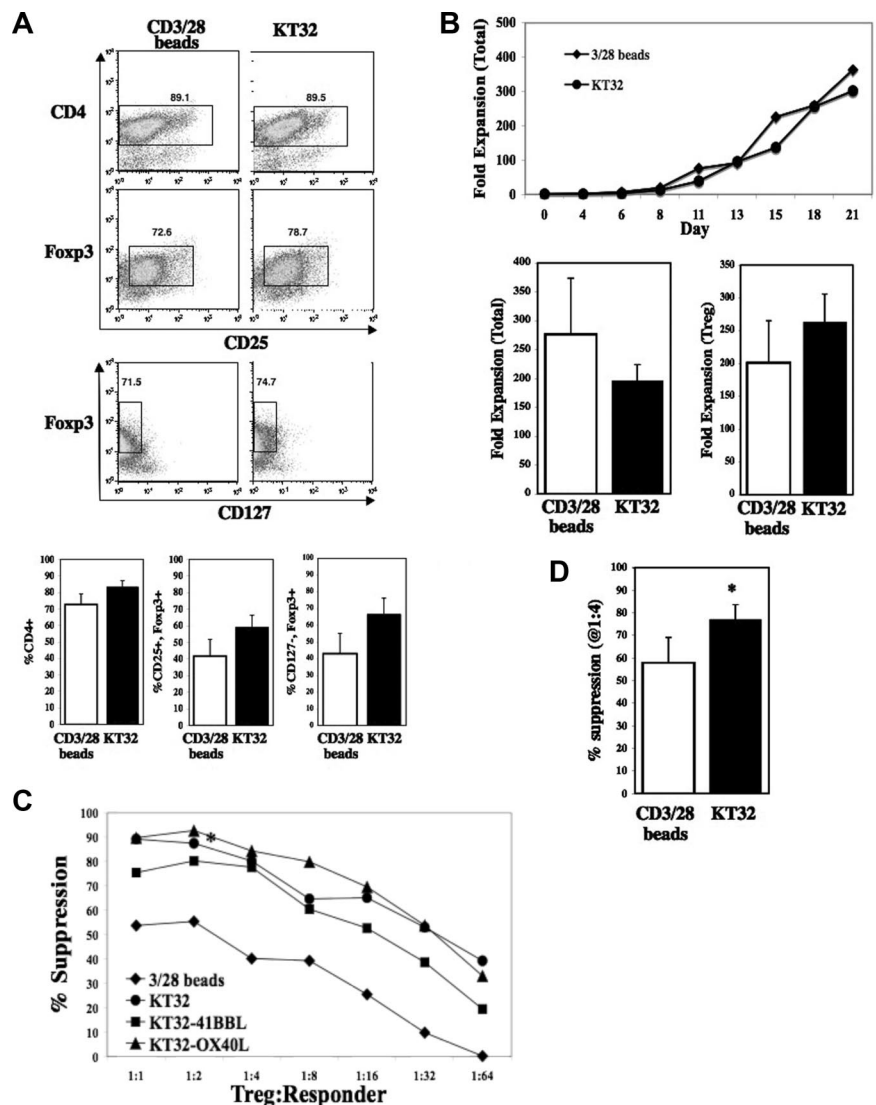
Results

Increased Treg suppressor cell function of UCB Tregs in KT32 aAPCs versus bead-based expansion cultures

Previously, we have demonstrated an approximately 100-fold expansion of UCB-derived Tregs using anti-CD3/28 mAb–coated beads and high-dose IL-2 after 3 weeks of culture.[31] Since cell-based aAPCs have been shown to be superior to a bead-based system for polyclonal CD4$^+$ T-cell expansion, we compared the effects of anti-CD3/28 mAb–coated beads to KT32 cells loaded with anti-CD3/28 mAbs.[29,36] To more closely mimic likely clinical trial protocols, cryopreserved UCB units were used as a source of Tregs, in contrast to our prior studies using fresh UCB units.[31] Whereas only 12% of UCB cells were CD4$^+$ before the bead- and column-based isolation procedure, the mean column-purified T-cell product placed into culture was 70% CD4$^+$, of which a mean of 68% were CD25$^+$FoxP3$^+$ or CD127$^-$Foxp3$^+$ and phenotypically classified as Tregs (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article; Figure 1A). CD8- and CD19-expressing cells constituted, on average, less than 3% and 1% of the initial Treg prep, respectively,

along with less than 20% neutrophils. After 18 to 21 days of culture using anti-CD3/28 beads or KT32 cells with anti-CD3/28 mAbs, cells were phenotyped and fold expansion quantified. Expansion with KT32 cells or beads in a total of 7 experiments resulted in a higher mean percent CD4$^+$ cells (83% ± 4% vs 73% ± 7%, $P = .008$; Figure 1A), with the remaining cells principally being CD8$^+$CD4$^-$ (data not shown). KT32 versus bead-expanded cultures had a higher percentage of Tregs as indicated by CD25$^+$, Foxp3$^+$, or CD127$^-$, Foxp3$^+$ (59% ± 8% versus 42% ± 10%, and 67% ± 9% versus 43% ± 12%, respectively). Bead-based versus KT32 cultures had a higher overall fold T-cell expansion (276 ± 97 vs 197 ± 27, respectively) but modestly lower overall UCB Treg (CD4$^+$, CD127$^-$, Foxp3$^+$) expansion (278- ± 50- vs 199- ± 59-fold, respectively; Figure 1B). Consistent with the higher Treg content, Tregs generated from KT32 versus bead-based cultures and added at T-responder cells (1:4 ratio) in an MLR culture resulted in a significantly higher average suppression index (77% ± 6% vs 58% ± 11%, $P = .01$; Figure 1C). A ratio of 1:4 (Treg/Tresp) was chosen for this analysis because it was uniformly illustrative, but it should be noted that 4 of 6 Treg cultures expanded with aAPCs had at least 50% suppression at ratios of 1:16 or lower. Compared with bead-based cultures, we conclude
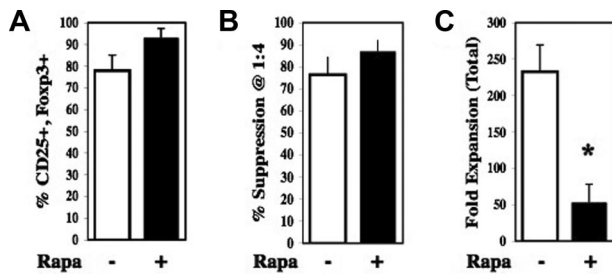
Figure 2. Rapamycin inhibits, rather than aids, the in vitro expansion of UCB Tregs. Data from a series of 3 experiments showing that rapamycin does not significantly increase the purity (A) or suppressive function (B) of UCB Treg cultures. (C) Rapamycin significantly inhibits the expansion of UCB Treg cultures ($P \leq .05$).

that KT32 aAPC cultures favor the expansion of UCB Tregs with suppressor cell function.

The addition of the mTOR inhibitor rapamycin to bead-based UCB Treg cultures lowers Treg expansion without increasing suppression potency

Studies have demonstrated that the addition of rapamycin to expansion cultures preferentially promoted the outgrowth of functional CD4+CD25+Foxp3+ Tregs at the expense of CD4+CD25− T-effector cells in both murine and human CD4+ T-cell cultures.[37-40] Rapamycin did not significantly increase the mean percentage of CD25+Foxp3+ Tregs (Figure 2A) or the degree of Treg suppression (Figure 2B). However, Treg expansion (Figure 2C) was significantly reduced by rapamycin. Similar data were obtained using Tregs isolated from fresh UCB units (n = 3 experiments; data not shown). We conclude that adding rapamycin to bead-based UCB Treg cultures was not advantageous under these conditions in increasing the overall number of functional Tregs present after expansion.

KT32 aAPCs coexpressing 4-1BBL or OX40L provided superior UCB Treg expansion compared with KT32 aAPCs alone or bead-based cultures

Since the KT32 cell-based versus bead-based aAPCs preferentially expanded highly suppressive Tregs and addition of rapamycin to bead-based cultures did not augment UCB Treg suppression, we focused on improving the aAPC system to permit better Treg expansion without loss of suppression using aAPCs that coexpressed OX40L or 4-1BBL. In 6 experiments, KT32/4.1BBL and KT32/OX40L aAPCs supported the preferential outgrowth of UCB Tregs with a CD25+FoxP3+ and CD127−FoxP3+ phenotype (Figure 3A). Total cell expansion was significantly higher with KT32/4.1BBL and KT32/OX40L compared with beads (2.9- and 3.4-fold, respectively) or KT32 cells alone (4.5- and 5.3-fold, respectively; Figure 3B). More importantly, KT32/4–1BBL or KT32/OX40L versus KT32 cells resulted in a significant increase in the expansion of Tregs characterized as CD4+25brFoxP3+ (1268- ± 317- and 1007- ± 102- vs 316- ± 30-fold, respectively; $P < .03$ for each) or CD4+127−Foxp3+ (1340- ± 337- and 1133- ± 82- vs 342- ± 34-fold, respectively; $P < .04$ for each). Compared with bead-based expansion cultures, UCB Tregs expanded with the 3 different KT32 lines provided greater suppression ($P \leq .04$ for each comparison), which was comparable in magnitude for all 3 KT32 lines, as expected based on Foxp3 staining (Figure 3C).

Because UCB Tregs expanded with KT32/4.1BBL were more suppressive on a per-cell basis, we assayed the UCB Treg cultures at days 6 and 17 for the secretion of cytokines (including IL-4, -5, -10, IFN-γ, and TGF-β). IL-4 and IL-10 were present at very low or undetectable levels whereas similar amounts of IL-5 were produced by all cultures (data not shown). Whereas TGF-β secretion was similar at day 6, KT32/4.1BBL-stimulated Treg cultures had remarkably higher levels of TGF-β by day 17 (Figure 3D). It is also interesting to note that KT32/4.1BBL-stimulated UCB Tregs secreted very high levels of IFN-γ on day 6, but returned to near background levels by day 17 (Figure 3E). Similar results were found for UCB Treg cultures expanded with KT32 and KT32/OX40L (data not shown).

Comparable responder frequency and proliferative capacity with increased survival of aAPC- versus bead-expanded Tregs assessed by an in vitro allogeneic suppression assay

Since UCB Treg suppression potency was increased by the use of aAPC- versus bead-based expansion cultures, we sought to analyze UCB Treg proliferation and accumulation during a suppression assay. This type of analysis is incompatible with our standard MLR assay, so we used a suppression assay in which Tregs suppress anti-CD3 bead-stimulated allogeneic PBMNCs. However, instead of labeling PBMNCs, UCB Tregs were CFSE-labeled to permit analysis of Treg responder cell frequency, proliferative capacity, and Treg recovery at the end of the assay. Tregs were added with or without unlabeled allogeneic PBMNCs (ratios 1:2-1:16). While anti-CD3 mAb–coated beads alone do not induce Treg proliferation (1:0 ratio), Tregs did proliferate in response to anti-CD3 mAb–coated beads plus allogeneic PBMNCs (Figure 4A). In all instances, UCB Treg proliferation progressively increased as Treg-to-PBMNC ratios decreased. Importantly, the total number of UCB Tregs at the end of the 4-day assay was significantly greater using UCB Tregs from aAPC- versus bead-based cultures at higher (eg, 1:2) ratios that are typically optimally suppressive (Figure 4B). However, neither the proliferative capacity (total cells produced) nor the frequency of cells capable of responding to the mitogenic signals differed between groups (data not shown).

KT32/4.1BBL aAPC-expanded Tregs have decreased Bim expression

Differential survival could also explain the increased Treg cell numbers in the in vitro assay. To test for a molecular mechanism that would confer a survival advantage on UCB Tregs expanded with aAPCs, we isolated RNA from UCB Treg cultures expanded with either anti-CD3/28 beads or KT32/4.1BBL and tested for the expression of genes related to apoptosis.

Although acute 4.1BB stimulation of T cells is known to increase the expression of antiapoptotic bcl-2 members, we found no significant increase in bcl-xL, and even a slight decrease in bcl-2 expression over anti-CD3/28 bead controls (Figure 4C). However, analysis of BH3 proapoptotic genes revealed that expression of Bim was reduced approximately 2-fold in KT32/4.1BBL versus anti-CD3/28 bead-expanded cultures (Figure 4C).

Increased in vivo persistence and suppression of xenogeneic GVHD-induced lethality by aAPC- versus bead-expanded UCB Tregs

To determine whether expanded Tregs were effective in suppressing human PBMNC-induced xenogeneic GVHD, UCB Tregs were
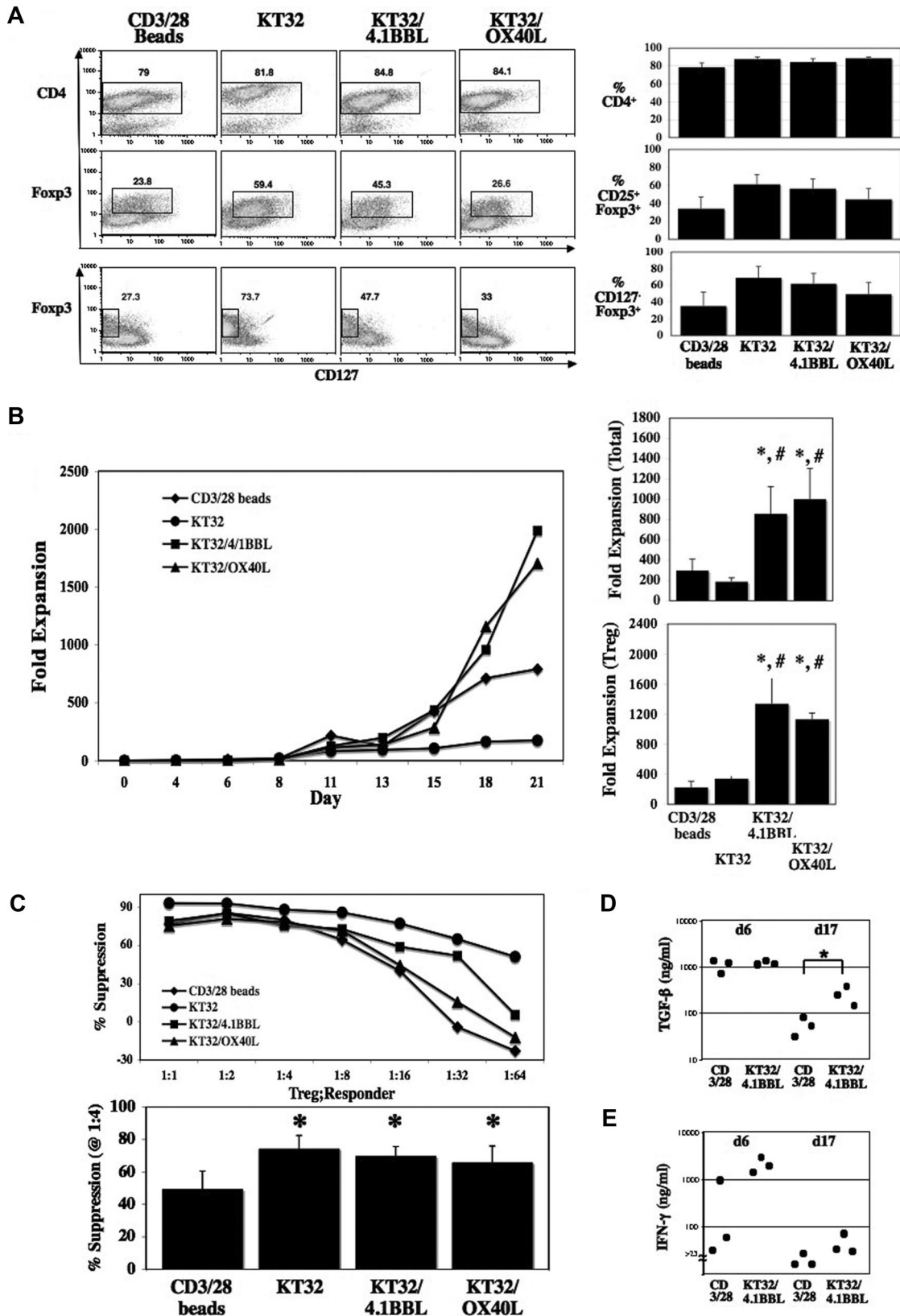
Figure 3. Treg lines expanded with cell-based aAPCs have equivalent purity and expansion, with increased suppressive function. (A) Representative example (i) and summary (ii) of the CD4, CD25 versus Foxp3 (CD4-gated), and CD127 versus Foxp3 (CD4-gated) profiles for purified cord blood cells expanded 21 days in vitro with bead- or cell-based aAPCs. (B) Representative example (i) and summary (ii) of in vitro Treg expansion demonstrating the increased effectiveness of 4-1BBL or OX40L costimulation. (C) Representative example (i) and summary (ii; average values for Treg-to-Tresponder ratio of 1:4) demonstrating cord blood Tregs expanded with aAPCs potently suppress an in vitro MLR assay, and cell-based aAPCs are even more effective than bead-based aAPCs. Tregs were cultured for 18 to 21 days, and each summary is presented as the mean plus or minus SEM, with n = 6. TGF-β (D) or IFN-γ (E) present in day 6 or 17 supernatants from UCB Treg cultures expanded with anti-CD3/28 beads or KT32/4.1BBL. Note that the limit of detection for IFN-γ was 25 ng/mL. *$P \leq .05$ for comparison with CD3/28 beads; #$P \leq .05$ for comparison with KT32.
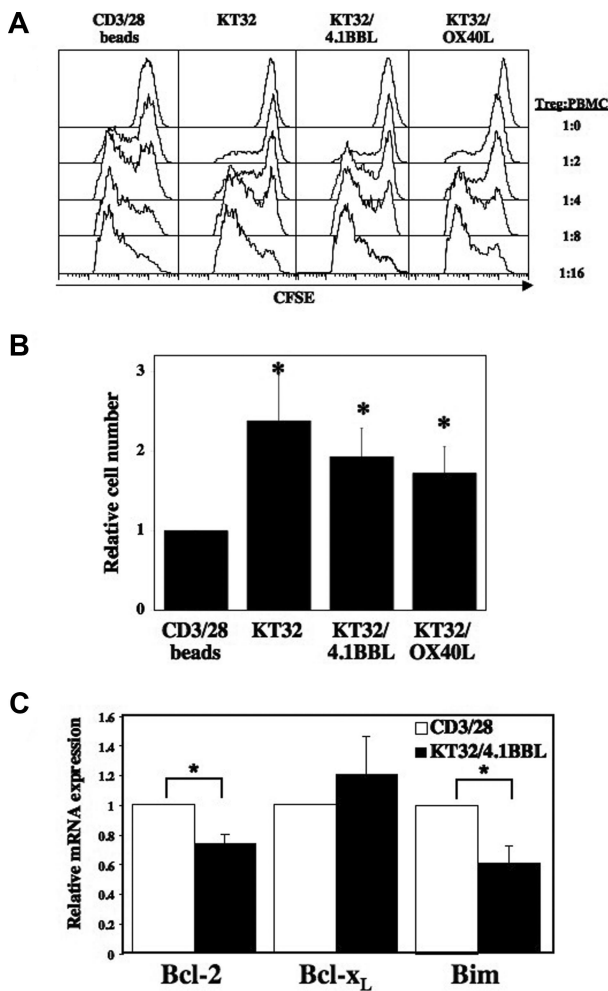
Figure 4. Tregs expanded with cell-based aAPCs have increased survival and accumulation in in vitro suppression assays. Cord blood Tregs were expanded in vitro using bead-or cell-based aAPCs with or without 4.1BBL or OX40L costimulation, then CFSE-labeled and incubated with allogeneic PBMNCs and anti-CD3 beads for 4 days and Treg cell division monitored by CFSE-dye dilution. (A) Representative example of CFSE dilution in Tregs during bead-based suppression assays. (B) Quantitation of Treg cell number (CD4$^+$, CFSE$^+$) on day 4 of suppression assay at Treg-to-PBMNC ratio of 1:2 as assessed by flow cytometry using counting beads and CFSE-dilution. (C) Pro- and antiapoptotic gene expression in UCB Tregs expanded with CD3/28 beads versus KT32/4.1BBL. Data are mean plus or minus SEM for 5 independent cultures. (E) Data in panel A is representative of a single experiment, while data in panels B and C represent the mean plus or minus SEM for 3 and 5 independent experiments, respectively. *$P <$ .05.

cultured with beads or aAPCs and then cotransferred at a 1:1 ratio with PBMNCs (30×10^6 each) into sublethally irradiated, macrophage-depleted, Rag$^{-/-}$, IL2R$\gamma_c^{-/-}$ recipients. UCB Treg cultures stimulated with KT32/4.1BBL or KT32/OX40L resulted in a higher frequency of Tregs (Figure 5A) and an overall T cell– (2.8- and 2.0-fold, respectively) and Treg-specific (4.8- and 3.4-fold, respectively) expansion (Figure 5B) compared with bead-based cultures. aAPC-expanded UCB Tregs were more suppressive in vitro than those generated using beads (Figure 5C). UCB Tregs from each expansion method given at a 1:1 ratio with PBMNCs significantly reduced GVHD-induced lethality versus PBMNC controls (survival rates for n = 8 or 9 per group: beads [44%]; OX40L or 4-1BBL [75%]; controls [0%]; $P <$ .002 Tregs vs PBMNC controls). Because survival rates even in all Treg groups were markedly superior to controls, in vivo superiority of cell-versus bead-based aAPCs was not demonstrated. However, only

KT32/4.1BBL or KT32/OX40L but not bead-expanded Tregs provided similar survival compared with the cohort receiving no PBMNCs (Figure 5), whereas the survival for mice receiving anti-CD3/28 bead-expanded Tregs was significantly lower than mice receiving no human PBMNCs ($P =$ .037). Body weights were consistent with survival data, and showed that KT32/4.1BBL- and KT32/OX40L-expanded Tregs were superior to CD3/28 bead-expanded Tregs in preventing GVHD-induced weight loss (days 8-73 for 4-1BBL; days 10-31 and 49-80 for OX40L; $P \leq$ .05; Figure 5E). By in vitro phenotypic and functional assays as well as in vivo mean weight curves and survival comparisons to saline-only controls, UCB Tregs expanded with cell-based aAPCs were superior to those expanded with beads.

A potential mechanism for the increased GVHD prevention efficacy of cell-based aAPC expansion is through enhanced in vivo UCB Treg persistence. Using HLA-A2 expression to distinguish HLA-A2$^+$ UCB Tregs from the HLA-A2$^-$ GVHD-causing PBMNCs, the relative peripheral blood Treg frequencies were monitored. In all groups, the highest Treg percentages were present at day 7 and then progressively declined through day 19 (Figure 6A). The percentage of Tregs at day 7 was approximately 4- and 6-fold higher after the infusion of UCB Tregs expanded with KT32/4.1BBL and KT32/OX40L cells, respectively, compared with anti-CD3/28 bead expansion. Whereas 10 of 16 (63%) recipients that had less than or equal to 16% day-7 circulating Tregs died during the 80-day observation period, 0 of 10 recipients with higher percent Tregs succumbed to GVHD (10/16 vs 0/10; $P =$ .001 by χ^2; Figure 6B). Notably, the majority of recipients of KT32/OX40L- or KT/4.1BBL-expanded Tregs had more than or equal to 16% circulating Tregs (8 of 10 for each group) and both groups had an 80% survival rate. In contrast, none of 10 recipients of bead-expanded Tregs had more than 10% Tregs and survival for this group was only 40%. These data indicate that TNFR-modified aAPCs versus bead-expanded Tregs resulted in a higher day-7 circulating percent Tregs and survival rate.

To determine the extent to which Treg treatment affected GVHD-induced injury, mice were assessed for the level of circulating PBMNCs and the extent of bone marrow (BM) and parenchymal organ injury. Analysis of blood revealed that moribund animals at the time of death, whether or not they received Tregs, had a higher human percent of CD4$^+$ T cells compared with surviving mice, ranging from 21% to 84% in 10 of 10 mice succumbing to GVHD, versus 0% to 14% in 16 of 16 mice that survived (Figure 6C). Similar data were found for human CD8$^+$ T cells in blood (data not shown). Moribund mice were anemic with hematocrit readings below 17% in 10 of 12 mice, in contrast to hematocrit values greater than 29% in 16 of 16 Treg-treated mice surviving to the end of the experiment, similar to animals not receiving PBMNCs (Figure 6D). Histologic examination of moribund animals revealed significant perivascular cuffing and infiltration into the parenchyma proper of the liver, perivascular/peribronchiolar cuffing in the lung, injury to mucosal crypts, abscesses, goblet cell destruction, and inflammatory cell infiltrates in the ileum (Figure 6E; data not shown). Significantly higher GVHD pathologic scores were noted in the liver, lung, and colon of mice receiving PBMNCs alone versus saline-treated controls at the time of death or elective killing (Figure 6F). Compared with PBMNC-only controls, Treg-treated recipients had significantly lower liver, lung, and ileum GVHD scores ($P <$ .05 for each group, and for each tissue). No significant differences were seen when comparing individual Treg groups to saline controls. Treg-treated mice were not GVHD-free, as evidenced by the significantly higher

Figure 5. In vitro–expanded cord blood Tregs decrease mortality in a xenogeneic model of GVHD. Tregs were purified from frozen cord blood and expanded with a single stimulation of bead- or cell-based aAPCs for 18 days, then were cotransferred with allogeneic PBMNCs into clodronate-treated, irradiated Rag2$^{-/-}$, $\gamma_c^{-/-}$ mice. (A-C) Day-18 flow phenotype showing percent CD4$^+$ and percent CD25$^+$Foxp3$^+$ (CD4-gated), fold expansion, and suppression index (respectively) of the cell lines used. (D) Kaplan-Meyer survival curve showing increased survival of clodronate-treated, irradiated Rag2$^{-/-}$, $\gamma_c^{-/-}$ mice receiving human PBMNCs with or without groups of Tregs in a 1:1 ratio (ie, 30×10^6 each). For groups PBMNCs, CD3/28 beads, KT32/4.1BBL, KT32/OX40L, and PBS, n = 8, 9, 8, 8, and 4, respectively. $P \le .05$ for each Treg-treated group compared with PBMNCs. (E) Average weight (% of initial) for mice surviving on given day for different groups of mice. *$P \le .05$ from days 8 to 73 for 4.1BBL and days 10 to 31 and 49 to 80 for OX40L. Data are representative of 4 experiments ameliorating XGVHD with Tregs.
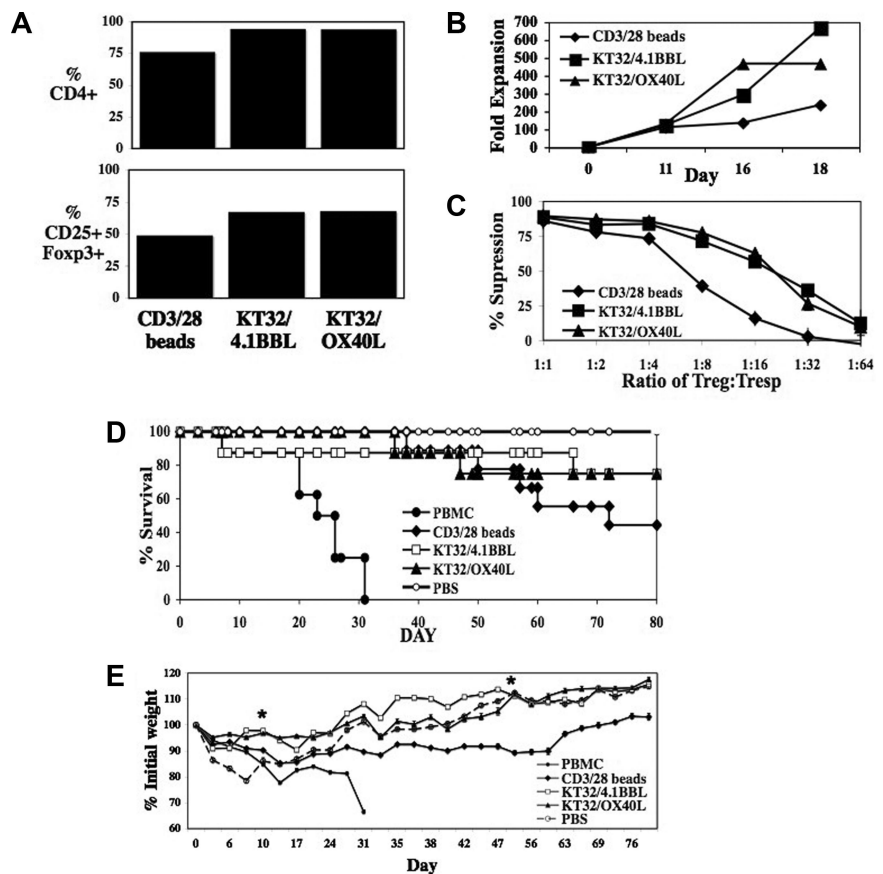
mean GVHD scores in liver and lung compared with saline-treated controls ($P \le .01$). Analysis of tissues for human CD4$^+$ and CD8$^+$ cells via immunohistochemistry confirmed the presence of human T cells in areas of tissue pathology (Figure 6G and data not shown). Although HLA-A2$^+$ Tregs were not found in blood after day 28, it is possible that long-term survival correlates with Treg persistence in GVHD target tissues. Therefore, tissues from representative recipients (moribund or at the end of the experiment) of aAPC-expanded UCB Tregs (n = 5) were stained with antibodies to HLA-A2 and CD4. No HLA-A2$^+$ cells were observed, even when serial sections documented the presence of human CD4$^+$ T cells (data not shown).

Since UCB Tregs obtained using TNF sub-family–modified aAPCs versus beads resulted in higher survival, additional studies sought to determine whether nonmodified KT32 cells also would be advantageous compared with beads. UCB Tregs were prepared and infused as described for Figure 6. On day 3, peripheral blood mean UCB percent Tregs greater than or equal to 16% in both Treg-treated groups (data not shown). Both UCB Treg sources completely prevented GVHD-related deaths, while only 2 of 6 animals from the PBMNC-only group survived through the day-38 evaluation period ($P \le .04$ vs PBMNCs for each Treg group). In a second experiment, the Treg-to-PBMNC ratio was lowered to 1:3 to accentuate differences between Treg groups. UCB Tregs expanded using KT32 cells versus beads resulted in a high proportion of CD25$^+$FoxP3$^+$ (72% vs 63%) and FoxP3$^+$CD127$^-$ (94% vs 92%), similar total cell (437- vs 365-fold) and CD25$^+$FoxP3$^+$ Treg (271- vs 257-fold) expansion, and

were modestly more suppressive in 2 distinct MLR assays (1:8 ratio: 84% vs 51%; 79% vs 72%; data not shown). Compared with controls, UCB Tregs expanded using KT32 cells increased long-term survival (0% vs 70%; $P \le .003$), whereas Tregs expanded using beads had a more modestly improved survival (0% vs 30%; $P = .04$), which was significantly lower than with KT32 cells ($P < .05$; Figure 7A). UCB Treg content in the blood was quantified on days 10 and 17 after transfer. On day 10, KT32 versus bead-expanded Tregs resulted in a 5-fold higher circulating Treg number, which declined to undetectable levels by day 17 (Figure 7B, $P \le .04$). Conversely, analysis of peripheral blood and spleen at the time of death or elective sacrifice (day 67) revealed significantly lower numbers of PBMNC-derived human CD4$^+$ T cells in mice receiving KT32-expanded Tregs (Figure 7C; $P \le .007$ for each).

Discussion

We have shown that cell-based aAPCs were more effective than microspheric beads in favoring the expansion of CD4$^+$25$^+$FoxP3$^+$ Tregs with the capacity to potently suppress in vitro allogeneic responses, which correlated with increased TGF-β secretion. By genetically modifying aAPCs to coexpress OX40L or 4-1BBL, a greater than 1250-fold expansion of UCB Tregs could be achieved in 2.5 to 3 weeks without the need for cell sorter purification and without the loss of in vitro suppressor cell potency. The addition of rapamycin to the expansion culture was neither required nor desirable. In vivo adoptive transfer of expanded UCB Tregs
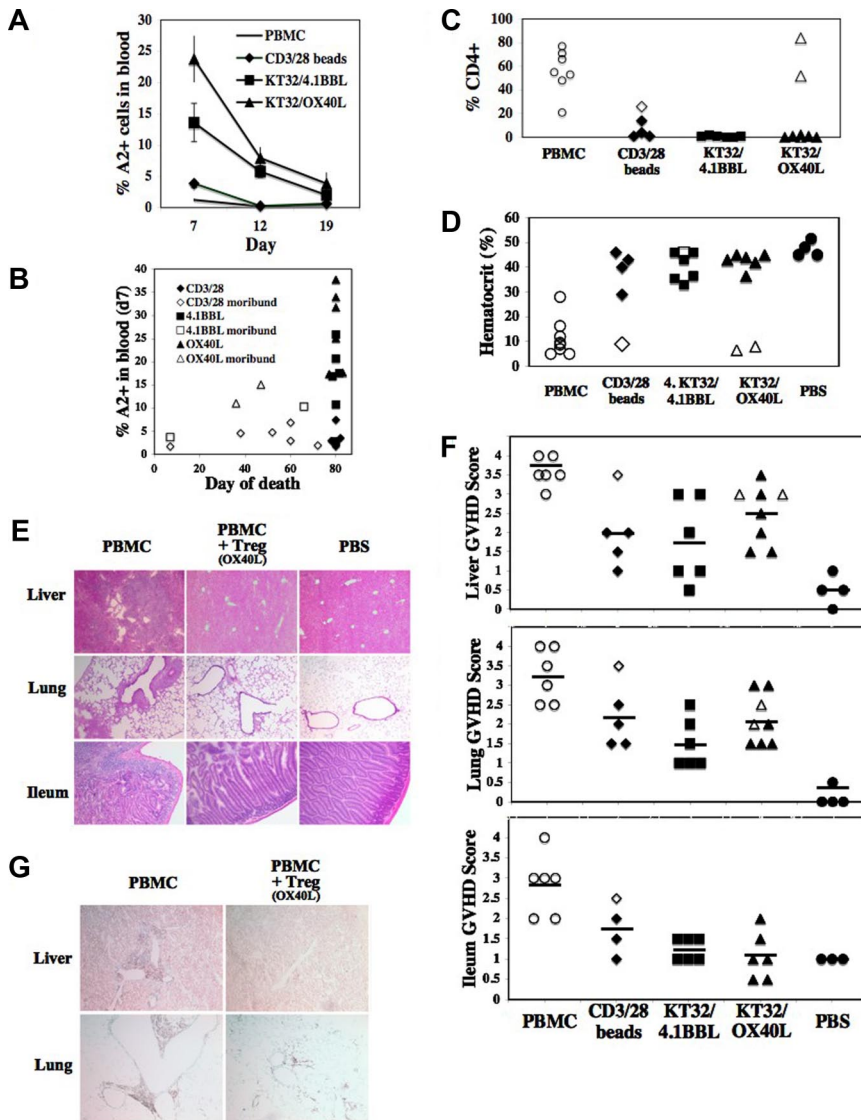
Figure 6. Cotransfer of Tregs leads to amelioration of GVHD pathology. (A) Differential persistence of Tregs in blood of animals on the indicated days after transfer. (B) Comparison of Treg (A2$^+$) numbers in circulation on day 7 versus day of death due to disease (open symbols) or conclusion of the experiment (closed symbols). (C,D,F) Disease severity was assessed at time of death (open symbols) or at the conclusion of the experiment (closed symbols). (C) Decreased percentage of human CD4$^+$ cells was observed in blood from Treg-treated animals that survived to day 80. (D) Moribund animals were found to have decreased hematocrit readings, except for the one peri-transplant, non–GVHD-related death observed in the 4.1BBL group. Representative example (E) and disease severity scores (F) for H&E staining of liver, lung, and ileum from animals with or without PBMNCs and Tregs. (G) Representative examples of anti-hCD4 peroxidase staining of liver and lung from animals with or without PBMNCs and Tregs expanded with KT32/OX40L. Pathology data from same experiment as Figure 5, and the pathology is representative of 3 experiments analyzed. Images were acquired at room temperature with an Olympus BX51 microscope, Olympus U-plan Apo and 10× objective lens (aperture = 0.40; Olympus, Hamburg, Germany) with an RTT Spot camera and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI).

indicated that cell-based aAPC expansion cultures were superior to beads in suppressing xenogeneic GVHD. Thus, these studies suggest a novel and more effective strategy for UCB Treg expansion than the currently widely used bead-based expansion approach.

Prior reports indicated that rapid and sustained polyclonal human CD4$^+$ growth is possible using CD32-expressing K562 cells as aAPCs.[36] By coexpressing 4-1BBL in CD32$^+$ K562 aAPCs, more rapid and sustained polyclonal human CD8$^+$ T cells were observed than when using anti-CD3/28 mAb–

Figure 7. Tregs expanded with cell-based aAPCs offer increased protection from GVHD mortality and pathology over Tregs expanded with bead-based aAPCs. Cord blood Tregs were purified, expanded in vitro using bead- or cell-based aAPCs, and injected along with allogeneic PBMNCs into clodronate-treated, irradiated Rag2$^{-/-}$, $\gamma_c^{-/-}$ mice as in Figure 5. (A) Kaplan-Meyer survival curve showing increased survival of mice receiving human PBMNCs with or without groups of Tregs in a 3:1 ratio (ie, 30 × 10^6 PBMNCs and 10 × 10^6 Tregs). For groups PBMNCs, CD3/28 beads, and KT32, n = 8, 10, and 10, respectively. $P \le .05$ for each Treg-treated group compared with PBMNCs. (B) Enumeration of Tregs in blood of animals on the indicated days after transfer, showing increased persistence for KT32-expanded Tregs. $P < .04$ on day 10. (C) Decreased percentage of human CD4$^+$ cells were observed in blood and spleen from Treg-treated animals that survived to day 67.
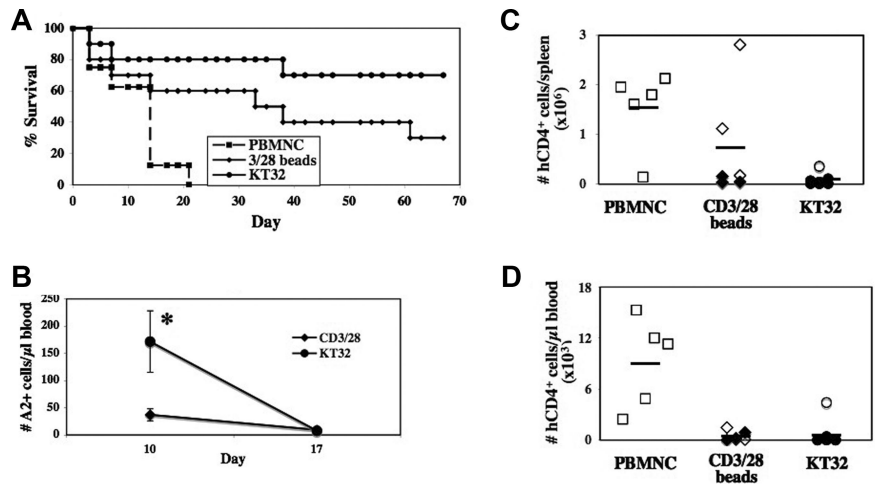
coated beads.[29] Polyclonal human NK cell expansion has been reported using K562 aAPCs that coexpressed 4-1BBL and IL-15.[41] Since genetically modified (GM-CSF transduced) K562 cells[42] have been injected into cancer patients to provide a bystander response for vaccination,[43-45] the choice of human K562 as aAPCs should be more readily approvable for Treg production under good manufacturing practice (GMP) conditions. The lack of major histocompatibility complex (MHC) expression by K562 aAPCs has been shown to permit retention of a broad repertoire of CD4+ and CD8+ T cells. Compared with bead-based aAPCs, K562 aAPCs may permit better formation of the immunologic synapse as a result of the fluidity of the APC membrane,[29,46] extending a study reporting the use of a human CD32 transfected murine L929 to present anti-CD3/28 mAbs for expanding flow sorter–isolated adult peripheral blood Tregs.[47] Alternatively, K562 cells may endogenously express other molecules that provide costimulation to Tregs. For example, it is interesting to note the expression of CD30 and CD30L on UCB Tregs and K562, respectively (data not shown), especially in light of recent reports demonstrating that CD30 signaling is critical for Treg-mediated acute GVHD protection.[48]

While our data clearly demonstrate that 4.1BB or OX40 signaling is beneficial for expansion without compromising in vitro or in vivo suppressor cell function, results from other studies have varied greatly depending on system and organism involved. Therefore, either human UCB Tregs or the aAPC and culture conditions used to expand these cells in the initial presence of 4.1BBL or OX40L signals are biologically distinct from murine Tregs expanded with splenic APCs and anti-CD3 mAb. Finally, since K562 aAPCs are lethally irradiated and are not detectable after day 4 of culture (data not shown), it is interesting to note that the initial exposure of UCB Tregs to 4.1BBL or OX40L prolonged effects on expansion of CD4+25+FoxP3+ Tregs with potent suppressor function. While this effect could potentially be explained by changes in surface expression of the respective costimulatory receptors on stimulated UCB Tregs, we have found no differences in 4.1BB or OX40 expression in aAPC- versus bead-expanded UCB Treg cultures (data not shown), consistent with and extending our previous report that OX40 expression is up-regulated in UCB Tregs in response to restimulation of anti-CD3/28 bead cultures.[31]

Similar to other TNFRs, the primary signal generated from 4.1BB or OX40 ligation is TRAF 2 activation, which leads to Bcl-x$_L$ up-regulation.[16,49] While no change in Bcl-x$_L$ was observed, we show that Tregs expanded with 4-1BBL-expressing aAPCs had an approximately 2-fold decrease in the expression of the mRNA for Bim, a BH3-only, proapoptotic protein. Bim is the rate-limiting step in controlling lymphocyte apoptosis induced by cytokine deprivation or clonal deletion, and small differences in Bim expression (eg, Bim$^{+/-}$ mice) can have significant effects on apoptosis.[50-53] Although Bim$^{-/-}$ mice have a profound autoimmune phenotype, a potential link between Bim expression and Treg development and survival has not been explored. These data extend our prior report of the high degree of apoptosis observed after anti-CD3/28 mAb–induced restimulation of long-term, bead-expanded UCB Treg lines that correlated with lack of up-regulation of the antiapoptotic protein bcl-x$_L$.[54]

In contrast to adult peripheral blood Tregs, in which isolation of CD45RA+ or CD4+25+CD127^{lo} cells or addition of rapamycin is required for reproducible expansion, UCB Tregs isolated by MACS selection and expanded with cell- or bead-based aAPCs are more than 90% CD45RA+ and are mostly FoxP3+ and CD127^{lo} at the end of culture. Consistent with the low potential for overgrowth of UCB Treg cultures with activated Teff cells (even in the absence of rapamycin), no exacerbation of XGVHD was seen even when infusing cells from a culture that contained as few as 28% CD4+, CD127−, Foxp3+ cells (data not shown).

It has recently been reported that only Tregs stimulated in vitro with directly and indirectly presented alloantigens were able to prevent chronic graft rejection.[55] However, this is not a requirement for the polyclonally expanded Tregs described herein, as they effectively suppressed T-cell activation both in vitro and in vivo even when specifically mismatched for HLA-A2.

In addition to suppression assays with HLA-A2–mismatched Treg/Tresp, the fact is that we have observed similar Treg suppression curves for over 300 MLR experiments using unrelated responders and stimulators. Therefore, polyclonal expanded UCB Tregs appear to be capable of substantial suppression of proliferative responses without the prerequisite for selecting responder cells that are HLA class I or II matched. While these data suggest that UCB Tregs can be used as an "off the shelf" product, it is likely that such usage would be done with at least partial HLA matching with the recipient, thereby having the potential added benefit of further increasing suppression levels. An important consideration in using Tregs as a cellular therapy to inhibit GVHD is that, unlike murine models, human memory cells retain alloreactivity. Our MLR suppression assays were performed on PB CD4+ T cells, which contained a mixture of naive and memory T cells. A sampling of 10 such preps, which were all suppressible, shows that at least 37% plus or minus 5% of cells are CD45RO+ and at least 43% plus or minus 6% are CD62L^{lo}, CD44^{hi} (data not shown). Likewise, the xenogeneic GVHD model employs whole PBMNCs, which would also be a mixture of naive and memory T cells.

In conclusion, we have shown that aAPCs are more efficient than anti-CD3/28 mAb–coated beads in expanding functional UCB Tregs that retain full suppressor cell potency in vitro and in vivo without the need for cell sorter isolation or the addition of rapamycin. The coexpression of OX40L or 4-1BBL can provide more than 1000-fold expansion in 3 weeks. Since K562 aAPC culture expansion conditions are adaptable to GMP conditions, these data not only have important implications for clinical trials but also provide novel insights into the biologic effects of OX40L and 4-1BBL on human Treg expansion and function.

Authorship

Contribution: K.L.H. and P.H.-M. designed the research, performed the experiments, interpreted the data, and wrote the

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