ASBMT American Society for Blood and Marrow Transplantation

BIOLOGY

Cotransplantation of Ex Vivo Expanded and Unexpanded Cord Blood Units in Immunodeficient Mice Using Insulin Growth Factor Binding Protein-2–Augmented Mesenchymal Cell Cocultures

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Ex vivo expansion of cord blood (CB) hematopoietic stem cells and cotransplantation of 2 CB units (CBUs) could enhance the applicability of CB transplantation in adult patients. We report an immunodeficient mouse model for cotransplantation of ex vivo expanded and unexpanded human CB, showing enhanced CB engraftment and provide proof of concept for this transplantation strategy as a means of overcoming the limiting cell numbers in each CBU. CBUs were expanded in serum-free medium supplemented with stem cell factor, Flt-3 ligand, thrombopoietin, and insulin growth factor binding protein-2 together with mesenchymal stromal cell coculture. Unexpanded and expanded CB cells were cotransplanted by tail vein injection into 45 sublethally irradiated nonobese diabetic SCID-IL2$\gamma^{-/-}$ (NSG) mice. Submandibular bleeding was performed monthly, and mice were sacrificed 4 months after transplantation to analyze for human hematopoietic engraftment. Expansion of non-CD34$^+$ selected CB cells yielded 40-fold expansion of CD34$^+$ cells and 3.1-fold expansion of hematopoietic stem cells based on limiting dilution analysis of NSG engraftment. Mice receiving expanded grafts exhibited 4.30% human cell repopulation, compared with 0.92% in mice receiving only unexpanded grafts at equivalent starting cell doses, even though the unexpanded graft predominated in long-term hematopoiesis ($P = .07$). Ex vivo expanded grafts with lower initiating cell doses also showed equivalent engraftment to unexpanded grafts with higher cell dose (8.0% versus 7.9%; $P = .93$). In conclusion, ex vivo expansion resulted in enhanced CB engraftment despite eventual rejection by the unexpanded CBU.

Biol Blood Marrow Transplant 18: 674-682 (2012) © 2012 American Society for Blood and Marrow Transplantation

KEY WORDS: Hematopoietic stem cells, Double cord blood transplants, Ex vivo expansion, Graft versus host disease, Murine transplantation, Engraftment

INTRODUCTION

More than 22,000 hematopoietic stem cell (HSC) transplantations are performed annually worldwide for patients with leukemia, bone marrow failure, and other disorders of the blood and immune system [1]. In the last 2 decades, cord blood (CB) has been increasingly used as an alternative to the more common HSC sources (ie, bone marrow and peripheral blood). This is due to its many advantages, including permissiveness to HLA mismatching, lower risk of graft-versus-host disease (GVHD), ready availability, and the superior proliferative capacity of CB HSCs compared with marrow or peripheral blood [2]. However, CB transplantation is generally limited to children and smaller adults, given that the number of cells in each CB unit (CBU) is insufficient for most adults.

Double CB transplantation (DCBT) involving the cotransplantation of 2 CBUs could partially overcome the barrier of insufficient cell dose. Coinfusion of 2

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Financial disclosure: See Acknowledgments on page 682.

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Received August 6, 2011; accepted January 3, 2012

1083-8791/$36.00
doi:10.1016/j.bbmt.2012.01.001

Table 1. Experimental Design for NSG Mice Transplantation

Group	Total Initial Cell Dose, $\times 10^6$ Cells	Cells Used for Each CBU	
		CB-A, $\times 10^6$ Cells	CB-B, $\times 10^6$ Cells
1	2	2	-
2	2	-	2
3	2	1	1
4	2	0.4	1.6
5	2	1.6	0.4
6	1	0.5	0.5*
7	1	0.5*	0.5
8	2	1	1*
9	2	1*	1

*Represents the number of input CB cells prior to expansion.

CBUs does not always provide sufficient cells for engraftment, however [3]. To surmount this problem, a modified version of DCBT involving cotransplantation of an ex vivo expanded CBU and an unexpanded CBU was postulated to be beneficial. Ex vivo expansion of 1 of the 2 CBUs could potentially enhance engraftment by increasing the number of HSCs and hematopoietic progenitor cells, and the unexpanded unit provides a ready source of HSCs, should the ex-vivo expansion fail. To date, many preclinical studies have been performed, but none has truly mimicked the conditions under which these DCBT ex vivo expansion CB clinical trials are being conducted [4-6].

Recently, a combination of cytokines, including insulin growth factor binding protein-2 (IGFBP2) and angiopoietin-like 3, was shown to result in a 20-fold net expansion of repopulating human CB HSCs with $CD133^+$ selected CB cells [7]. Mesenchymal coculture has also been shown to augment ex vivo CB expansion [8,9].

In the present study, we used a combination of mesenchymal coculture and IGFBP2 supplementation in our unselected CB expansion protocol, with cotransplantation of expanded CB cells into NOD/SCID-IL2$\gamma^{-/-}$ (NSG) mice together with an unexpanded CBU in a DCBT setting. Engraftment results show that DCBT with 1 expanded CBU and 1 unexpanded CBU enhanced human hematopoietic engraftment in NSG mice compared with CBT with single or double unexpanded CBU.

MATERIALS AND METHODS

Preparation of Cells

Fresh umbilical CB was obtained from Singapore Cord Blood Bank. The use of CB was reviewed and approved by the Singapore Cord Blood Bank Research Advisory Ethics Committee, as well as the Institutional Review Boards of National University of Singapore and Singapore General Hospital. Mononuclear cells (MNCs) were isolated from fresh CBUs by Ficoll-Histopaque-1077 (Sigma-Aldrich, St Louis, MO) density gradient centrifugation. No phenotypic cell selection process was performed for the CB cells. HuES9.E1, a human embryonic stem cell-derived mesenchymal stem cell (ESMSC) cell line, was maintained on gelatin-coated tissue culture plate surface in DMEM as described previously [10].

CB-MNC Culture with or without Addition of IGFBP2

For this experiment, 4×10^5cells/mL of freshly thawed CB MNCs were cocultured with ESMSCs in StemSpan serum-free medium (Stemcell Technologies, Vancouver, Canada) supplemented with 100 ng/mL of stem cell factor (SCF), 50 ng/mL of Flt-3 ligand (Flt-3L), and 100 ng/mL of thrombopoietin (TPO) (all from PeproTech, Rocky Hills, NJ), with or without 20 ng/mL of IGFBP2 (R&D Systems, Minneapolis, MN). One day before seeding of CB cells, ESMSCs were seeded at a confluent density of 4×10^4 cells/cm^2. After 11 days of culture, the expansion folds for viable total nucleated cells (TNCs) and $CD34^+38^-90^+$ cells (ie, HSCs) were measured by flow cytometry.

Ex Vivo Expansion of CB Cells for NOD/SCID-IL2$\gamma^{-/-}$ Mice Transplantation

When ESMSCs reached 90% to 100% confluence, CB MNCs were cocultured with ESMSCs at a density of 8×10^5 cells/mL using StemSpan medium supplemented with the aforementioned cytokines. On day 7, the medium volume was topped up by adding an equivalent volume (as on day 0 of coculture) of fresh StemSpan medium supplemented with cytokines. Cells were cultured at 37°C in 5% CO_2 and normal O_2 and harvested on day 11.

NSG Mice Transplantation

NSG mice were purchased from Jackson Laboratory (Bar Harbor, ME) via the SingHealth Experimental Medicine Centre and were maintained in the Duke–National University of Singapore vivarium. All animal experiments were conducted with the approval of SingHealth Institutional Animal Care and Use Committee. Unexpanded or expanded CB cells were injected i.v. via the tail vein into sublethally irradiated (240 cGy) 9- to 12-week old NSG mice. Two CBUs (designated CB-A and CB-B) were used. Cotransplantation with 1 ex vivo expanded CBU and 1 unexpanded CBU was defined as "transplanted with expanded unit," and transplantation with either 1 single unexpanded CBU or double unexpanded CBUs was defined as "transplanted with unexpanded unit." A total of 45 NSG mice were divided into 9 groups (Table 1). The mice were maintained for 4 months, with submandibular bleeding for blood assays performed each month.

Harvesting of Bone Marrow and Spleen

At the end of the fourth month posttransplantation, the mice were sacrificed in a CO_2 chamber. Femurs and tibias were harvested and immediately placed into cold

RPMI medium (Invitrogen/Life Technologies, Grand Island, NY). Joint ends were cut, and bone marrow was flushed out with 10 mL of 2% FBS-RPMI. Then, spleens were harvested and immediately placed into 0.5 mL of Dulbecco's phosphate-buffered saline (Invitrogen) over ice. To harvest HSCs, the harvested spleens were mechanically sheared through a 40-μm cell strainer (BD Falcon; BD Biosciences, San Jose, CA). Both the bone marrow and spleen cell suspension were washed, after which contaminated red blood cells were lysed by ammonium chloride-based buffer before flow cytometry analysis.

Flow Cytometry

All data were acquired using a Cytomic FC500 Flow Cytometer (Beckman Coulter, Marseille, France), and 10,000 events per sample were collected. Acquired data were subsequently analyzed with CXP analysis software (Beckman Coulter). To analyze ex vivo expansion of CB cells, the cells were stained for viability, multipotential HSCs, T cells, B cells, regulatory T cells, naïve T cells, and effector T cells. Hematopoietic cell viability was identified by staining with CD45-PC7, Annexin V-FITC, and 7-AAD (all from Beckman Coulter). Multipotential HSCs were identified by staining with CD45-PC7, CD34-PE, CD38-APC, and CD90-FITC. B cells, T cells, and regulatory T cells were identified by staining with CD45-PC7, CD3-ECD, CD19-PE, CD4-FITC, CD25-APC, and FoxP3-PE antibodies (all from BD Biosciences except CD3-ECD and CD19-PE, from Miltenyi Biotec, Bergisch Gladbach, Germany). Naive T cells and effector/effector memory T cells were identified by staining with CD45-FITC, CD3-PE, CD4-Percp Cy5.5, CD8-ECD, CCR7-PC7, and CD45RO-APC antibodies (all from BD Biosciences except CD8-ECD, from Miltenyi Biotec).

To analyze bone marrow and spleen HSCs, the harvested bone marrow and spleen cells were stained phenotypically for myeloid and lymphoid lineage. For myeloid lineage analysis, the cells were stained using human CD45-APC, CD71-PE, CD15-FITC, CD66-FITC (all from Miltenyi Biotec) as well as mouse CD45-PE (BD Biosciences) and mouse CD45 FITC (Miltenyi Biotec). For common myeloid and lymphoid lineage analysis, the cells were stained using human CD45-APC, CD34-PE, CD19-FITC, CD20-FITC (all from Miltenyi Biotec) and CD3-ECD (BD Pharmingen), as well as mouse CD45-PE and mouse CD45 FITC (Miltenyi Biotec).

DNA Extraction and Chimerism Studies

Chimerism of each mouse was determined by a variable number tandem repeat (VNTR) assay. Genomic DNA from mouse bone marrow and spleen were extracted using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD). PCR amplification of human-specific locus D1S80 [11] was performed using primers 5′-GAAACTGGCCTCCAAACACTGCCCGCCG3′ and 5′-GTCTTGTTGGAGATGCACGTGCCCC TTGC-3. PCR was performed using DyNAzyme EXT DNA polymerase (Finnzymes, Espoo, Finland) under the following conditions. DNA was first denatured at 95°C for 2 minutes, then amplified for 5 cycles of 95°C for 30 seconds and 65°C annealing for 30 seconds, followed by 1 cycle of elongation at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. Genomic DNA samples from human CB (positive control) and bone marrow cells of nontransplanted mice (negative controls) were processed in parallel. Amplified PCR products were electrophoresed in 2.6% agarose gels, stained with ethidium bromide (Sigma-Aldrich, St Louis, MO), and visualized under UV light.

IFN-γ Intracellular Staining

Peripheral blood (100-150 μL) was collected from NSG mice transplanted with double CBUs by submandibular bleeding on day 45 posttransplantation. Collected peripheral blood from each group (Table 1) was pooled, and 6×10^5 peripheral blood cells were stimulated with 1.5×10^5 $CD3^-$ selected mononuclear cells from either donor unit for 5 hours in 3 mL of HEPES (Invitrogen) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and 35 U/mL of penicillin/streptomycin (Invitrogen) at 37°C in a humidified 5% CO_2 incubator. For IFN-γ intracellular staining, 5 μg/mL of brefeldin A (Sigma-Aldrich) was added after a 1-hour incubation. Unstimulated controls were included as well. The cells were stained with live/dead fixable violet stain–ECD (Invitrogen) and CD3-PE, CD4-Percp Cy5.5, CD8-ECD, CCR7-PC7, and CD45RO-APC surface antibodies (all from BD Biosciences) for 20 minutes at 4°C. After fixation/permeabilization (Cytofix/Cytoperm; BD Biosciences), cells were washed and subjected to IFN-γ FITC intracellular staining for 30 minutes.

Statistical Analysis

Results are reported as mean ± standard deviation. The significance of difference between 2 groups was determined using the 2-tailed Student *t*-test. Analysis of variance was used to determine the significance of difference among 3 or more groups. A P value $<.05$ was considered to indicate statistical significance. Data processing and statistical analysis were performed with OriginPro 7.5 (OriginPro, Northampton, MA).

RESULTS

IGFBP2 Selectively Expands HSCs in Whole Cord Blood by 21-Fold in a Mesenchymal Stromal Coculture System

IGFBP2 did not significantly increase the expansion of viable TNCs ($P = .08$), but it increased

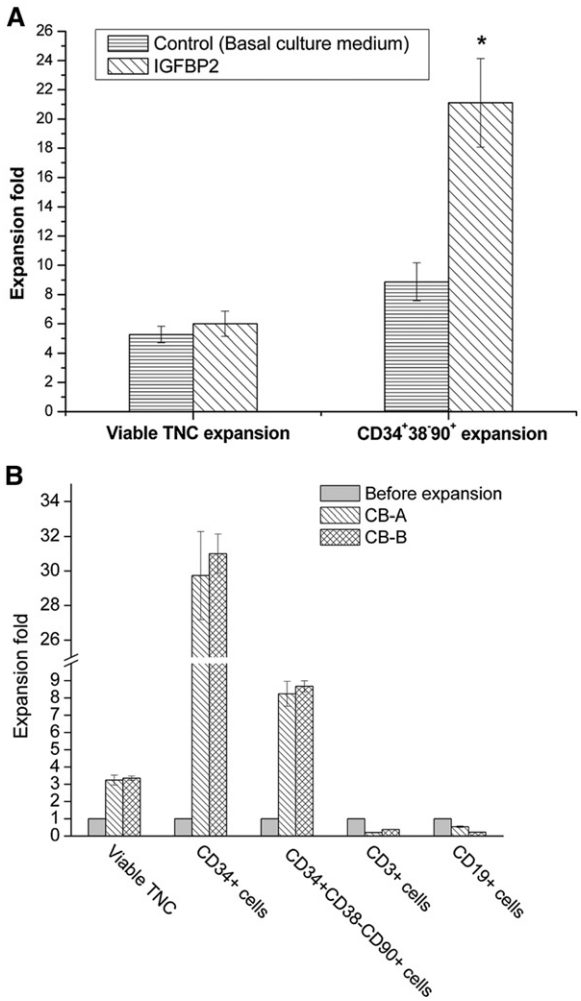

Figure 1. Ex vivo expansion of cord blood units using cytokines. (A) Viable TNCs and $CD34^+38^-90^+$ expansion fold of CB cells in the presence of IGFBP2. Cord blood cells were inoculated at the density of 4×10^5cells/mL and cocultured with mesenchymal stromal cells for a total of 11 days. Basal culture medium for control groups were cultured in the presence of 50 ng/mL of SCF, Flt-3 50 ng/mL and 100 ng/mL TPO. There is no significant difference for the expansion of viable TNCs between control and IGFBP2 group ($P = .08$). With the addition of 20 ng/mL of IGFBP2, there is a 21-fold $CD34^+38^-90^+$ (ie, HSCs) expansion fold compared to control ($P = .001$) (n = 2). (B) Expansion profile of the 2 cord blood units used for NSG mice transplantation. Cord blood cells cocultured with mesenchymal stromal cells in the presence of 100 ng/mL of SCF, 50 ng/mL of Flt-3, 100 ng/mL of TPO and 20 ng/mL of IGFBP2 for 11 days. There is an overall 3.2-fold expansion of viable TNC, 30-fold expansion of $CD34^+$ cells, and eight fold expansion of $CD34^+CD38^-CD90^+$ cells. $CD19^+$ (B cells) and $CD3^+$ (T cells) were reduced by three to five fold with this expansion protocol. Results are mean ± SD of expansion fold from a single experiment with triplicate assay (n = 2).

expansion of $CD34^+38^-90^+$ cells (ie, HSCs) by 21-fold ($P < .001$) (Figure 1A). To assess the reproducibility of the IGFBP2 effect, we expanded 2 CBUs (CB-A and CB-B) with IGFBP2 supplementation. Expansion of both CBUs was similar, with a 3.2-fold expansion of viable TNCs, a 30-fold expansion of $CD34^+$ cells, an eight fold expansion of $CD34^+CD38^-CD90^+$ cells, and a two- to fivefold reduction in $CD19^+$ and $CD3^+$ cells (Figure 1B). Therefore, ex vivo culture of CB MNCs with IGFBP2 supplementation and MSC coculture selectively expanded HSCs.

Superior Human Hematopoietic Engraftment in Mice Receiving Expanded CB Cells

We performed an NSG repopulation assay to test whether the ex vivo expanded CB cells would lead to increased and sustained hematopoietic engraftment compared with unexpanded CB cells. The percentage of human CD45 cell repopulation was compared in the mice receiving unexpanded 2×10^6 CB cells and those receiving expanded 2×10^6 CB cells. At the same initial starting dose, transplantation of an expanded CBU resulted in a higher percentage of CD45 cell repopulation, although the differences in mean values were not statistically significant for peripheral blood (4.30% ± 2.72% versus 0.92% ± 1.14%; $P = .07$), bone marrow (14.67% ± 19.79% versus 1.61% ± 2.48%; $P = .23$), and spleen (59.27% ± 19.76% versus 27.05% ± 29.00%; $P = .16$) (Figure 2A). However, at a starting dose of 1×10^6 for expanded CBUs and 2×10^6 for unexpanded CBUs, the percentages of CD45 cell repopulation were equivalent for peripheral blood (8.0% ± 13.2% versus 7.9% ± 10.0%; $P = .93$), bone marrow (4.5% ± 7.0% versus 10.5% ± 14.3%; $P = .30$), and spleen (37.5% ± 32.9% versus 39.1% ± 23.9%; $P = .90$) (Figure 2B). Based on limiting dilution analysis of NSG mice engraftment results (with a positive result defined as >1% human HSC engraftment), mice undergoing transplantation with 1×10^6 of expanded CB cells had a similar percentage of HSCs (competitive repopulation unit [CRU] = 1/1.76e6) compared with those receiving 2×10^6 unexpanded CB cells (CRU = 1/1.68e6). There was a 3.1-fold expansion of HSCs during the ex vivo expansion when the 3.2-fold expansion of TNCs was taken into account (Figure 2C).

Comparable Multilineage Profile between Unexpanded and Expanded CBUs

Multilineage engraftment was compared in NSG mice receiving unexpanded CB cells and those receiving expanded CB cells at 4 months posttransplantation (Figures 2D and 3). There was no significant difference in the mean percentage of repopulation for the human myeloid lineage (CD34, CD71, and CD66b/CD15) and lymphoid lineage (CD19/CD20 and CD3) between the mice that received unexpanded and expanded CB cells (CD71, $P = .06$; CD6615, $P = .23$; CD34, $P = .09$; CD1920, $P = .13$; CD3, $P = .78$).

Unexpanded CBU Predominating in Final Donor Chimerism Due to the Immune Reaction between the 2 Donor CBUs

DNA extraction was performed on the collected bone marrow and spleen for chimerism studies. All of

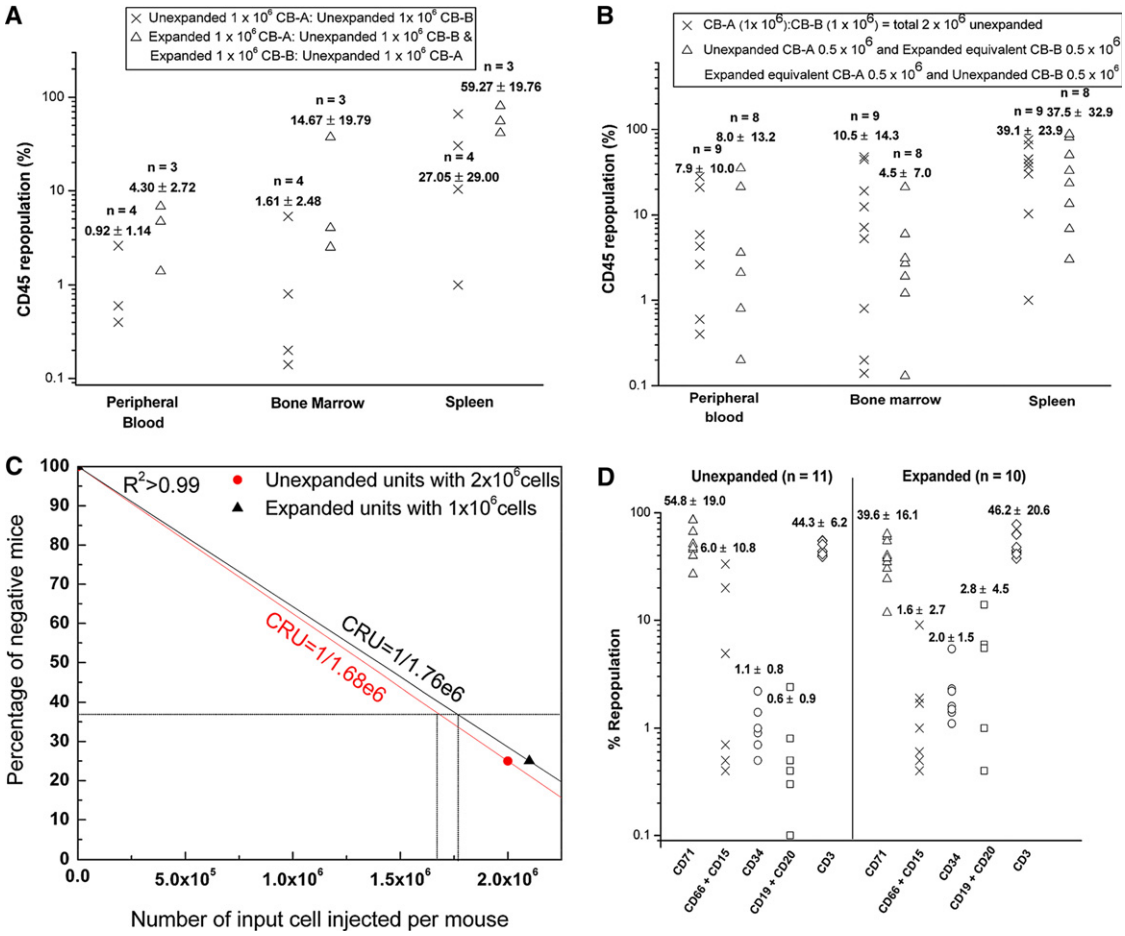

Figure 2. Human CD45 repopulation in the peripheral blood, bone marrow, and spleen of NSG mice. (A) Mice that underwent a transplantation of 2 $\times 10^6$ unexpanded versus a transplantation of 2 $\times$ 10^6 expanded CB cells (cell dose 1:1). (B) Mice that underwent a transplantation of 2 $\times$ 10^6 unexpanded versus a transplantation of 1 $\times 10^6$ expanded CB cells (cell dose 2:1). Each symbol represents engraftment of a single mouse that underwent transplantation assayed at 4 months after transplantation. Results are expressed as mean $\pm$ SD of percentage of engraftment from a single experiment. (C) Limiting dilution analysis of double cord blood transplantation performed in NSG mice. Negative engraftment was defined by less than 1% human CD45 engraftment in the bone marrow. (D) Multilineage engraftment in NSG mice that underwent a transplantation of unexpanded or expanded cord blood cells. Each symbol represents engraftment of a single mouse that underwent transplantation assayed at 4 months after transplantation. Some mice had 0% donor repopulation, and these are not plotted. Results are expressed as mean $\pm$ SD of percentage of engraftment from a single experiment (n = 1).

the mice that underwent DCBT had single CBU domination. On VNTR analysis, the unexpanded CBU was shown to dominate over the expanded CBU at 4 months after transplantation. We also investigated the potential role of an immune reaction in mediating final CBU dominance after DCBT in mice. We performed a multiparameter flow cytometry assay designed to detect whether IFN-γ–secreting T cells that developed in the recipient mice after DCBT were capable of specific recognition of cells derived from either CBU. After a 5-hour stimulation of peripheral blood with CD3-selected mononuclear cells from either donor CBU, a significant $CD8^+CD45RO^{+/-}$ $CCR7^-$ IFN-γ–secreting cell population, effector $CD8^+$ T cells, was detected in the mice stimulated with nonengrafted CBUs (1.5% $\pm$ 1.1%), whereas IFN-γ response was undetectable in the group stimulated with engrafted CBUs (0%) (Figure 4A). Furthermore, FACs analysis of CB cells before cotransplantation showed that 70% of unexpanded grafts consisted of $CD3^+$ cells, compared with only 4% of expanded grafts (Figure 4B). These data provide a possible explanation of why the unexpanded CBU achieves eventual unit dominance in recipient mice. Furthermore, greater human cell engraftment was seen in the mouse peripheral blood at 1 month post-transplantation in the mice receiving expanded CB cells compared with those receiving unexpanded CB cells (21.4% $\pm$ 3.8% versus 10.9% $\pm$ 4.3%; P = .08) (Figure 4C), suggesting that the expanded cells contribute to early stages of engraftment.

Possible GVHD-Related Deaths Due to High Proportion of $CD3^+$ T Cells in the Unexpanded CB Unit

In the mouse experiment described earlier, 14 of the 34 mice died during the first 2 months after transplantation. Before death, those mice exhibited hair loss, weight loss, lack of vigor, and a hunchback appearance.

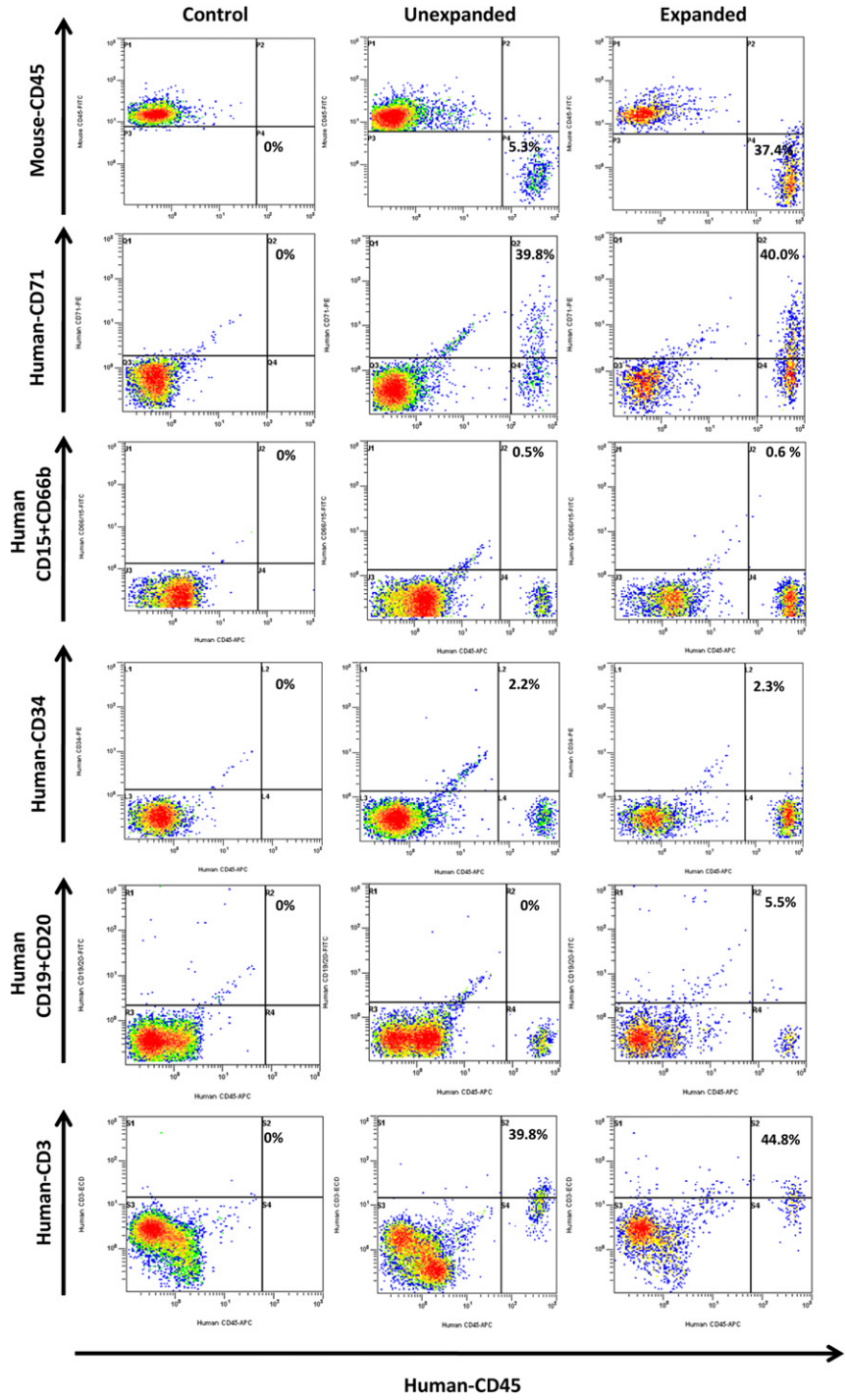

Figure 3. Representative fluorescence-activated cell sorting plots of bone marrow cells. Flow cytometric data from 3 representative mice. Lane 1, control; lane 2, mouse injected with unexpanded 1×10^6 cells CB-A and unexpanded 1×10^6 cells CB-B; lane 3, mouse injected with unexpanded 1×10^6 cells CB-A and expanded 1×10^6 cells CB-B at 4 months after transplantation. Mice with best engraftment were chosen for unexpanded and expanded group. Percentages of cells in each quadrant are listed.

Interestingly, the survival rate was 80% in the mice receiving expanded CB cells, compared with 50% from mice receiving unexpanded CB cells (Figure 5A). T cell population analysis results before and after ex vivo expansion from another independent mouse experiment found ~20-fold, 37-fold, and six fold increases in $CD3^+$ T cells, $CD3^+CD4^+$ helper T cells, and $CD3^+CD8^+$ cytotoxic T cells, respectively, whereas a three fold decrease in $CD4^+CD25^+Foxp3^+$ regulatory T cells in the unexpanded CBUs compared with the expanded CBUs (Figure 5B). In the helper T cell population, there were 73.5% naïve T cells in the unexpanded CBUs versus 7.5% in the expanded CBUs and 25% effector T cells in the unexpanded CBUs versus

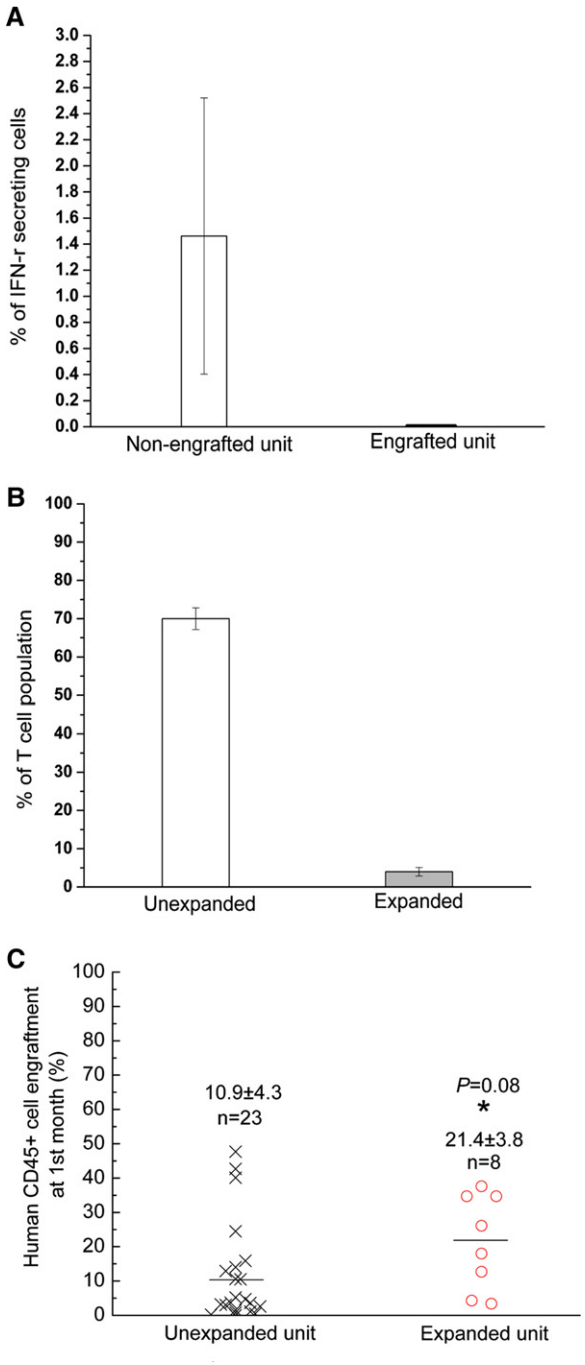

Figure 4. Immunoreaction between dual CBUs and proportion of $CD3^+$ T cells within expanded and unexpanded units. (A) Mouse peripheral blood collected 45 days after transplantation shows presence of $CD8^+CD45RO^{+/-}CCR7^-$ IFN-γ–secreting cells that are reactive against $CD3^-$ selected cells from the nonengrafted CBU and the engrafted CBU. IFN-γ–secreting $CD8^+$ T cells are also detectable among 8 groups of mice establishing single donor dominance (n = 1). (B) $CD3^+$ T cell population analysis before and after ex vivo expansion. (C) Human cell engraftment in NSG mice peripheral blood at 1 month posttransplantation with 2×10^6 expanded and unexpanded cells.

77.5% in the expanded CBUs (Figure 5C). In the cytotoxic T cell population, there were 48% naïve T cells in the unexpanded CBUs versus 2% in the expanded CBUs and 49% effector T cells in the unexpanded CBUs versus 95.5% in the expanded CBUs (Figure 5C). When a 3.2-fold ex vivo expansion of TNCs was taken into account, CB expansion resulted in six fold lower concentration of $CD3^+$ T cells, a 12-fold lower concentration of $CD3^+CD4^+$ helper T cells, a two fold lower concentration of $CD3^+CD8^+$ cytotoxic T cells, and a 10-fold higher concentration of $CD4^+CD25^+Foxp3^+$ regulatory T cells. The higher regulatory T cell and lower $CD3^+$ cell content in the expanded cells help explain the higher survival rate in mice undergoing transplantation with at least one ex vivo expanded CBU. Of note, despite a higher proportion of naïve T cells in the unexpanded CBUs, most of these cells (95%-98%) transformed to effector T cells after infusion (Figure 5D).

DISCUSSION

In this study, we found that IGFBP2 enhanced ex vivo CB expansion in a mesenchymal stromal coculture system, resulting in a 21-fold increase in HSCs. Furthermore, in a DCBT model, we found superior human hematopoietic engraftment in mice receiving expanded CB cells, although the unexpanded CBU predominated in final donor chimerism.

Both the cytokines and the mesenchymal stromal coculture system used in this study likely contributed to this enhanced human hematopoietic engraftment. Various cytokines dictate the cellular fates of HSCs, including self-renewal, differentiation, apoptosis, and quiescence. Out of the 4 cytokines studied, SCF, Flt-3L, and TPO are widely used for ex vivo expansion of CB cells, but IGFBP2 is not. The addition of IGFBP2 has been shown to expand CB HSCs through an as-yet-unknown mechanism [7,12]. Fei et al [8] reported that coculture of CB $CD34^+$ cells with mesenchymal stromal cells could enhance rapid engraftment of CB cells in NOD/SCID mice [8]. This is consistent with Schofield's "niche hypothesis" [13], in which mesenchymal coculture could simulate the native bone marrow microenvironment, restoring the original interaction between the marrow stroma and the HSCs [14-18].

In comparisons of human cell engraftment rate with unexpanded CBUs and with expanded CBUs, all results reported here are based on the initial cell dose. The initial cell dose before expansion is a valid basis for comparison, being a major determinant of CB selection in CB trials [19,20]. We found greater mean human cell repopulation in the group receiving expanded CBUs, with difference approaching statistical significance ($P = .07$). This finding was consistent in all 3 tissues analyzed (peripheral blood, bone marrow, and spleen). Furthermore, the expanded CBUs provided a similar engraftment rate despite a lower initial cell dose, suggesting that this ex vivo expansion protocol could make CB transplantation a viable option for

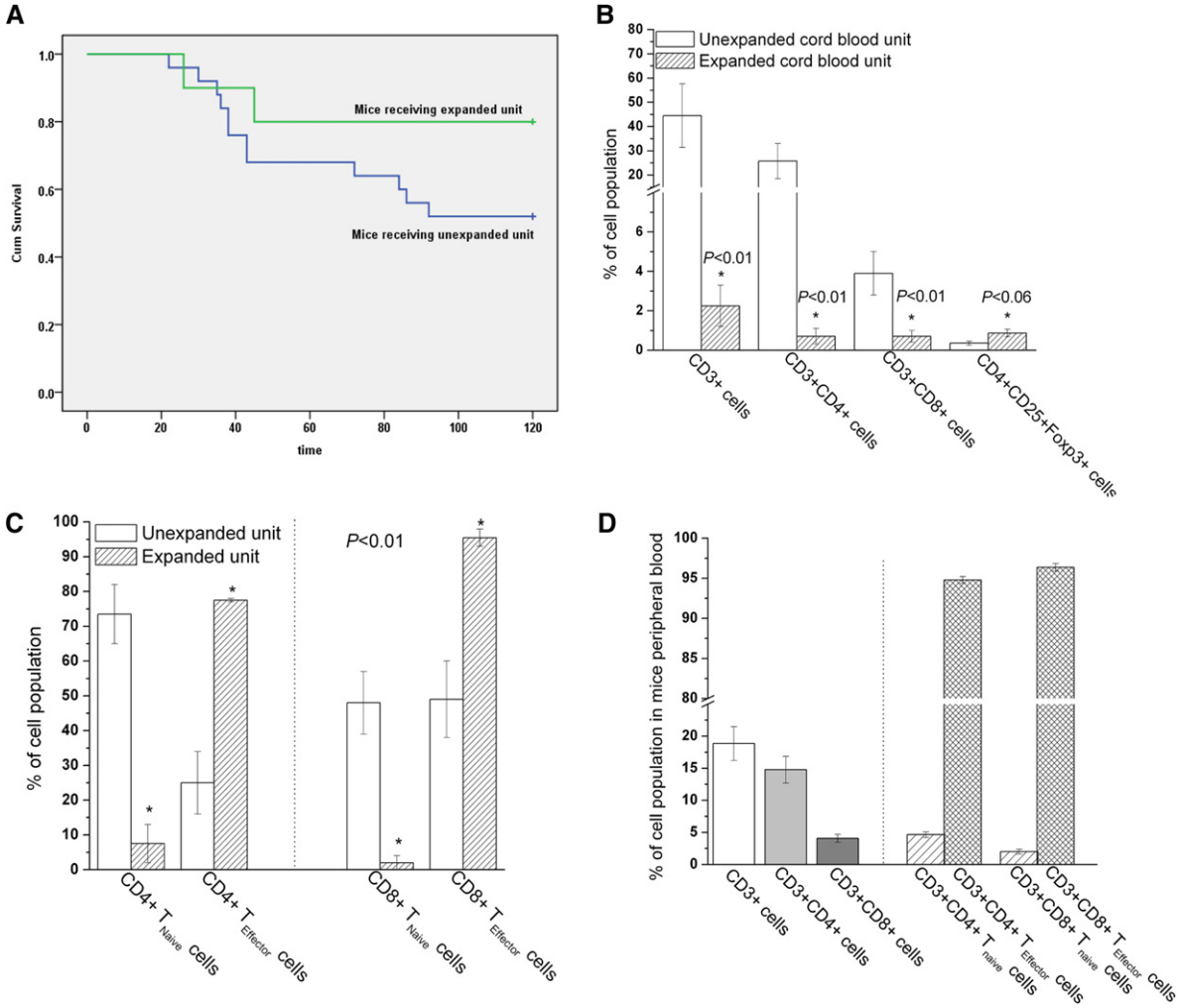

Figure 5. Low survival relates to high population of $CD3^+$ T cells in the unexpanded unit. (A) Kaplan-Meier survival estimates of the probability of survival. A total of 24 NSG mice underwent transplantation with 2×10^6 of double unexpanded units, and 10 NSG mice underwent transplantation with double CBUs comprising 0.5×10^6 unexpanded and 1.5×10^6 expanded cord blood cells. (B and C) Analysis of T cell populations before and after ex vivo expansion. (D) Analysis of T cell populations of the NSG mice peripheral blood on day 45 posttransplantation.

more adult patients by overcoming the barrier of insufficient cell dose.

Despite the superior human hematopoietic engraftment in mice receiving expanded CB cells, it was interesting to note that the unexpanded CBU predominated eventually, as has been reported previously. In 10 subjects with high-risk acute leukemia, Delaney et al. [21] performed clinical ex vivo expansion using immobilized engineered Notch ligand with cytokines (SCF, Flt3 ligand, TPO, IL-6, and Il-3), followed by the infusion of one unexpanded CBU and one Notch-expanded CBU. At 80 days posttransplantation, they found that the expanded CBU contributed to the initial myeloid engraftment, but eventual donor engraftment was 100% from the unexpanded CBU. Although we did not have sufficient DNA to perform the first month's VNTR analysis, the greater human cell engraftment in peripheral blood in the NSG mice that received expanded CBUs suggested that the expanded CBUs, with greater numbers of stem/progenitor cells, can enhance early-stage engraftment. Nonetheless, given that CB transplantation often results in delayed engraftment compared with other HSC sources [22], the rapid engraftment provided by cotransplantation of expanded CBUs could lead to significant reduction in mortality.

The lack of in vivo persistence of the expanded CBUs might possibly be related to a loss of stem/progenitor cell renewal potential or immune rejection. We believe that decreased stem/progenitor cell renewal potential is not responsible, for 3 reasons. First, our expansion results show a 21-fold increase in $CD34^+38^-90^+$ cells (ie, HSCs) compared with controls. Second, our limiting dilution analysis data showing a 3.1-fold increase in HSCs do not appear to support the connection. Finally, our single CB transplantation study (unpublished data) found significantly higher CD34 (stem cell progenitor) repopulation in NSG mice receiving expanded CBUs compared with those receiving unexpanded CBUs.

Based on our data, we believe that the mechanism of single CBU dominance after DCBT involves an immune reaction of a specific $CD8^+$ T cell response against the nonengrafted CBU. This assumption is consistent with findings reported by Gutman et al [23], who demonstrated in an in vitro assay that single-unit dominance resulted from an IFN-γ response by $CD8^+CD45RO^{+/-}CCR7^-$ effector memory T cells in the winning unit against the rejected unit. Yahata et al. [4] reported that when mononuclear cells (ie, B, T, and NK cells) from 2 CBUs were cotransplanted with $CD34^+$ cells into immunodeficient NOD/SCID/γc^{null} mice, engraftment was dominated by one of the CBUs, whereas when only purified $CD34^+$ cells were transplanted, both CBUs engrafted as expected. Of note, our expansion findings show a ~17.5-fold higher $CD3^+$ cell fraction in unexpanded CBUs than in expanded CBUs. Furthermore, although the majority of T cells in the unexpanded CBUs before infusion were naïve T cells, 95% to 98% of these transformed to effector T cells after infusion. As such, the expanded CBU, with a much lower T cell content (<1%), could be rejected by the unexpanded CBU through an immune reaction.

To the best of our knowledge, this is the first report of DCBT in the NSG mouse model using 1 ex vivo expanded CBU and 1 unexpanded CBU, based on an expansion protocol using the novel cytokine IGFBP2 in a mesenchymal stromal coculture system. We have shown that ex vivo expansion resulted in greater engraftment in the immunodeficient mice, whereas the unexpanded CBU predominated in long-term hematopoiesis, possibly due to immune-mediated rejection of the expanded CBU by the unexpanded CBU.

ACKNOWLEDGMENTS

The authors thank Professor Harvey Lodish (Whitehead Institute, MIT) for his advice on mouse transplantation and cord blood expansion protocol and Dr How Gee Fung for her advice on VNTR analysis. The authors also thank Professor John Rush and Dr John Allen for reading the manuscript.

Financial disclosure: This work was supported by National Medical Research Council, the Singapore Stem Cell Consortium, and the Biomedical Research Council. The authors have no conflicts of interest to disclose.

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