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Mesenchymal Stem Cells Secreting Angiopoietin-Like-5 Support Efficient Expansion of Human Hematopoietic Stem Cells Without Compromising Their Repopulating Potential

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Clinical and preclinical applications of human hematopoietic stem cells (HSCs) are often limited by scarcity of cells. Expanding human HSCs to increase their numbers while maintaining their stem cell properties has therefore become an important area of research. Here, we report a robust HSC coculture system wherein cord blood $CD34^+$ $CD133^+$ cells were cocultured with mesenchymal stem cells engineered to express angiopoietin-like-5 in a defined medium. After 11 days of culture, SCID repopulating cells were expanded ~60-fold by limiting dilution assay in NOD-scid $Il2rg^{-/-}$ (NSG) mice. The cultured $CD34^+$ $CD133^+$ cells had similar engraftment potential to uncultured $CD34^+$ $CD133^+$ cells in competitive repopulation assays and were capable of efficient secondary reconstitution. Further, the expanded cells supported a robust multilineage reconstitution of human blood cells in NSG recipient mice, including a more efficient T-cell reconstitution. These results demonstrate that the expanded $CD34^+$ $CD133^+$ cells maintain both short-term and long-term HSC activities. To our knowledge, this ~60-fold expansion of SCID repopulating cells is the best expansion of human HSCs reported to date. Further development of this coculture method for expanding human HSCs for clinical and preclinical applications is therefore warranted.

Introduction

Hematopoietic stem cells (HSCs) are the most studied, best understood stem cells in the human body and are used extensively to treat congenital immunodeficiencies and some hematologic cancers. Human HSCs for clinical use are derived from umbilical cord blood, bone marrow, and mobilized peripheral blood, where HSCs have been stimulated to migrate from bone marrow into the blood by growth factors or drugs. Despite the established use of HSCs from several sources, mismatches in the major histocompatibility complex (MHC) antigens and low absolute numbers obtained from any single donor limit clinical applications. One way to overcome the latter problem is to expand HSCs in vitro, which is especially useful with cord blood samples where cell numbers are strictly limited, but MHC mismatches have reduced impact [1]. There have been 2 recent clinical trials assessing the safety and effectiveness of these approaches, one using mesenchymal stem cells (MSCs) [2] and one using a mixture of 2 cord blood samples one of which had been expanded [3]. Both trials were successful, with 5 of 6 patients who received the HSCs expanded using MSCs in complete remission 1 year after the transplantation [2] and patients who received HSCs from 2 cords, one of which was expanded, showing a significantly reduced period of neutropenia after transplantation [3]. However, by the end of this reported study, the cultured cells had largely vanished from the patients. These findings highlight the clinical benefits already available by injecting cultured cells, on the other hand, the short-term persistence of the in vitro-expanded and engrafted cells critically highlights the need for expansion methods that leave cells able to compete with and eventually replace unmanipulated cells in the long term. Although the short-term benefits of cultured cells have now been demonstrated, long-term persistence is a key requirement that still needs improvements in expansion procedures.

Interest in human HSCs and their expansion has led to the development of a range of assays to determine the stemness or stem cell activity of populations of cells. The key assays of stem cells are functional—they must show self-renewal and

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the ability to generate the appropriate tissue. The best assays available to study this property in putative human HSCs involve xenotransplantation into immune-deficient mice. Demonstration of both long-term (2–3 months) multilineage reconstitution of human blood in a murine host and the ability of the putative HSCs to mediate reconstitution of a secondary host upon re-isolation from the primary mouse are generally accepted as the gold standard for demonstrating the presence of human HSCs. The quantification of HSCs is best done by another sensitive in vivo assay—limiting dilution where small numbers of HSCs are used to minimally reconstitute mice and the failure rate of the assay used to derive the frequency of SCID repopulating cells (SRCs). Finally, to assess the relative fitness of different populations of HSCs, competition assays in a single host are used.

The techniques described above have been used to assess many specific procedures for their ability to isolate or expand HSCs. Two types of approaches have been generally used to expand HSCs in vitro: feeder cell-free (FCF) culture in the presence of specific growth factors and coculture with feeder cells, such as MSCs. In FCF culture, the best in vitro expansion of human HSCs achieved to date has resulted in ~16–20-fold increases in SRCs. This has been achieved with 2 distinct cocktails of growth factors. First, Delaney et al. used a cocktail-based around notch ligand, which achieved an ~16-fold increase in SRCs and was used in the successful clinical trial with 2 cord blood samples described above [3]. Second, Zhang et al. used thrombopoietin (TPO), stem cell factor (SCF), fibroblast growth factor-1 (FGF-1), insulin-like growth factor binding protein 2 (IGFBP2), and angiopoietin-like-5 (Angptl5), and reported an ~20-fold expansion of SRCs after a 10–11-day culture [4,5].

Coculture with feeder cells to mimic HSCs' physiological microenvironment has also been extensively characterized. MSCs from bone marrow [6], Wharton's jelly of umbilical cord segments [7], cord blood [8], and other fetal tissues [9–11] have been shown to support expansion of human HSCs [6–8,11–16]. Depending on the coculture conditions, 80–800-fold increase in total cell number and 4–100-fold increase in $CD34^+$ cells were reported [17–19]. The highest expansion was achieved by culturing human HSCs with MSCs in a 3D configuration using a woven mesh where ~100-fold expansion of $CD34^+$ cells was achieved in 10 days [5]. However, most studies with coculture did not rigorously examine the SCID repopulating activity of expanded cells in mice using long-term reconstitution, limiting dilution or competitive reconstitution assays. In the few cases where expanded cells were tested in mice, only one dose of expanded progeny was injected into NOD-scid mice and reconstitution was assayed 6 weeks later in the bone marrow [5,18,20]. For example, when the progeny of $CD34^+$ cells cocultured with human brain endothelial cells were tested in mice, a 3.4-fold increase in SRCs was obtained in NOD-scid mice [21].

We reasoned that combining the best FCF culture conditions with the extra support from feeder cells would result in better expansion, both in terms of SRC number and long-term competitive fitness. We therefore combined the FCF culture method developed by Zhang et al. with support from a feeder layer of primary human MSCs. After in vitro culture we characterized the cells using CD34 and CD133 coexpression as a marker for stem cell activity, which we have previously demonstrated correlates with human HSC activity in culture (Drake et al. manuscript submitted). This immediate readout of culture was used to identify optimal culture conditions that we rigorously evaluated using long-term, serial and competitive reconstitutions. Our results show robust HSC growth, achieving ~60-fold expansion of $CD34^+$ $CD133^+$ cells, and most critically ~60-fold expansion of SRCs in an 11-day coculture. The expanded HSCs from the improved coculture give rise to multilineage engraftment of hematopoietic cells in NSG mice, with enhanced T-cell reconstitution as compared to expanded HSCs from FCF culture. Critically expanded HSCs result in serial reconstitution and show equal fitness to uncultured cells in competition assays. These findings suggest that coculture in the presence of selected growth factors in the medium is an efficient method to expand human HSCs in vitro. The enhanced T-cell reconstitution by such expanded HSCs may be of particular interest in instances where a rapid T-cell reconstitution is desirable.

Materials and Methods

Cell purification

Purified human $CD133^+$ cord blood cells were purchased from AllCells. Alternatively, umbilical cord blood was obtained from the National Disease Research Interchange or the Singapore Cord Blood Bank. $CD34^+$ cells were purified with the RosetteSep system using the CD34-positive selection kit (Stem Cell Technologies). The purity of $CD34^+$ cells was 90%–99%. After in vitro expansion, $CD133^+$ cells were purified by staining cells with PE-conjugated anti-CD133 (E-Biosciences) followed with a PE-positive selection kit (Stem Cell Technologies).

FCF culture of $CD34^+$ $CD133^+$ cord blood cells

Cryopreserved or freshly isolated $CD34^+$ $CD133^+$ cord blood cells were cultured in vitro as described [4]. Briefly, StemSpan medium (Stem Cell Technologies) was supplemented with 10 ng/mL SCF (R&D), 20 ng/mL TPO (R&D), 10 ng/mL FGF-1 (Gibco), 100 ng/mL IGFBP2 (R&D), 500 ng/mL Angplt5 (Abnova), 10 μg/mL of heparin (Sigma), and 1× penicillin and streptomycin (Gibco) to obtain the expansion medium. About 10^4 $CD34^+$ $CD133^+$ cord blood cells were plated in each well of a 96-well round-bottomed plate, in 200 μL/well of the expansion medium.

Angplt5-expressing MSCs

The human *Angptl5* gene (DNA NM_178127.2; Origene) was cloned into the pLB2 lentiviral vector. The resulting vector encodes both Angplt5 and green fluorescent protein (GFP) under the same EF1α promoter [kindly provided by Patrick Stern and Richard Hynes of Massachusetts Institute of Technology (MIT)]. To produce lentivirus, 293FT cells were cotransfected with lentiviral vectors, the HIV-1 packaging vector Delta8.9 and the VSVG vector. Human MSCs from the bone marrow of adult donors were purchased commercially (Lonza or Stem Cell Technologies). MSCs were cultured in MesenCult MSC Basal Medium (Stem Cell Technologies) and transduced with the lentivirus expressing GFP alone or both GFP and Angplt5 at a multiplicity of infection (MOI) of 5. Four to 5 days post-transduction, 30%–45% of cells were GFP^+ by

flow cytometry analysis. The expression of Angptl5 was confirmed by quantitative PCR and Western blot (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/scd).

Coculture of $CD34^+$ $CD133^+$ cord blood cells

MSCs expressing Angptl5 (referred to as MSC-A5) or MSCs expressing GFP only (referred to as MSC-GFP) were plated in a 24-well plate at 5×10^4 cells/well overnight. About 10^4 $CD34^+$ $CD133^+$ cells were then added to the MSC culture per well (at a ratio of 1:5) in the expansion medium similar to FCF conditions but without Angptl5. At different time points, hematopoietic cells were resuspended, counted, and analyzed by flow cytometry to obtain the total cell number and the number of double-positive (DP) cells. Because early passages of MSCs are superior at in vitro expansion of $CD34^+$ $CD133^+$ cord blood cells [6], we used MSCs that have passaged only 4–8 times in this study.

Mice and intracardiac injection

NSG mice were obtained from the Jackson Laboratory and bred in the animal facilities at Massachusetts Institute of Technology, Nanyang Technological University, and National University of Singapore. Pups within 48 h of birth were sublethally irradiated (100 rad) and engrafted with HSCs by intracardiac injection. About 10^5 unexpanded or expanded $CD34^+$ $CD133^+$ cells or total expanded cells containing 10^5 $CD34^+$ $CD133^+$ cells were injected per recipient. All research with human samples and mice was performed in compliance with the institutional guidelines.

Limiting dilution, serial, and competitive reconstitution assays

For limiting dilution assays, 5,000, 1,000, and 400 unexpanded or expanded $CD34^+$ $CD133^+$ cells from the same cord blood were injected into sublethally irradiated pups. A nontoxic green food dye [Apple Green Liquid Dye (containing tartrazine E142), Bake King; Gim Hin Lee (Pte) Ltd.] was mixed with cells just before injection to monitor the success of the injection. SRC frequency in limiting dilution assays was determined by the method of maximum likelihood with L-CALC software (StemCell Technologies). Serial reconstitution was performed as follows: 14 weeks after the primary reconstitution, bone marrow cells were harvested from both femurs and tibia and human $CD34^+$ cells were purified by anti-CD34 selection (Stem Cell Technologies). Purified $CD34^+$ cells were pooled from different primary mice reconstituted with the same expanded cells and 10^4 cells were injected into sublethally irradiated adult NSG mice. For the competitive reconstitution assay, the expanded and unexpanded cells from HLA-$A2^+$ donor were mixed in equal number with unexpanded or expanded cells from HLA-A^- donor and injected into sublethally irradiated newborn pups.

Analysis of reconstituted mice

Single-cell suspensions from blood, spleen, and/or bone marrow were prepared, counted, and stained with antibodies specific for human CD3, CD11c, CD14, CD15, CD19, CD33, CD34, CD45, CD56, HLA-DR (Biolegend), CD133 (Miltenyi or EBiosciences), and murine CD45.1 (Biolegend). Stained cells were analyzed on FACScalibur, FACS-Canto, or LSR II cytometers (Beckton Dickinson). Analyses were performed with FlowJo Software (Tree-Star). Hematoxylin and eosin (H&E) staining was performed on frozen spleen sections. For immunofluorescence staining of spleen sections of both reconstituted and nonreconstituted NSG mice, monoclonal antibody to CD20 (clone L26; Abcam) and polyclonal antibodies to CD3 (Abcam) were used as primary antibodies. Alexa Fluor647 donkey anti-mouse IgG (Invitrogen) and Alexa Fluor546 donkey anti-rabbit IgG (Invitrogen) were used as secondary antibodies. H&E stains were observed with a light microscope and immunofluorescence stains were observed using a slide scanner (Mirax Midi; Zeiss).

Immunization and ELISPOT assay

Sixteen weeks after engraftment, mice were immunized with tetanus toxoid (TT) vaccine (Tetavax; Sanofi Pasteur). Specifically, 10 μL of the vaccine, representing 1/50 of the recommended vaccination dose for an adult human, was mixed with 90 μL PBS and then injected intraperitoneally. Mice were boosted twice with the same dose in 3-week intervals. Two weeks after the last immunization, single-cell suspensions were prepared from spleens for ELISPOT assay (EBiosciences). About 5×10^5 $CD3^+$ cells were plated per well in a 96-well flat-bottomed plate (Multiscreen-IP; Millipore) in the medium alone or in the presence of either 10 ng/mL of phorbol myristate acetate (PMA) or 0.5 μg/mL of a TT peptide (Genscript). Spots were counted using an ImmunoSpot S5 Versa Analyzer (Cellular Technology Ltd.) and analyzed with ImmunoCapture software (Analysis Software).

Results

Combination of coculture and selected growth factors enhances expansion of $CD34^+$ $CD133^+$ cord blood cells

To enhance HSC expansion, we investigated culture conditions that combine an MSC feeder layer and the HSC growth factors described by Zhang et al. [4]. Human Angptl5 was used at high concentrations in this FCF culture; however, MSCs produce it at low levels (data not shown). We reasoned that ongoing production of Angptl5 by the feeder cells might be superior to bolus doses but that given the large amounts needed for HSC expansion in vitro, the endogenous production might be inadequate. We therefore transduced MSCs with lentiviral vectors expressing either GFP (MSC-GFP) or both GFP and Angptl5 (MSC-A5). MSC-GFP served as a baseline for coculture, whereas MSC-A5 allowed us to test the effect of higher levels of Angptl5 on HSC expansion. Purified $CD133^+$ cord blood cells, which were also $CD34^+$, were cultured with a confluent MSC-A5 or MSC-GFP feeder layer at a starting ratio of 1:5 and in the FCF culture as a control. All cultures included SCF, FGF, IGFBP2, and TPO in the medium; the FCF culture was also supplemented with Angptl5 as described [4]. For direct comparison, purified $CD133^+$ cells from the same cord blood were cultured in all conditions. At the start of the coculture, hematopoietic cells attached to the MSC feeder layer (Fig. 1A). By day 5, the density of hematopoietic cells was noticeably increased. By day 7, proliferation

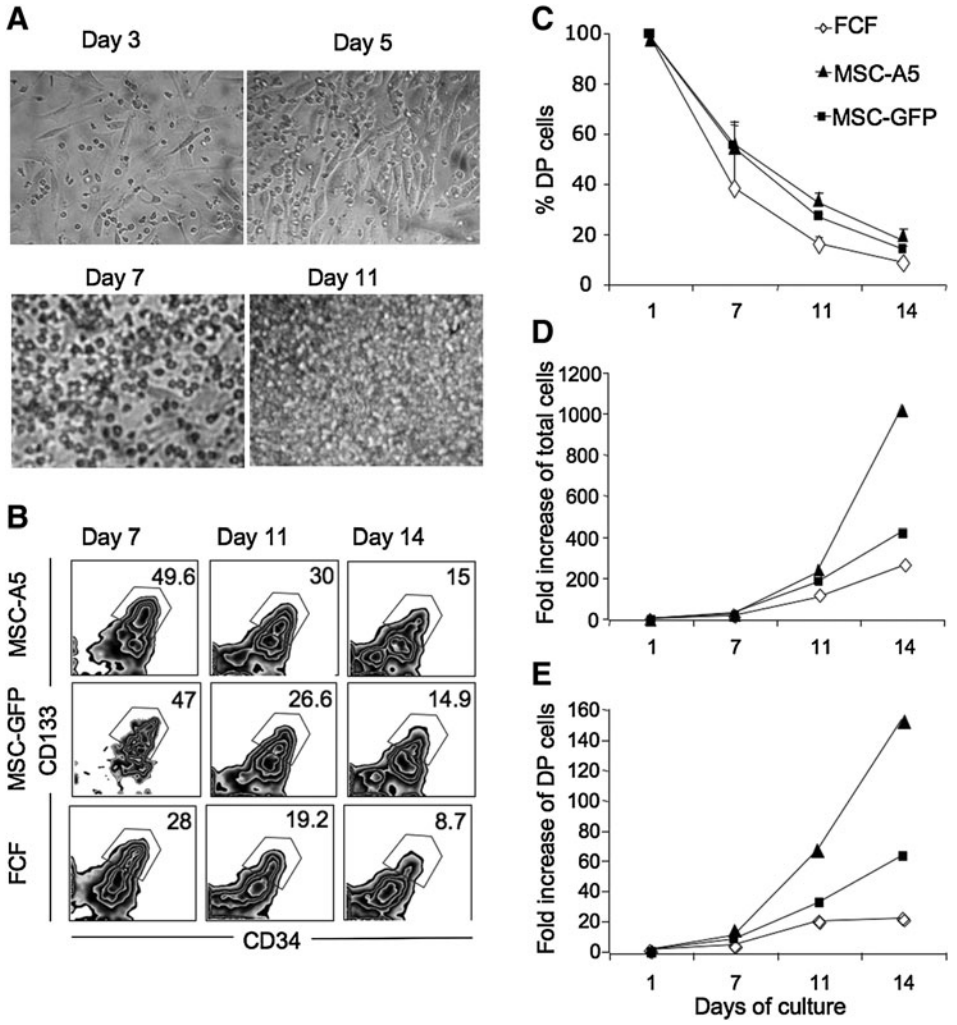


FIG. 1. Combination of coculture and growth factors enhances expansion of $CD34^+$ $CD133^+$ cord blood cells. Purified $CD34^+$ $CD133^+$ cord blood cells were cocultured with MSC-A5 or MSC-GFP in the presence of SCF, FGF, IGFBP2, TPO, and heparin or cultured in the FCF culture. **(A)** Coculture with MSC-A5 was observed at indicated time points using an inverted microscope (10× magnification). **(B–E)** Nonadherent cells in MSC-A5 and MSC-GFP cocultures and the FCF culture were harvested at 7, 11, and 14 days, counted, and analyzed for CD34 and CD133 expression. **(B)** Representative CD34 versus CD133 staining profiles of cultured cells. The number indicates the percentage of DP cells in the gated region. **(C)** The average of percentages of DP cells in the cultures at different time points from 3 experiments. **(D)** Fold increase of total hematopoietic cells during the course of the cultures. **(E)** Fold increase of $CD34^+$ $CD133^+$ DP cells during the course of the cultures. Representative data from 1 of 4 experiments (Supplementary Table S1) are shown. DP, double positive; FCF, feeder cell-free; FGF, fibroblast growth factor-1; IGFBP2, insulin-like growth factor binding protein 2; MSC, mesenchymal stem cell; SCF, stem cell factor; TPO, thrombopoietin.

of hematopoietic cells was evident (Fig. 1A, D). By day 11, hematopoietic cells had completely covered the MSC-A5 feeder layer (Fig. 1A).

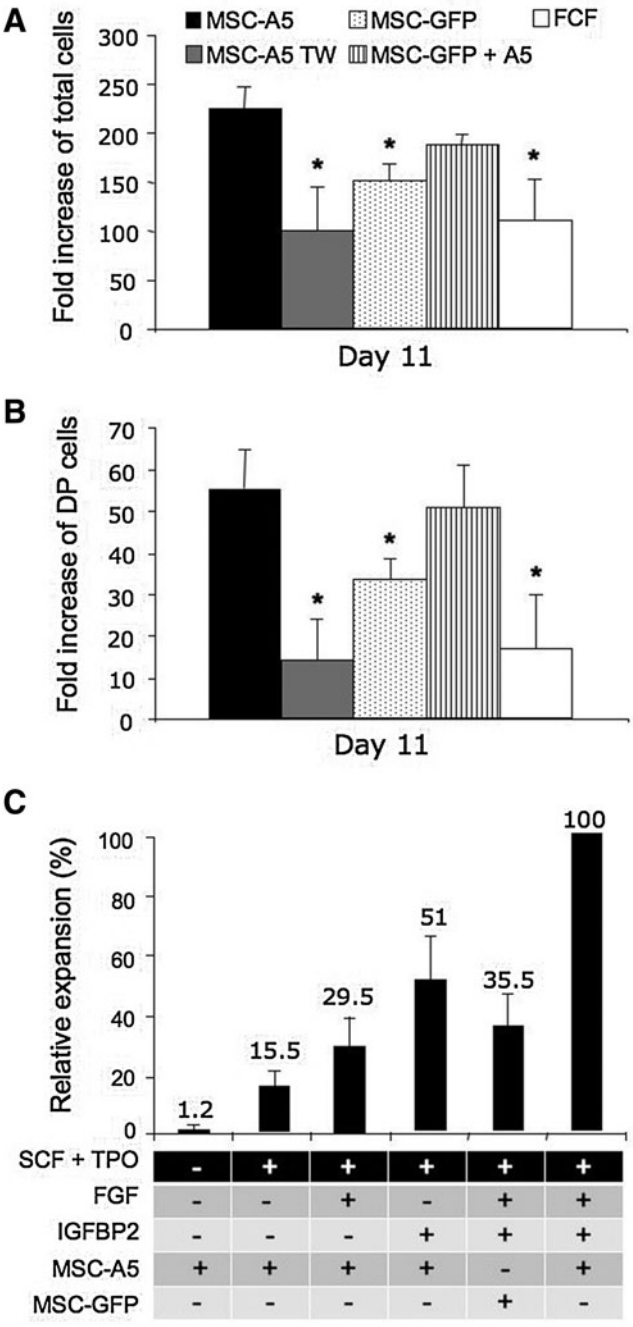
Recently, we have shown that SRCs reside in the $CD34^+$ $CD133^+$ DP fraction of in vitro-expanded cells and that the expansion of HSCs in culture correlates linearly with the DP cell expansion (Drake et al., submitted). At the beginning of the coculture, >95% of the input cells were $CD34^+$ and $CD133^+$ (Supplementary Table S1). After culture for 7 days, the percentage of DP cells decreased to ~50% in MSC-A5 or MSC-GFP coculture and ~30% in FCF culture (Fig. 1C). The percentages continued to decrease and by day 14 to ~15% in the cocultures and ~10% in the FCF culture. Despite the decrease of the percentages of DP cells in the cultures, because the total cell numbers increased dramatically (Fig. 1D), the actual numbers of DP cells were increased ~60-fold (46–66-fold) in MSC-A5 coculture, ~29-fold (22–37-fold) in MSC-GFP coculture, and ~20-fold (16–26-fold) in FCF culture at day 11 (Fig. 1E). By day 14, the number of DP cells did not increase significantly in the FCF culture, whereas the number of DP cells increased to ~150 and ~60-folds in MSC-A5 and MSC-GFP cocultures, respectively. The study was repeated 4 times and the data are summarized in Supplementary Table S1. These results show that combination of MSCs and the growth factors supports a much more robust expansion of $CD34^+$ $CD133^+$ HSCs as compared to FCF culture. Although lack of ELISA antibodies prevented measurement of the precise concentration of secreted Angptl5 in these experiments, our results clearly show that the optimal HSC expansion requires a level of Angptl5 above that normally produced by MSCs.

Both cell-to-cell contact and soluble factors contribute to the enhanced expansion of $CD34^+$ $CD133^+$ cord blood cells

We performed the cocultures using transwells to prevent direct contact between MSC-A5 and $CD34^+$ $CD133^+$ cord blood cells. During 11 days of culture, both the fold increase of total hematopoietic cells and fold increase of DP cells were similar in the transwell coculture and the FCF culture. In contrast, coculture with MSC-GFP led to a greater expansion of both total cells and DP cells and coculture with MSC-A5 led

to an even greater expansion of total cells and DP cells (Fig. 2A, B). Addition of recombinant Angplt5 to MSC-GFP coculture resulted in further expansion of both total cells and DP cells to a level similar to that seen in MSC-A5 coculture, confirming that greater than physiological expression of Angptl5 enhances in vitro culture. These results suggest that contact between MSCs and $CD34^+$ $CD133^+$ cells is a significant factor contributing to the enhanced expansion of DP cells.

We also performed HSC cocultures without adding specific growth factors. For ease of comparison, DP cell expansion in culture was normalized to the fully supplemented MSC-A5 coculture condition, which gave the highest level of expansion (Fig. 2C). When $CD34^+$ $CD133^+$ cells were cocultured with MSC-A5 without the addition of any of the growth factors, they did not expand at all. Addition of just SCF and TPO, which are often used together for HSC expansion [22,23], to the above coculture led to expansion of DP cells, reaching 19% of that obtained under the fully supplemented coculture condition. With further addition of FGF or IGFBP2, the relative expansions increased to 36% and 61%, respectively. Without exogenously added Angplt5 (coculture with MSC-GFP in the presence of SCF, TPO, FGF, and IGFBP2), the relative expansion was 43% (Fig. 2A, B). These results suggest that all 5 growth factors contribute to the expansion of DP cells in the coculture as in the FCF culture. We therefore focused on MSC-A5 culture (the best new condition identified) and compared it to FCF culture (the starting point for the improvement) and uncultured cells (as a universal baseline).



Expanded DP cells maintain repopulating capacity in vivo

We assessed the SCID repopulating activity of the expanded cells by limiting dilution. $CD34^+$ $CD133^+$ cells were expanded in either MSC-A5 coculture or FCF culture and purified. Sublethally irradiated NSG pups were each engrafted with 5,000, 1,000, and 400 unexpanded or expanded $CD34^+$ $CD133^+$ cells from the same cord blood and analyzed 8 weeks later. A frequently used threshold of 0.05% was selected as the minimum chimerism in the bone marrow for the engraftment to be considered successful. When 5,000 cells were injected, 100% of the mice were chimeric; when 1,000 and 400 cells were injected the percentages of chimeras decreased. Additionally, all 3 conditions showed no significant difference in the percentages of human $CD45^+$ cells in the bone marrow of reconstituted mice at each cell dose (Fig. 3A). The frequency of SRCs in unexpanded $CD34^+$ $CD133^+$ cells was calculated, by limiting dilution analysis using Poisson statistics, as 1 in 1,024 with 95% confidence interval (CI) of 1/535–1/1,958 (Fig. 3B). The frequency of SRCs in DP cells from MSC-A5 coculture and FCF culture was 1 in 726 (with 95% CI of 1/334–1/1,578) and 1 in 506 (with 95% CI of 1/233–1/1,096), respectively. Because DP cells were expanded 58 and

FIG. 2. Both cell-to-cell contact and soluble factors contribute to the enhanced expansion of $CD34^+$ $CD133^+$ cord blood cells. **(A, B)** Purified $CD34^+$ $CD133^+$ cord blood cells were cocultured with MSC-A5 or MSC-GFP with SCF, TPO, FGF, and IGFBP2 added in the medium, or cultured under the FCF condition, or cocultured with MSC-A5 in transwells (MSC-A5 TW) with the 4 growth factors, or cocultured with MSC-GFP with the 4 growth factors plus recombinant Angplt5 (MSC-GFP + A5). At day 11 of the culture, the fold increase of total and DP cells were determined. **(A)** Fold increase of total hematopoietic cells during the course of the culture. **(B)** Fold increase of DP cells during the course of the culture. **(C)** $CD34^+$ $CD133^+$ cord blood cells were cocultured with MSC-A5 or MSC-GFP with or without the indicated growth factors for 11 days. The expansion of DP cells in different cultures was normalized to that under the standard condition where $CD34^+$ $CD133^+$ cord blood cells were cocultured with MSC-A5 in the presence of SCF, TPO, FGF, and IGFBP2 in the medium. Shown are relative percentages of DP cells at day 11 of culture under the different conditions. The MSC-A5 condition is the reference group for *P* values. Angplt5, angiopoietin-like-5. $^*P < 0.05$.

18-fold in MSC-A5 coculture and FCF culture, respectively, total SRCs were therefore expanded 82 [(1,024/726)×58] and 36 [(1,024/506)×18] fold.

We further assessed the reconstitution capacity of expanded DP cells using a competitive repopulation assay. To track transferred cells from different donors within the same recipient, 2 cord blood samples differing in expression of MHC class I gene HLA-A2 were used. Expanded $CD34^+$ $CD133^+$ cells from 1 cord was mixed with equal number of unexpanded cells from the other cord or vice versa. Unexpanded $CD34^+$ $CD133^+$ cells from both cords were also mixed in equal proportion and transferred. Eight weeks postengraftment, ~50% of human $CD45^+$ cells in the bone marrow were HLA-$A2^+$ and ~50% HLA-$A2^-$ in all 3 groups of mice (Fig. 3C). These results further suggest that in vitro expansion does not significantly diminish the ability of DP cells to reconstitute NSG recipients.

Expanded cells are capable of stable long-term and efficient secondary reconstitution

The expanded cells were further tested for their ability to give rise to stable long-term reconstitution in NSG recipients. Total cultured cells containing 10^5 DP cells were injected into each recipient mouse. Twelve weeks after engraftment, the percentage of human leukocytes in the blood was ~40% (Fig. 4A). This percentage increased slightly or were maintained at 24 weeks after engraftment (Fig. 4A, B). No difference in reconstitution was observed between expanded cells from MSC-A5 coculture or FCF culture in the blood and spleen (Fig. 4A). These results suggest that expanded cells from both protocols possess similar levels of long-term reconstitution activity.

We also assessed the presence of long-term HSC activity in the expanded cell population by serial transfer. Expanded cells from day 11 MSC-A5 coculture were transferred into irradiated NSG pups. Fourteen weeks after engraftment, human $CD34^+$ cells were magnetically enriched from the bone marrow of primary recipient mice and injected into sublethally

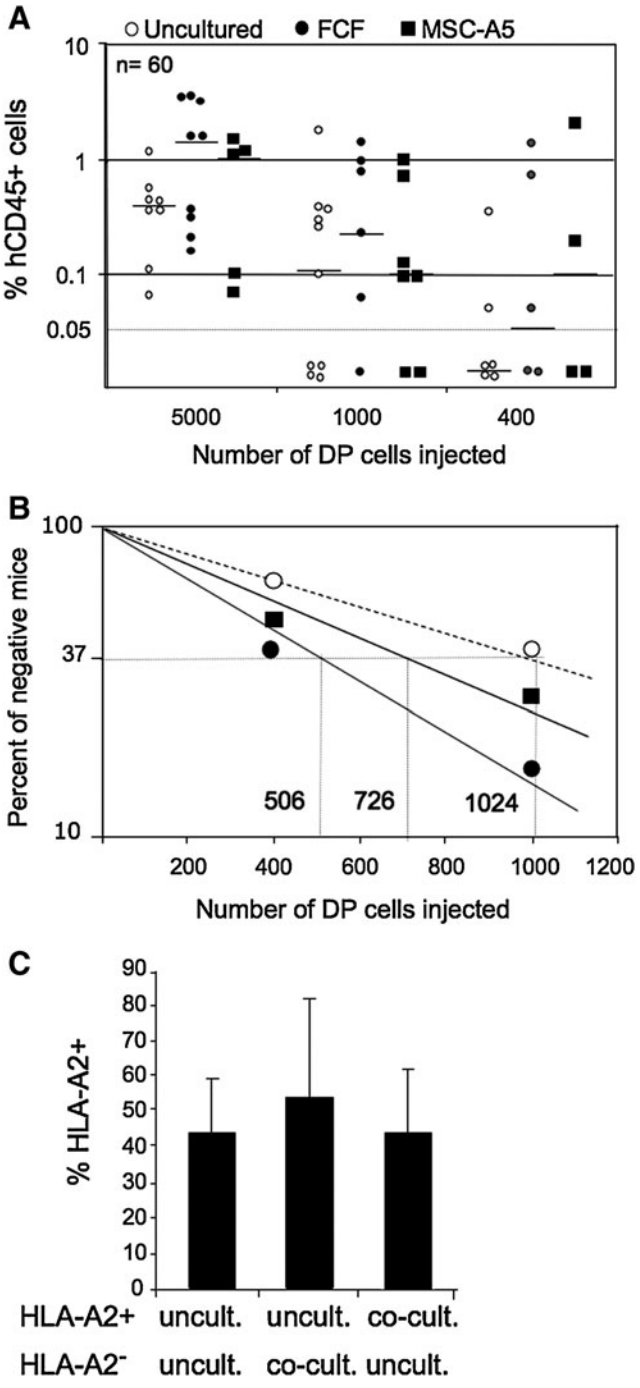


FIG. 3. Expanded DP cells have the same repopulation capacity as unexpanded cells. **(A, B)** Limiting dilution assay, $CD34^+$ $CD133^+$ cord blood cells were expanded by coculture or FCF culture for 11 days, and DP cells were purified by immunomagnetic cell isolation (95% purity). About 5,000, 1,000, or 400 unexpanded $CD34^+$ $CD133^+$ cells or expanded DP cells from the same cord blood donor were engrafted into irradiated newborn pups. A nontoxic green food dye was mixed with cell solutions just before the injection to monitor whether the intracardiac injection was successful (pups turn green immediately after the injection). Mice with failed injections were excluded from further analysis. Eight weeks later, mice were analyzed for human $CD45^+$ cells in the bone marrow. **(A)** Comparison of percentages of human $CD45^+$ cells in the bone marrow of recipient mice injected with different numbers of unexpanded or expanded DP cells. Mice were considered as nonchimera if the percentage of human $CD45^+$ cells in the bone marrow is below 0.05%. The data were from 1 of 3 experiments. Each symbol represents 1 mouse. The horizontal lines indicate the mean. **(B)** Limiting dilution analysis for estimating the frequency of SCID repopulating cells. The numbers shown indicate the calculated frequency of SCID repopulating cells using the maximum likelihood estimator. **(C)** Comparison of unexpanded and expanded DP cells in competitive repopulation assay. $CD34^+$ $CD133^+$ cord blood cells were purified from a HLA.$A2^+$ and a HLA-$A2^-$ cord blood. A portion of the cells from each donor was expanded on MSC-A5 coculture for 11 days and $CD34^+$ $CD133^+$ cells purified. Unexpanded or expanded cells from HLA-$A2^+$ donor were mixed in equal proportion (5×10^4 from each) with unexpanded or expanded cells from HLA-$A2^-$ donor and transferred into irradiated newborn pups. Eight weeks after injection, peripheral blood mononuclear cells. (PBMCs) were analyzed for human CD45 and HLA-A2 by flow cytometry. The average percentages of HLA-$A2^+$ cells among human $CD45^+$ cells are shown ($n = 5$ per group).

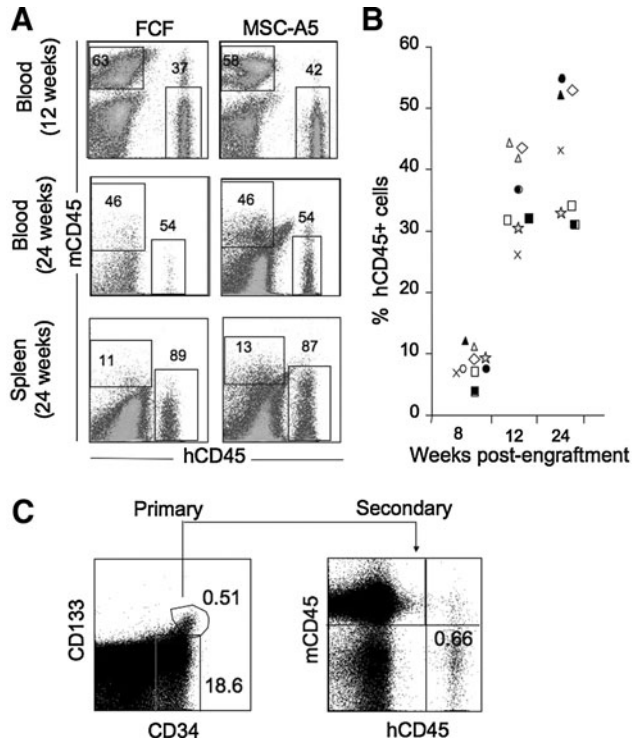


FIG. 4. Expanded cells are capable of stable long-term and efficient secondary reconstitution. **(A)** Stable long-term reconstitution by expanded cells. Day-11-expanded cells (of the same cord blood donor) from either MSC-A5 coculture or FCF culture were engrafted into sublethally irradiated newborn pups (10^5 DP cells per recipient). Mice were bled at the indicated time points and PBMCs were analyzed for human CD45 (hCD45) and mouse CD45 (mCD45). Reconstitution in the spleen was also assayed in some mice 24 weeks after engraftment. Dot plots show mCD45 versus hCD45 staining profiles of PBMCs at 12 and 24 weeks, or spleen at 24 weeks of the same mouse engrafted with either expanded cells from coculture or FCF culture. **(B)** Comparison of percentages of human CD45 cells in PBMCs of mice engrafted with expanded cells from MSC-A5 coculture at 8, 12, and 24 weeks after engraftment. Each symbol represents 1 mouse. The same symbol represents the same mouse at different time points. **(C)** Serial transfer of human $CD34^+$ cells. Day-11-expanded cells from MSC-A5 coculture were transferred into irradiated newborn pups (10^5 DP cells per recipient). Fourteen weeks later, bone marrow cells were harvested from primary mice and human $CD34^+$ cells were enriched and transferred into sublethally irradiated adult NSG mice. Twelve weeks after secondary transfer, the presence of human $CD45^+$ cells in the bone marrow was analyzed. *Left panel*, CD133 versus CD34 staining profile of bone marrow cells is shown for 1 primary mouse gating on $hCD45^+$ cells. *Right panel*, mCD45 versus hCD45 staining profile is shown for a representative secondary recipient mouse. The experiments were done twice, using cells from 2 different cord blood samples.

irradiated adult NSG mice. Twelve to 16 weeks later, among the 14 secondary recipient mice, 11 had 0.1%–2% of human $CD45^+$ cells in the bone marrow, 1 had 11%, and the other 2 had $\sim$0.03%. A representative mouse is shown in Fig. 4C. These data show that the expanded $CD34^+$ $CD133^+$ cells from MSC-A5 coculture retain secondary reconstitution potential, suggesting the presence of long-term HSC activity.

Expanded $CD34^+$ $CD133^+$ cells give rise to multiple lineages of blood cells in NSG mice

We tested the ability of expanded cells from the cocultures to reconstitute different lineages of human blood cells in NSG mice. The chimerism, as defined by the percentage of human $CD45^+$ cells among both human and mouse $CD45^+$ cells, in the bone marrow, spleen, and peripheral blood was similar among mice engrafted with cells from either FCF culture or MSC-A5 coculture. Among human blood lineage cells analyzed, T cells ($CD3^+$), B-lineage cells ($CD19^+$), macrophages ($CD14^+$ $CD33^+$), myeloid precursor cells ($CD33^+$), hematopoietic stem/precursor cells ($CD34^+$, $CD34^+$ $CD133^+$, or $CD19^+IgM^+$), dendritic cells ($CD11c^+$ $HLA\text{-}DR^+$), and natural killer cells ($CD56^+$) were detected in the blood, spleen, or bone marrow (Fig. 5A and data not shown). In mice reconstituted with coculture-expanded cells, a significant increase in $CD3^+$ T cells was observed in blood and spleen ($P < 0.05$). The higher percentages of $CD3^+$ T cells were already detectable in the peripheral blood 8 and 12 weeks after reconstitution (Fig. 5B). The reconstitution of all other lineages was similar (Table 1). These results demonstrate that expanded cells from the cocultures are capable of differentiating into multiple lineages of blood cells in NSG mice.

H&E staining revealed a restoration of the splenic architecture with the appearance of the white pulp in mice reconstituted with coculture-expanded cells but not in unreconstituted NSG mice (Fig. 5C, D). Further, T cells and B cells were found in the same follicles, with $CD3^+$ T cells residing predominantly in the inner region of the follicle and surrounding the central arteriole, whereas $CD20^+$ B cells reside in the outer region of the follicle (Fig. 5E, F). These results suggest that there is a partial recovery of secondary lymphoid structure.

Human T cells are functional in the reconstituted mice

The enhanced T-cell development with coculture-expanded cells prompted us to test T-cell functionality. $CD34^+$ $CD133^+$ cord blood cells were expanded for 11 days in either the coculture or the FCF culture and injected into sublethally irradiated newborn pups. Similarly, reconstituted mice with expanded cells from either coculture or FCF culture were immunized with TT thrice. Two weeks after the last immunization, splenocytes were assayed for IFN-γ expression by ELISPOT assay after stimulation with either PMA or a TT peptide. With PMA stimulation, significant numbers of IFN-γ immunospots were detected in all splenocyte samples (Fig. 5G). With peptide stimulation, significant numbers of spots were detected only in TT immunized mice. However, the average numbers of spots were significantly higher in mice reconstituted with cells from the coculture than the FCF culture (104 ± 37 vs. 22 ± 14, $P < 0.005$). Further, the distribution of $CD3^+$ cells and $CD20^+$ cells in the spleen of the immunized animals was changed. The rearrangement of these 2 cell populations was mixed, suggesting possible interaction between T and B cells (Supplementary Fig. S2).

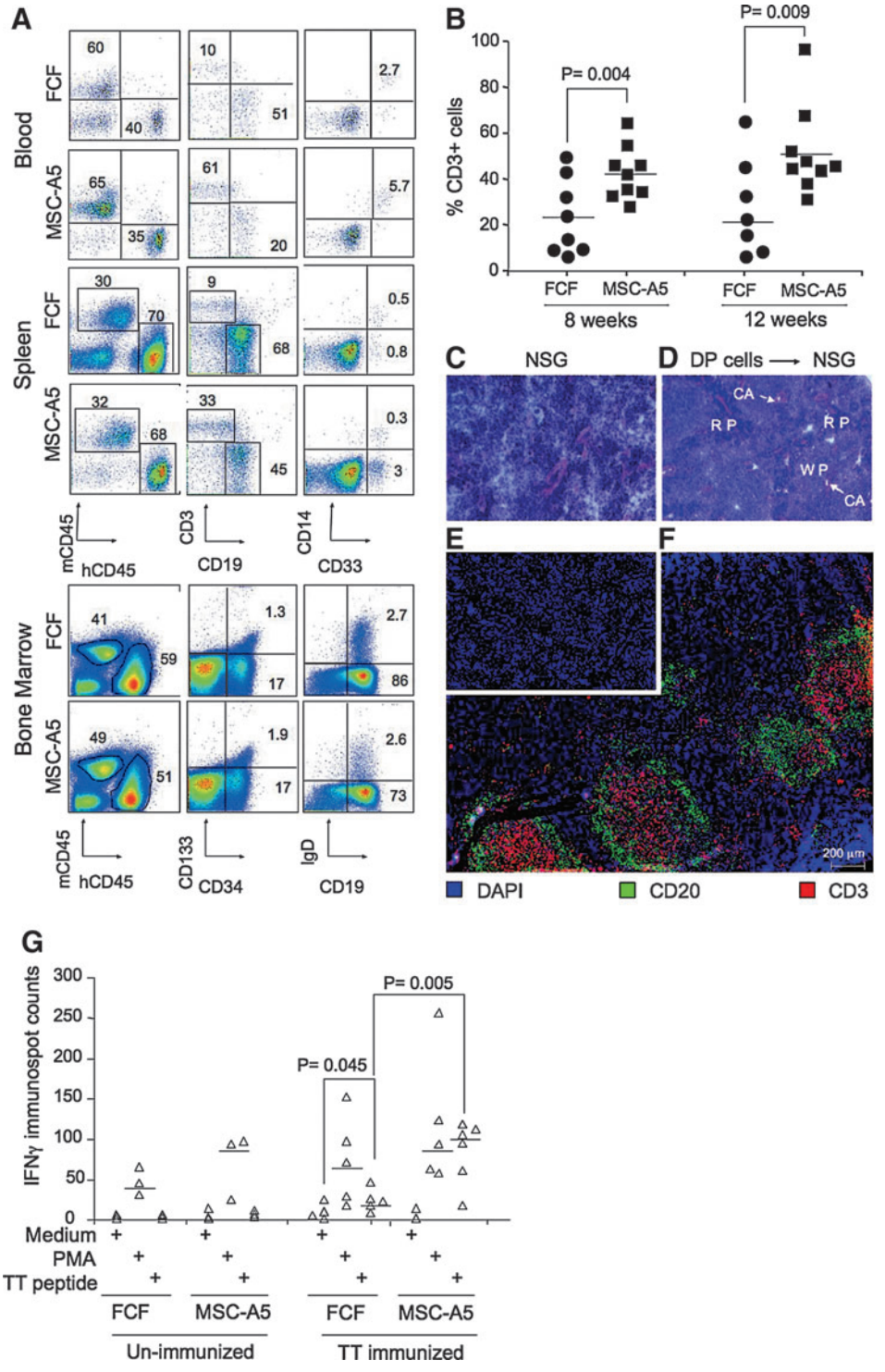


FIG. 5. Expanded cells are capable of differentiating into multiple lineages of hematopoietic cells in NSG mice. **(A)** $CD34^+$ $CD133^+$ cells from the same donor were either expanded in the MSC-A5 coculture or the FCF culture for 11 days. Total expanded cells, containing 10^5 DP cells, were engrafted into sublethally irradiated NSG neonates. Fourteen weeks after injection, the presence of various lineages of human hematopoietic cells in the blood, spleen, and bone marrow were analyzed by flow cytometry. All staining profiles were gated on human $CD45^+$ cells, except for mCD45 versus hCD45 staining profiles, which were gated on total live cells from the individual tissues. The percentage of human $CD45^+$ cells is calculated by [%$hCD45^+$/(%$hCD45^+$ + %$mCD45^+$)] and the percentage of mouse $CD45^+$ cells is calculated by [%$mCD45^+$/(%$hCD45^+$ + %$mCD45^+$)]. Representative dot plots are shown from 1 set of mice reconstituted with cells from the same cord blood. Cord blood from 3 different donors were used. **(B)** Blood was sampled at 8 and 12 weeks postengraftment and mononuclear cells were stained for human CD45, CD3, and CD19. The percentages of $CD3^+$ cells among $CD45^+$ cells are shown for mice reconstituted with expanded cells from MSC-A5 cocultures or FCF culture. Each symbol represents 1 mouse. The horizontal line indicates the median value. **(C–F)** Comparison of hematoxylin and eosin, or CD3 and CD20 immunohistochemical staining of spleen sections of NSG mice **(C, E)** and NSG mice engrafted with expanded cells from MSC-A5 cocultures **(D** and **F,** 20 weeks after engraftment**). (C, D)** Hematoxylin and eosin staining, showing spleen architecture with the red pulp (RP), the white pulp (WP), and the *arrows* pointing at the central arterioles (CA). Magnification 4×. **(E, F)** Anti-CD3 and anti-CD20 staining. Scale bar is 200 µm. **(G)** T-cell responses in mice engrafted with coculture-expanded cells. Mice with similar human leukocyte reconstitution were immunized with TT thrice with 3 weeks' interval. Two weeks after the third immunization, the percentages of human T cells from the spleen were determined by flow cytometry. For ELISPOT assay, the same number (5×10^5) of human T cells from different samples were seeded into wells coated with anti-human IFN-γ antibody and cultured for 48 h under 3 conditions: medium alone (control), in the presence of phorbol myristate acetate, or in the presence of a TT-specific peptide. A comparison of the numbers of IFN-γ immunospots among different samples is shown. Each symbol represents 1 mouse. Data shown are from 1 of 2 independent experiments. IFN-γ, interferon gamma; TT, tetanus toxoid.

TABLE 1. COMPARISON OF RECONSTITUTION LEVELS IN NSG MICE ENGRAFTED WITH EXPANDED CELLS OF THE SAME CORD BLOOD DONOR BY EITHER THE MSC-A5 COCULTURES OR THE FEEDER CELL-FREE CULTURES

	$hCD45^+$	$CD33^+$	$CD3^+$	$CD19^+$	$CD33^+$ $CD14^+$	$CD11c^+HLA\text{-}DR^+$	$CD15^+$	$CD56^+$	$CD34^+$
	Median (range)	*Median (range)*	*Median (range)*	*Median (range)*	*Median (range)*	*Median (range)*	*Median (range)*	*Median (range)*	*Median (range)*
Blood									
FCF	40 (33–50)	5.6 (3–8)	11.5 (10–44)	36 (21–51)	4.6 (1.5–5.2)	5 (4.7–5.9)	5.67 (3.9–6.4)	1.12 (1–1.20)	ND
MSC-A5	34 (26–46)	5.7 (3–9.2)	44 (21–68)	21 (7–54)	5.63 (3.8–9.7)	8.9 (3.2–16.5)	9.69 (3.9–12.7)	0.98 (0.5–1.16)	ND
Spleen									
FCF	80 (76–87)	1.1 (0.4–1.1)	17.18 (5.3–29)	54 (20–87)	0.13 (0.07–0.19)	1 (1–2.9)	1 (0.7–1)	1.4 (1.2–2.1)	ND
MSC-A5	75 (63–87)	1 (0.8–3.2)	30 (8.3–54)	55 (25.8–85)	0.35 (0.2–1.1)	1.44 (0.7–2.3)	0.85 (0.5–1.5)	0.69 (0.6–1)	ND
BM									
FCF	44 (41–48)	3.35 (3–5.2)	2.68 (0.4–4.9)	69.5 (60–79)	1.9 (1–2.1)	1.3 (1.2–2)	3.7 (1.5–6)	ND	16.8 (8.6–33)
MSC-A5	45 (19–74)	4.7 (2.1–9.8)	2.57 (0.8–7.8)	72.3 (67–76)	1.8 (1.3–4.6)	1.5 (1.2–4.2)	2.33 (0.4–4.6)	ND	20.75 (10–32)

See Fig. 5A legend for detailed description of experimental procedures and analysis. Shown are the median percentages and the range of the percentages of different human blood lineage cells in the blood, spleen, and bone marrow (BM). Data shown are from 9 and 3 mice engrafted with expanded cells from the MSC-A5 coculture and the FCF culture, respectively. Representative data from 1 of the 2 experiments are shown.

FCF, feeder cell-free; MSC, mesenchymal stem cell; ND, not detectable.

These results suggest that enhanced T-cell development leads to an enhanced T-cell response in mice reconstituted with coculture-expanded cord blood cells.

Discussion

Here we report an improved method to expand cord blood HSCs by combining feeder cell coculture with growth factors from the FCF culture. Based on in vitro criteria we have assessed the most promising coculture condition (MSC-A5) extensively in vivo and this improved coculture method is superior to FCF culture in expanding human HSCs. In the 2 best expansions reported to date using other FCF culture systems, Delaney et al. showed ~15-fold and Boitano et al. [24] reported ~17-fold expansion of SCID repopulating activity. Our improved coculture method produced ~60-fold expansion of SRCs in 11 days, as well as robust engraftment for ~6 months and secondary reconstitution assessed 12 weeks after the secondary recipients were engrafted. Differences in culture conditions, HSC donors, laboratory techniques, and recipient mice make it difficult to strictly compare the fold expansion of putative HSCs between studies. Based on the SRC expansion in vivo, our coculture system is at least 3 times better at expanding cord blood HSCs than the other reported FCF cultures. Moreover, when $CD34^+$ cells isolated from fetal liver and mobilized peripheral blood were cultured under the same conditions, expansion of DP cells in MSC-A5 coculture was also significantly higher than that in the FCF culture (data not shown).

We also directly compared HSC expansion in coculture with our previously reported FCF culture. When the equal number of purified $CD34^+$ $CD133^+$ cells from the MSC-A5 coculture and FCF culture were compared in mice, there was no significant difference in the SRC frequency, suggesting that the DP cells from both cultures have similar stem cell activity. However, because addition of MSC-A5 feeder layer led to a greater expansion of DP cells, there is a ~2.3×increase in total SRCs in MSC-A5 coculture than FCF culture. Our coculture method is also superior to other human HSC cocultures reported in the literature. In the best studies reported to date, Zhang et al. showed an ~100-fold expansion of $CD34^+$ cells if MSCs were placed in a 3D matrix [4,5]; however, only a limited in vivo assessment was conducted, demonstrating successful engraftment of NOD-scid mice in all conditions examined, but no increase in SCID repopulating activity compared to uncultured cells. Our in vivo assessments showed robust expansions in SCID repopulating activity and we further assessed expanded cells by long-term, competitive, and serial reconstitution. To our knowledge, this is the first study where vigorous in vivo evaluation of expanded human HSCs in MSC coculture has been undertaken. Our ~60-fold expansion in SRCs with long-term, competitive, and serial reconstitution therefore sets a benchmark for coculture studies. While further characterization of other systems may exceed this benchmark, it represents the largest expansion of HSCs in any coculture reported to date.

In addition to robust expansion, the cocultured HSCs were capable of differentiating into a wide range of leukocyte lineages in NSG mice. Among the human cells that we assayed, there was no difference in reconstitution of NK cells, dendritic cells, monocyte/macrophage, and various precursor cells between expanded cells from the coculture and the FCF

culture. However, compared to unexpanded or expanded $CD34^+$ $CD133^+$ cells from the FCF culture that gave robust B cell but poor T-cell reconstitution initially, the coculture-expanded cells gave a significantly more rapid T-cell reconstitution, reaching the normal T to B cell ratio seen in both wild-type mice and healthy humans. The enhanced T-cell reconstitution was the opposite of the effect reported by Giassi et al. [25], where HSCs were expanded with a cocktail of growth factors in the absence of feeder cells. These results suggest a critical role of MSCs in mediating expansion and regulating differentiation in the coculture. We further show that the enhanced T-cell reconstitution is correlated with a stronger response to TT in reconstituted mice.

The first transplantation of ex vivo-expanded umbilical stem cells was reported by Shpall et al. [26]. Patients were safely coinjected with a fraction of cultured $CD34^+$ cells and a fraction of cytokine-cultured cells. Follow-up studies have shown that transplantation of in vitro-expanded HSCs improves the clinical outcome of HSC treatment of hematologic cancer, critically by shortening the period of neutropenia after infusion [3]. The improved coculture method reported here offers a more efficient way to expand HCSs for transplantation in the clinic, though the ability of these cells to engraft in humans has yet to be demonstrated. This promising improvement can also be combined with other approaches that aim at enhancing the engraftment of cells in patients, such as improving the homing of the transplanted cells by coinjection of MSCs or the administration of parathyroid hormone [27]. Our observation that cultured cells competed well with uncultured cells is of particular interest. A clinical trial similar to that reported by Delaney et al. is warranted. The enhanced T-cell reconstitution of the coculture-expanded HSCs, which occurred in the absence of any incidence of graft versus host disease, could be advantageous in transplantations as it would lead to faster recovery of adaptive immunity. In addition, there have been great interests in developing humanized mouse models for preclinical studies of infectious diseases and human hematologic diseases. Construction of humanized mice requires engraftment of NSG mice or other strains of mice with human HSCs. The improved coculture method reported here also offers the possibility of constructing large number of humanized mice using HSCs from the same cord blood source, as well as generating mice with superior T-cell function—helping to address one of the known weaknesses of the system [28].

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Author Disclosure Statement

The authors declare no competing financial interests.

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