STEM CELLS TRANSLATIONAL MEDICINE

CORD BLOOD

The official journal of CORD BLOOD ASSOCIATION

Ex Vivo Expansion of CD34$^+$CD90$^+$CD49f$^+$ Hematopoietic Stem and Progenitor Cells from Non-Enriched Umbilical Cord Blood with Azole Compounds

SUDIPTO BARI ,[a,b*] QIXING ZHONG,[b,c*] XIUBO FAN,[d] ZHIYONG POON,[a] ALVIN SOON TIONG LIM,[e] TSE HUI LIM,[e] NIRAJA DIGHE,[a] SHANG LI,[f] GIGI NGAR CHEE CHIU,[b†] CHRISTINA LI LIN CHAI ,[b†] WILLIAM YING KHEE HWANG [a,f,g,h†]

Departments of [a]Hematology, [d]Clinical Research, [e]Molecular Pathology, Singapore General Hospital, Singapore, Singapore; [b]Department of Pharmacy, National University of Singapore, Singapore, Singapore; [c]Genome Institute of Singapore, Agency for Science and Technology Research, Singapore, Singapore; [f]Cancer and Stem Cell Biology, Duke-NUS Medical School, Singapore, Singapore; [g]National Cancer Center Singapore, Singapore, Singapore; [h]Singapore Cord Blood Bank, Singapore, Singapore

*Contributed equally as first authors.

†Contributed equally as corresponding authors.

Correspondence: William Hwang Ying Khee, F.R.C.P., F.A.M.S., M.Med., M.R.C.P., M.B.B.S., National Cancer Center Singapore, Executive Office, Level 4, 11 Hospital Drive, Singapore 169610. Telephone: 65 9677 7142; e-mail: william.hwang.y.k@singhealth.com.sg

This article was published online on 2 February 2018. The publisher missed to include the Cord Blood logo. This notice is included in the online version to indicate this has been corrected 28 February 2018.

Received October 25, 2017; accepted for publication December 28, 2017; first published February 2, 2018.

http://dx.doi.org/10.1002/sctm.17-0251

Key Words. Umbilical cord blood • Hematopoietic stem and progenitor cells • Azole-based small molecules • Ex vivo expansion • Xenotransplantation with immunodeficient mouse model • Hematopoietic stem cell transplantation

ABSTRACT

Umbilical cord blood (UCB) transplants in adults have slower hematopoietic recovery compared to bone marrow (BM) or peripheral blood (PB) stem cells mainly due to low number of total nucleated cells and hematopoietic stem and progenitor cells (HSPC). As such in this study, we aimed to perform ex vivo expansion of UCB HSPC from non-enriched mononucleated cells (MNC) using novel azole-based small molecules. Freshly-thawed UCB–MNC were cultured in expansion medium supplemented with small molecules and basal cytokine cocktail. The effects of the expansion protocol were measured based on in vitro and in vivo assays. The proprietary library of >50 small molecules were developed using structure-activity-relationship studies of SB203580, a known p38-MAPK inhibitor. A particular analog, C7, resulted in 1,554.1 ± 27.8-fold increase of absolute viable CD45$^+$CD34$^+$CD38$^-$CD45RA$^-$ progenitors which was at least 3.7-fold higher than control cultures ($p < .001$). In depth phenotypic analysis revealed >600-fold expansion of CD34$^+$/CD90$^+$/CD49f$^+$ rare HSPCs coupled with significant ($p < .01$) increase of functional colonies from C7 treated cells. Transplantation of C7 expanded UCB grafts to immunodeficient mice resulted in significantly ($p < .001$) higher engraftment of human CD45$^+$ and CD45$^+$CD34$^+$ cells in the PB and BM by day 21 compared to non-expanded and cytokine expanded grafts. The C7 expanded grafts maintained long-term human multilineage chimerism in the BM of primary recipients with sustained human CD45 cell engraftment in secondary recipients. In conclusion, a small molecule, C7, could allow for clinical development of expanded UCB grafts without pre-culture stem cell enrichment that maintains in vitro and in vivo functionality. STEM CELLS TRANSLATIONAL MEDICINE *2018;7:376–393*

SIGNIFICANCE STATEMENT

This study identified a novel structural analog of SB203580 (p38-MAPK inhibitor) that expands hematopoietic stem and progenitor cells (HSPCs) from non-enriched umbilical cord blood–mononucleated cells. The expanded graft consists of primitive HSPC phenotype (CD34$^+$ CD90$^+$ CD49f$^+$), enhanced colony formation, and long-term severe combined immunodeficiency repopulation capacity in primary and secondary NSG mice recipients.

INTRODUCTION

Umbilical cord blood (UCB) transplantation has the distinct advantage of allowing human leukocyte antigen mismatched transplants to take place while having reduced graft versus host disease (GVHD) and retained graft versus tumor effects [1, 2]. However, delayed engraftment, especially when the cell dose of the UCB units is suboptimal, with consequent effects on post-transplant mortality, has limited its use [3]. Furthermore, concerns about low cell dose has also limited the use of UCB to only the ones with the largest cell counts in UCB banks, resulting in the underutilization of a sizable part of the global public UCB inventory [4].

Recent studies have suggested that ex vivo expansion of UCB could overcome this barrier by accelerating hematopoietic recovery and, thus,

reduce many of the risks of infectious complications post-transplant [1, 2]. These studies employ the use of novel agents like StemRegenin 1 (SR1) [5], nicotinamide (NAM) [6] and mesenchymal stromal cell (MSC) coculture [7], notch ligands [8] and have shown engraftment as early as within the second week of transplantation. However, current strategies for UCB expansion involve either MSC layers for coculture for expanding non-selected grafts or a prior hematopoietic stem and progenitor (CD34/CD133) cell (HSPC) selection step [9]. There are currently no reports of successful expansion of UCB HSPC in pre-clinical or clinical studies without the use of either of these strategies, which add to concerns about the cost, increased culture duration and additional manipulations required for obtaining the expanded UCB graft.

In an effort to expand HSPC from non-enriched UCB, we developed a proprietary library of approximately 50 small molecules using structure-activity-relationship studies of SB203580 (established is a p38α mitogen-activated protein kinases, p38α-MAPK inhibitor) and tested these compounds using phenotypic and functional studies. This particular compound was chosen since prior studies showed that SB203580 could expand both purified human CD133/CD34 UCB [10] and mouse bone marrow (BM) (Lin^- Sca^+c-kit^+) [11] HSPC. Several hallmark studies have also shown that reactive oxygen species limits the life span of HSPCs by activating the p38 MAPK pathway as a stress mediated response which could be overcome through addition of p38 inhibitors [12]. Further RNAi screening has also identified MAPK14 as a druggable suppressor for human HSPC expansion [13]. Preclinical studies investigating inhibition of p38 MAPK has been shown to restore hematopoiesis in myelodysplastic syndrome [14]. From the library of molecules, we report for the first time the use of a novel azole based small molecule, C7, which promotes successful expansion of HSPC from UCB–MNC without MSC coculture or a prior CD34/CD133 enrichment step; and demonstrate significant expansion of $CD34^+CD38^-CD90^+/CD49f^+$ HSPC supported by accelerated primary and sustained secondary engraftment of NSG mice.

Materials and Methods

UCB Collection, Processing, Thawing, and Culturing with Small Molecules

UCB was obtained through Singapore Cord Blood Bank (SCBB), from research consented donated units failing to meet the criteria for public clinical banking. Usage of the samples was approved by the Institutional Review Boards of National University of Singapore (NUS) and Singapore General Hospital (SGH) as well as the Research Advisory Ethics Committee of the SCBB. As described previously, mononuclear cells (MNC) were isolated from the fresh UCB by density gradient centrifugation using Ficoll-Histopaque Premium (GE Healthcare, U.K.) [15]. Counted UCB–MNCs were cryopreserved in 90% vol/vol autologous plasma with 10% vol/vol dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) for subsequent experimental usage. UCB–MNC was thawed using human serum albumin (25% vol/vol) (Health Sciences Authority, Singapore) and Dextran 40 (10% vol/vol) (Hospira, USA).

UCB–MNC were cultured at an empirically determined optimal density of 4.0×10^5 cells/ml without any cell surface marker dependent stem cell enrichment in StemSpan-Serum Free Expansion Media (SFEM) or StemSpan-Animal Component Free Media (ACF) (STEMCELL Technologies, Canada) supplemented with human cytokine cocktail of 100 ng/ml stem cell factor (SCF) (PeproTech, USA) and thrombopoietin (TPO) (PeproTech); 50 ng/ml FLT-3 Ligand (FLT-3L) (PeproTech); and 20 ng/ml insulin-like growth factor binding protein-2 (IGFBP-2) (R&D Systems, USA). Various cytokine combinations (at the respective concentrations described supra) were tested on UCB–MNC with and without the addition of the lead compound C7 or other structural analogs (at 5.0 μM) to determine their effects on expansion of total nucleated cell (TNC) and HSPC over 11 days in ACF/SFEM media. The substituted azole based small molecules (the structures of the synthesized analogs; design rationale; the synthetic pathway for lead compound C7 and its kinase inhibition profiling; and pharmacophore analysis of the compound library are described in Supporting Information) were dissolved in DMSO (Sigma Aldrich) and added to the cultures at an empirically determined optimal concentration of 5.0 μM. UCB–MNC cultures devoid of small molecules but supplemented with cytokines served as control while cultures supplemented with cytokines and DMSO (Sigma Aldrich) served as vehicle control. Cell cultures for comparison of C7 mediated expansion against current HSPC technologies (specifically commercially available small molecules; MSC coculture system; and $CD34^+$ selected expansion) and other small molecules (particularly, low molecular weight fragments of C7; and established inhibitors of specific kinases as determined from kinase inhibition profiling of C7) are described in Supporting Information. Expansion fold of phenotypically defined HSPC subsets were calculated by dividing the net number of "cell of interest" produced after 11 days by the net number of the "cells of interest" plated at day 0. Leukemic transformation or gross genomic changes of the UCB–MNC cultured with C7 were analyzed using specific probes for fluorescence in situ hybridization (FISH), karyotyping and leukocyte cytochemistry using routine clinical laboratory tests (briefly described in Supporting Information).

Cell cultures for in vitro experiments were done in 6- or 24-well plates (BD Falcon), while culturing for in vivo transplantation studies were carried out in T-175 flasks (Corning, USA). Cell cultures were maintained in a humidified, 5% carbon dioxide (CO_2) incubator at 37°C for the required duration. For UCB expansion evaluation and animal experimentation, an empirically optimized 10- to 11-day expansion protocol was used which included cytokine and small molecule replenishment on day 7. At completion of incubation, cells were aspirated from the culture-ware with subsequent rinsing by Dulbecco's phosphate buffered saline (DPBS) (Hyclone, USA). The extracted cells were counted using an automated differential hematology cell counter (COULTER $A^{c\cdot}T$ diff Hematology Analyzer, Beckman Coulter Inc., USA) and re-suspended in DPBS for subsequent in vitro (phenotypic or functional) analysis or in vivo xenotransplantation studies (details outlined in Supporting Information). Functional assays of expanded HSPCs are needed because of known limitation of phenotypic based results where the phenotypic definitions are mainly derived from studies involving primary non-cultured samples.

Animal Maintenance, Transplantation, and Procedures

Xenotransplantation studies were approved by the Singapore Health Services (SingHealth) Institutional Animal Care and Use Committee. NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ, better known as non-obese diabetic (NOD)–severe combined immunodeficient (SCID) gamma (NSG) mice, purchased from Jackson Laboratory (Bar Harbor, USA), were housed in cages of six of the same gender in

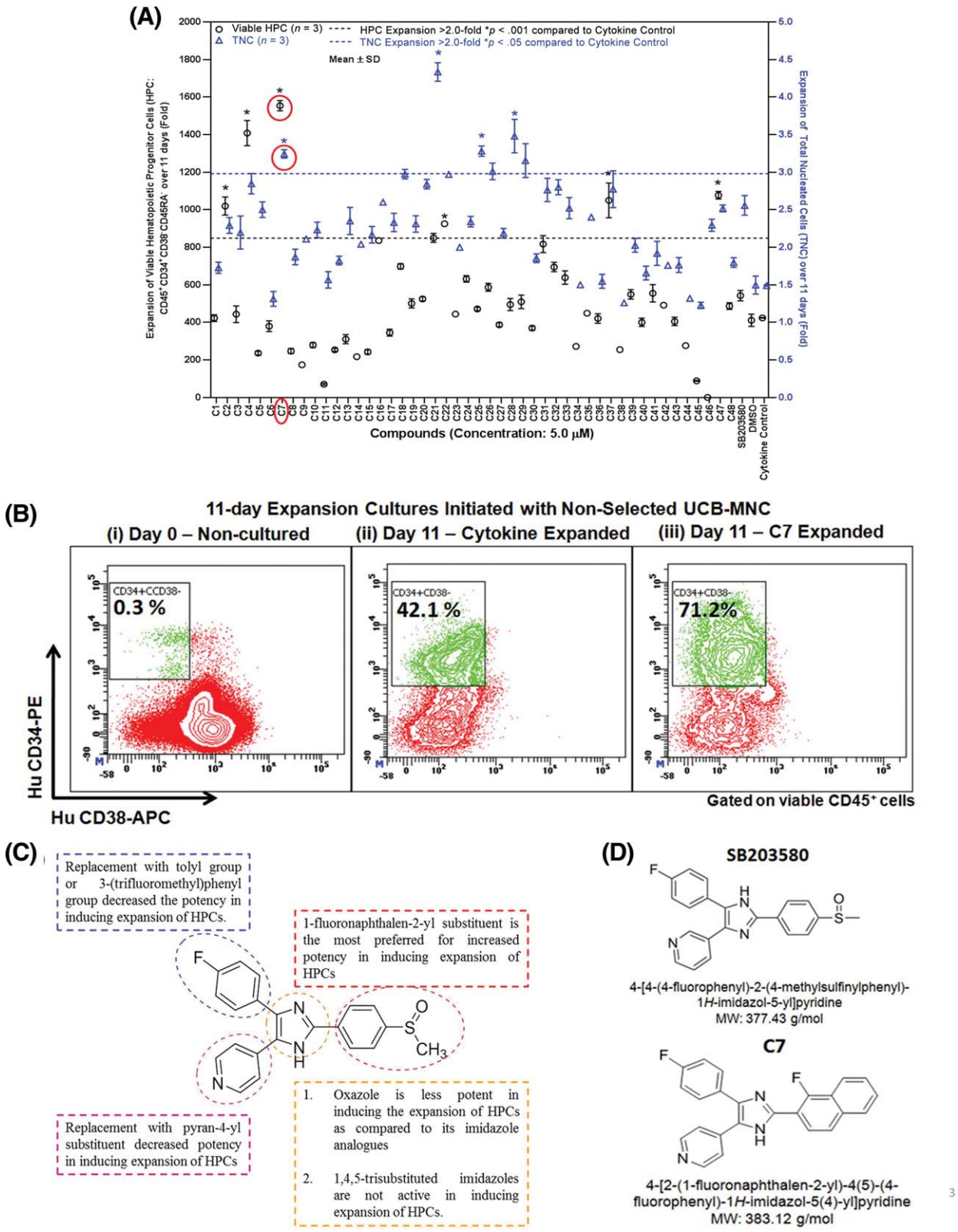


Figure 1.

SingHealth Experimental Medicine Centre. Sterilized food and water were accessible ad libitum. Following acclimation and successful breeding, the sub-lethally irradiated (240 cGy) 8–12 weeks old *primary* recipient mice were randomly divided into four experimental groups for tail vein administration of: (a) saline; (b) non-expanded UCB–MNC; (c) cytokine expanded UCB–MNC (fresh or cryo-preserved); and (d) C7 and cytokine expanded UCB–MNC (fresh or cryo-preserved). To investigate the in vivo human cell engraftment kinetics, expanded UCB–MNC (± C7) were transplanted at an empirically optimized equivalent dosage of 2.5×10^7, 5.0×10^7, 7.5×10^7, or 10.0×10^7 cells/kg while non-expanded UCB–MNC was transplanted at an absolute dosage of 2.5×10^7, 5.0×10^7, or 10.0×10^7 cells/kg. Expanded grafts were cryo-preserved in 90% fetal bovine serum (FBS) (Hyclone) and 10% DMSO (Sigma Aldrich) and were thawed using DPBS containing 20% FBS. Magnetic antibody labeled and column (Miltenyi Biotec, Germany) purified (as per manufacturer's protocol) human $CD45^+$ cells obtained from the BM of *primary* NSG recipients after 20 weeks of transplantation were administered (at dose of 1×10^6–2×10^6 cells/mouse) to *secondary* NSG recipients via tail vein injection for the following experimental groups: (a) non-expanded UCB–MNC; (b) cytokine expanded UCB–MNC; and (c) C7 and cytokine expanded UCB–MNC to monitor long-term human HSPC self-renewal/repopulation capacity. Preliminary in vivo studies comparing the performance of C7 expanded grafts against MSC coculture (expansion culture initiating cells: MNC) or expanded grafts generated by culturing purified $CD45^+CD34^+CD38^-$ in presence of C7 are described and reported in Supporting Information.

All NSG mice received prophylactic antibiotics and immunosuppressive drugs to minimize bacterial infection and reduce chances of GVHD respectively (Supporting Information). Assessment of human cell reconstitution after transplantation were done using either peripheral blood (PB) samples collected via the submandibular vein or from the BM of sacrificed mice at the stated time points (Supporting Information).

Flow Cytometric Analysis and Fluorescence Activated Cell Sorting

All data were acquired using the Cytomics FC500 Flow Cytometer (Beckman Coulter, Inc., USA), BD LSRII or LSRFortessa (Beckton Dickson, USA) for at least 10,000 events per sample. Acquired data were subsequently analyzed with CXP Analysis Software (Beckman Coulter, Inc.) or BD FACSDiva 8.0 Software (Beckton Dickson). Titration was performed to identify optimal antibody staining. Isotype controls or non-labeled cells were used for the purposes of gating out non-specific antibody binding during analysis. Detailed antibody labeling and flow cytometer panels are described in Supporting Information.

Statistical Analysis

Results are reported as either mean ± SEM; or mean ± SD; or geometric mean ± 95% confidence interval (CI) for the specified *n* value shown in the figures. The significance of difference between two groups was determined using the two-tailed Student *t* test (unless stated otherwise) or other appropriate tests such as Mann-Whitney *U* test (where maximum value of *U* represents product of the sample sizes for the two indicated groups being compared) at the *p* value shown in the figures. HSPC frequency in transplanted NSG mice was calculated using L-Calc (STEMCELL Technologies) and Extreme Limiting Dilution Analysis (Walter and Eliza Hall Institute Bioinformatics, Australia). Data processing and statistical analyses were performed with OriginPro 9.1 (OriginPro, USA), GraphPad Prism 6.0 (GraphPad Software, Inc., USA) and Microsoft Office Excel (Microsoft, USA).

RESULTS

Screening of the Structural Analogs of SB203580, Identified C7 as the Lead Compound to Expand HSPC from Non-enriched UCB–MNC

All the compounds were screened at a concentration of 5.0 μM since this has been shown to be the optimal working concentration for the parent compound, SB203580 in expanding HSPC from CD133/CD34-purified grafts [10, 11]. As shown in Figure 1A, only six compounds namely **C2** (4-[2-(1-fluoronaphthalen-2-yl)-4-[3-(trifluoromethyl)phenyl]-1*H*-imidazol-5-yl]pyridine), **C4** (4-[2-(1-fluoronaphthalen-2-yl)-4-(m-tolyl)-1*H*-imidazol-5-yl]pyridine), **C7** (4-[2-(1-fluoronaphthalen-2-yl)-4(5)-(4-fluorophenyl)-1*H*-imidazol-5(4)-yl]pyridine), **C22** 4-[4(5)-(*m*-tolyl)-1*H*-imidazol-5(4)-yl]pyridine), **C37** (4-[4(5)-(4-fluorophenyl)-1*H*-imidazol-5(4)-yl]pyridine) and **C47** (4-(4(5)-(4-fluorophenyl)-2-(7-methoxynaphthalen-2-yl)-1*H*-imidazol-5(4)-yl)pyridine) could expand viable HPC ($CD45^+CD34^+CD38^-CD45RA^-$) by at least twofold higher than the cytokine control or DMSO vehicle control ($p < .001$) when cultures were initiated with non-selected UCB–MNC. The addition of a particular compound, C7, resulted in a 1,554.1 ± 27.8-fold expansion of viable HPC compared to 542.4 ± 27.1-fold, 411.2 ± 32.5-fold, and 423.9 ± 1.9-fold in SB203580 (parent compound), DMSO (vehicle control) and cytokine control respectively in an 11-day expansion culture (Fig. 1A). When cell expansion was evaluated in terms of the change in TNC number, only four compounds namely **C7**, **C21** (4-[2-(2,6-difluorophenyl)-4(5)-(4-fluorophenyl)-1*H*-imidazol-5(4)-yl]pyridine), **C25** (4-[2-(2-chlorophenyl)-4(5)-(4-fluorophenyl)-1*H*-imidazol-5(4)-yl]pyridine) and **C28** (4-[4(5)-(4-fluorophenyl)-2-(2-

Figure 1. Identification of a novel small molecule, C7 that is a structural analog of the p38-MAPK inhibitor SB203580, which could expand HPC from non-enriched UCB–MNC. **(A):** Fold expansion of viable ($7AAD^-$) $CD45^+CD34^+CD38^-CD45RA^-$ HPC and TNC in cultures that lasted for 11 days with animal component free media (ACF) media, cytokine, and respective small molecule being replenished at day 7. SB203580, dimethyl sulfoxide, and cytokines alone in ACF media served as the reference compound, vehicle and blank control, respectively. The dashed black and blue line represents >2.0-fold expansion of HPC and TNC, respectively compared to cytokine control. Expansion data of HPC and TNC for lead compound C7 is highlighted with red circles. *, $p < .001$ compared to all other conditions for HPC expansion. *, $p < .05$ compared to all other conditions for TNC expansion. Data represents mean ± SD for $n = 3$. **(B):** Representative flow cytometer contour plots depicting $CD34^+CD38^-$ population which is a subset of the viable ($7AAD^-$) $CD45^+$ cells of (i) thawed non-selected UCB MNC at day 0 followed by culturing for 11 days in (ii) cytokine control and (iii) 5.0 μM of C7 supplemented with cytokines using serum free expansion media. Media, cytokines, and C7 were replenished at day 7. **(C):** Graphical summary of the structural activity relationship of the azole analogs investigated in inducing the ex vivo expansion of HPC from UCB–MNC. **(D):** Chemical structures, IUPAC name and molecular weight of SB203580 (parent compound) and C7 (top lead compound). Abbreviations: HPC, hematopoietic progenitor cells; MNC, mononucleated cells; TNC, total nucleated cell; UCB, umbilical cord blood.

nitrophenyl)-1*H*-imidazol-5(4)-yl]pyridine) could augment greater than twofold expansion compared to cytokine control ($p < .05$) (Fig. 1A). C21 supported the strongest expansion of TNC (4.3 ± 0.1-fold) compared to SB203580 (2.6 ± 0.1-fold) and cytokine control (1.5 ± 0.1-fold) ($p < .05$) (Fig. 1A). Although C21 expanded TNC at least 1.34-fold higher than C7 ($p < .001$) (Fig. 1A), it could only support 849.9 ± 26.3-fold expansion of HPC which is at least 1.8-fold lower than C7 ($p < .001$) (Fig. 1A). Based on these screening data, we identified C7 to be the lead analog of SB203580 that could expand HPC from frozen-thawed, non-selected UCB graft. C4 which could expand 1,408.3 ± 67.1-fold and 2.9 ± 0.1-fold of HPC and TNC respectively could be designated as the second best analog of SB203580 in supporting the expansion of UCB grafts. Representative flow cytometer contour plots showing the $CD45^+CD34^+CD38^-$ HPC population before and after expansion culture is shown in Figure 1B. The percentage of viable $CD45^+CD34^+CD38^-$ HPC in non-cultured or cultured MNC is shown in Supporting Information Figure 1B. A graphical summary of the structural activity relationship (SAR) of the azoles that are capable of inducing ex vivo expansion of HPCs

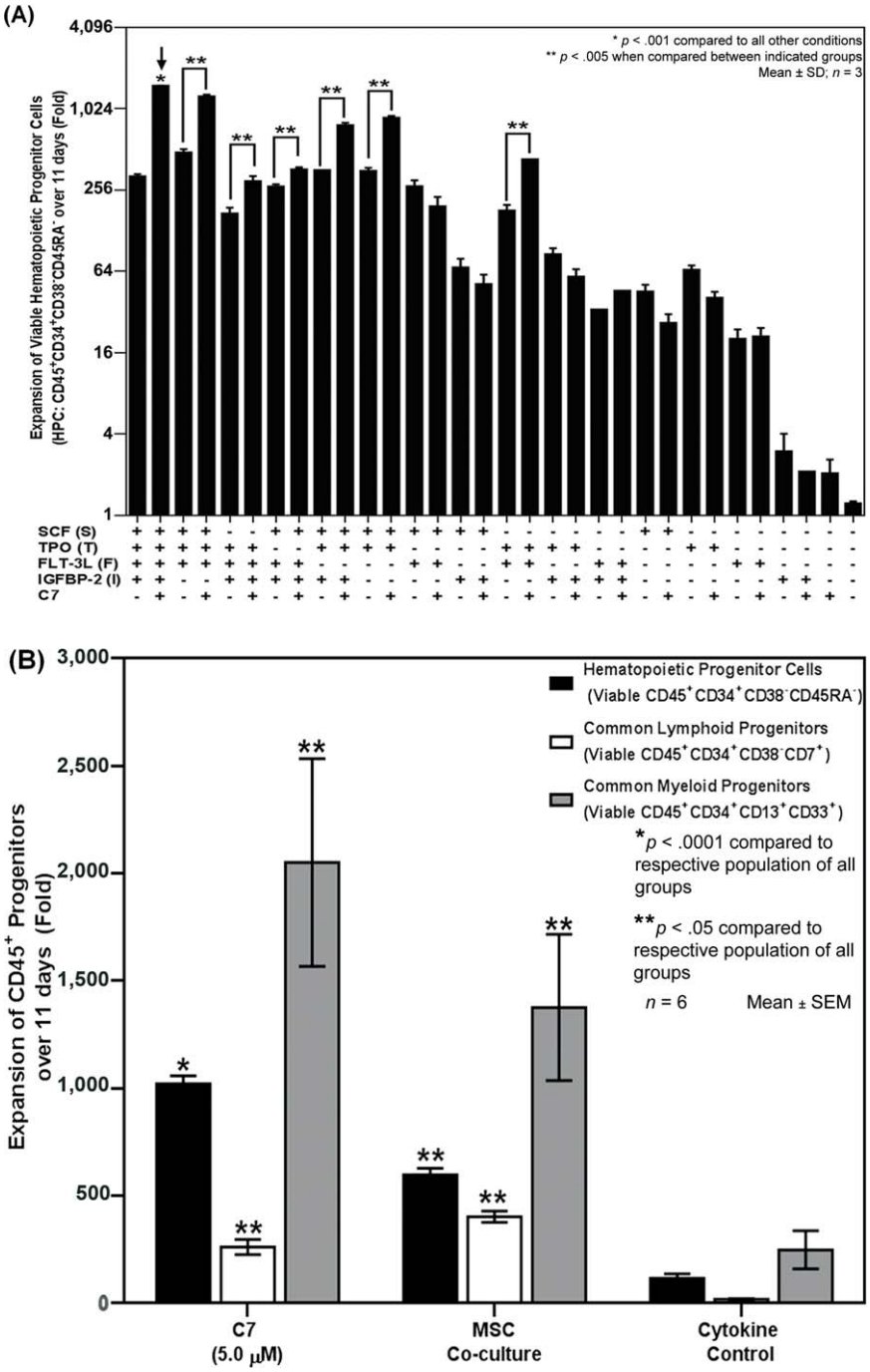


Figure 2.

is shown in Figure 1C with detailed results being described in Supporting Information. The chemical structures of C7 (lead compound) and SB203580 (parent compound) are shown in Figure 1D.

C7 Supported Phenotypic and Functional HSPC Population Enhancement from MNC in Absence of Specific Basal Cytokines and Out-Performed Current Expansion Technologies

As C7 is a novel small molecule, therefore, varying combination of cytokines were investigated to identify optimal cytokine cocktail. As shown in Figure 2A, optimal HPC expansion of 1,513.9 ± 6.4-fold was observed when cultures were supplemented with SCF (S), TPO (T), FLT-3L (F), IGFBP-2 (I), and C7 which was at least 4.7-fold higher than the four cytokines (S + T+F + I) culture ($p < .0001$). When the basal cytokine cocktail consisted of only three cytokines (such as combinations of S + T+F; T + F+I; F + I+S; and S + I+T), the addition of C7 could significantly ($p < .05$) boost expansion of HPC. For example, comparing between S + T+F (486.8 ± 27.2-fold) and S + T+F + C7 (1,265.2 ± 39.1-fold) we observed an expansion enhancement effect of 2.6-fold ($p < .05$). Similarly, a twofold augmentation of HPC expansion is observed when C7 is added to a basal cocktail of S + I+T ($p < .05$). Interestingly, C7 could support comparatively better expansion of HPC even when only two cytokines (example S + T or T + F) were added to the culture system. Minimal expansion was observed if C7 was used with either of cytokines (e.g., S + C7; T + C7; F + C7 or I + C7). The addition of C7 alone (i.e., without any cytokines) did not support the expansion of HPC, which was similar to growing the cells devoid of any growth factors. There were minimal acute cytotoxic effects of the compounds (at 5.0 μM) on leukocyte viability (Supporting Information Fig. 2A). Further experimentation with C7 showed that HSPC expansion from non-selected MNC were dependent on compound concentration (optimal concentration is determined to be 5.0 μM) (Supporting Information Fig. 2B, Experiment 1); culture duration (optimal expansion occurred with at least 9–11 days of culturing with media, cytokine, and small molecule replenishment being done on day 7) (Supporting Information Fig. 2B, Experiment 2); schedule of addition (essential to add C7 promptly at initiation of expansion culture) (Supporting Information Fig. 2C) but independent of type of basal growth media (equivalent performance of SFEM or ACF media) (Fig. 2F, Experiment 3).

As shown in Figure 2F, there was enhanced formation of granulocyte, monocyte (GM) colonies in colony forming unit (CFU) assay from cells expanded in presence of C7. Addition of C7 increased GM colonies by 2.5- and 5.7-fold compared to SB205380 or cytokine control ($p < .01$; Fig. 2F, Experiment 1), respectively. Similar to phenotypic HPC expansion, formation of CFU–GM was also dependent on the duration of the liquid cultures (Fig. 2F, Experiment 2). CFU–GM formed from UCB–MNC in culture with 5.0 μM of C7 for 7 days was only 38.1 ± 1.3-fold higher than non-cultured cells which increased to 136.2 ± 4.3-fold ($p < .001$ comparing day 7 and 11 CFU expansion in C7 cultures)

Figure 2. Expansion of HPC in presence of C7 with varying cytokine combination and in comparison to established expansion technologies. **(A):** Fold expansion of viable ($7AAD^-$) $CD45^+CD34^+CD38^-CD45RA^-$ HPC in cultures that lasted for 11 days with ACF media, different combinations of cytokines with and without 5.0 μM of C7. Media, group specific cytokine combinations and C7 were replenished at day 7. The concentrations of each cytokine are as follows: S represents SCF at 100 ng/ml; T represents TPO at 100 ng/ml; F represents FLT-3L at 50 ng/ml; and IG represents IGFBP-2 at 20 ng/ml. *, $p < .001$ compared to all other conditions. **, $p < .05$ when compared between indicated groups. Data represents mean ± SD for $n = 3$. **(B):** Ex vivo expansion of viable ($7AAD^-$) $CD45^+CD34^+CD38^-CD45RA^-$ HPC, lymphoid (CLP: $CD45^+CD34^+CD38^-CD7^+$) and myeloid progenitors (CMP: $CD45^+CD34^+$ $CD13^+$ $CD33^+$) from two separate UCB units were cultured with 5.0 μM of C7, cytokines and MSC. The expansion cultures lasted for 11 days with SFEM media, cytokine, and C7 replenishment being done on day 7. Cytokines alone in media served as the blank control. *, $p < .0001$ compared to respective population of all groups. *, $p < .05$ compared to respective population of all groups. Data represent mean ± SEM for $n = 6$. **(C):** Expansion of $CD34^+$ bright (early HPC: $CD45^+CD34(bright)^+CD38^-$ $CD45RA^-$) and $CD34^+$ dim (early HPC: $CD45^+CD34(dim)^+CD38^-$ $CD45RA^-$) progenitors when cultured in ACF media containing 5.0 μM of C7, or commercially available small molecules in presence of basal cytokines. The expansion cultures lasted for 11 days with ACF media, cytokine, and small molecules replenishment being done on day 7. Cytokines alone in media served as the blank control. The concentrations of each small molecule are as follows: S represents SR-1 at 1.0 μM; U represents UM171 at 50.0 nM; and N represents Nicotinamide at 5.0 mM. *, $p < .01$ compared to respective population in all groups. Data represents mean ± SD for $n = 3$. **(D):** Ex vivo expansion of viable ($7AAD^-$) $CD45^+CD34^+CD38^-CD45RA^-$ HPC when cultured in presence of 5.0 μM of each of the stated kinase modulators and basal cytokine cocktail. The expansion cultures lasted for 11 days with media, cytokine, and respective small molecule being replenished once on day 7. The control was SFEM media containing cytokines alone. The reference compound, SB203580, and vehicle control, DMSO, was also included. *, $p < .01$ compared to all other compounds and controls. Data represents mean ± SD for $n = 3$. **(E):** Expansion of viable $CD45^+CD34^+CD38^-CD45RA^-$ HPCs when cultures were initiated from magnetically purified $CD34^+$ grafts. The expansion cultures lasted for 11 days with ACF media, cytokine, and 5.0 μM of C7 replenishment being done on day 7. *, $p < .0001$ compared between the groups C7 and cytokines and cytokine control. Data represents mean ± SEM for $n = 6$. **(F): Experiment 1:** Ex vivo expansion of CFU–GM when two separate UCB units without pre-selection of stem cells were cultured in 5.0 μM of C7 and basal cytokines. The expansion cultures lasted for 10–11 days with SFEM, cytokine, and C7 replenishment being done on day 7. SB203580, DMSO, and cytokines alone in SFEM served as the reference compound, vehicle and blank control, respectively. *, $p < .01$ compared to SB203580, DMSO, and Cytokine control. Data represent mean ± SEM for $n = 6$. **Experiment 2:** Fold expansion of CFU–GM in cultures that lasted for 7 and 11 days. ACF media, cytokine, and 5.0 μM of C7 were replenished on day 7 for cultures lasting till day 11. Cytokines alone in ACF media served as the blank control. *p* values generated from Student's *t* test among indicated experimental groups are shown in the graph for the stated *n* values. Data represents mean ± SD for $n = 3$. **Experiment 3:** Ex vivo expansion of CFU–GM when UCB–MNC were cultured in SFEM or ACF media containing 5.0 μM of C7 in presence of basal cytokines. The expansion cultures lasted for 10–11 days with SFEM/ACF media, cytokine, and C7 replenishment being done on day 7. Cytokines alone in SFEM/ACF media served as the blank control. *p* values generated from Student's *t* test among indicated experimental groups are shown in the graph for the stated *n* values. Data represents mean ± SD for $n = 3$. **(G):** Ex vivo expansion of CFU–GEMM and BFU-E when UCB–MNC were cultured in SFEM media containing 5.0 μM of C7 in presence of basal cytokines. The expansion cultures lasted for 11 days with SFEM, cytokine, and C7 replenishment being done on day 7. Cytokines alone in SFEM served as the blank control. *, $p < .0001$ and **, $p < .05$ compared to cytokine control for CFU–GEMM and BFU-E expansion, respectively. Data represents mean ± SEM for $n = 6$. Abbreviations: ACF, animal component free media; BFU-E, erythroid burst-forming units; CFU, colony forming unit; DMSO, dimethyl sulfoxide; FLT-3L, FLT-3 Ligand; GEMM, granulocyte-erythrocyte-monocyte-megakaryocyte; GM, granulocyte, monocyte; HPC, hematopoietic progenitor cells; IGFBP-2, insulin-like growth factor binding protein-2; MNC, mononucleated cells; MSC, mesenchymal stromal cell; SCF, stem cell factor; SFEM, serum free expansion media; TPO, thrombopoietin; UCB, umbilical cord blood.

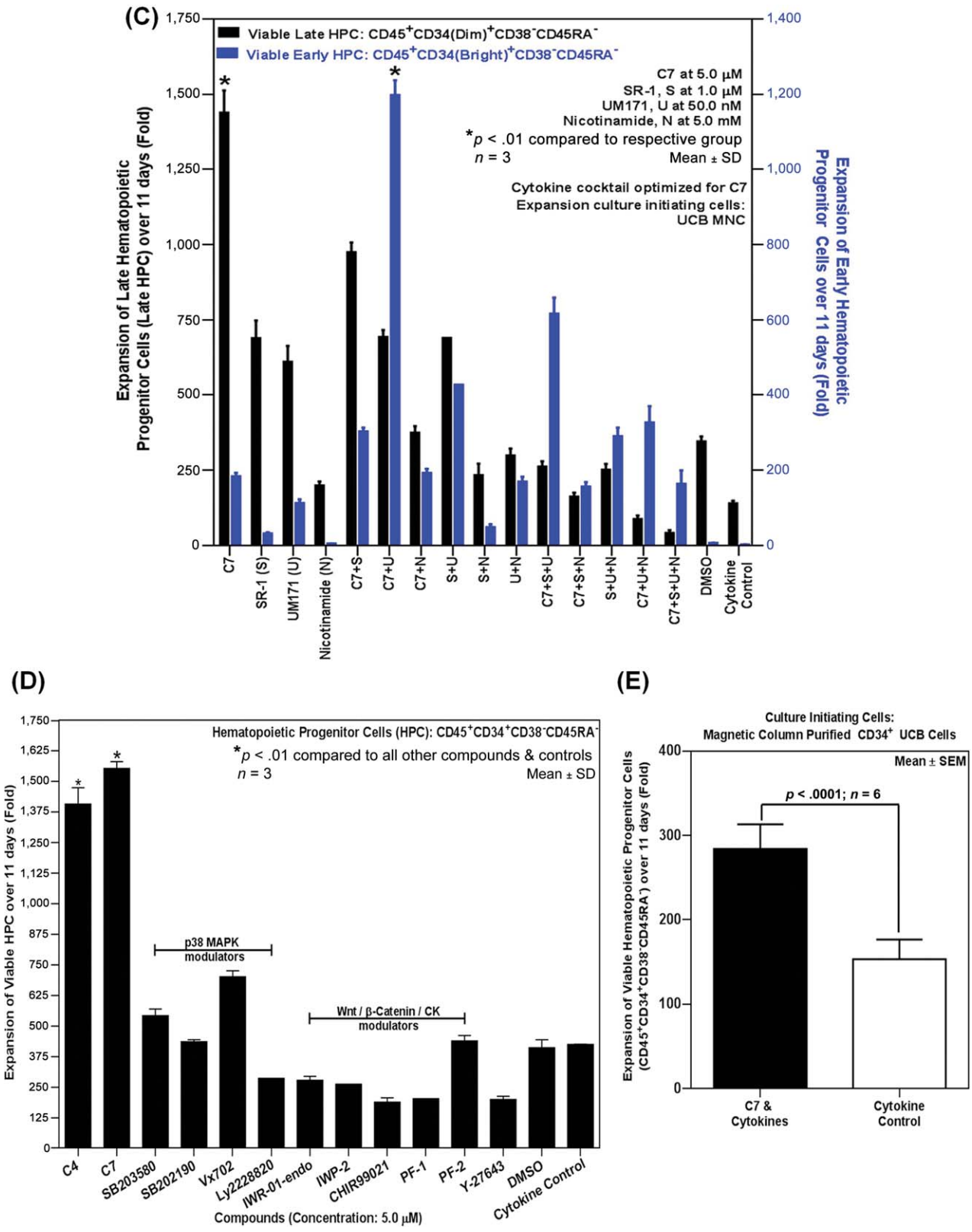


Figure 2. *Continued.*

from UCB–MNC cultured for 11 days (Fig. 2F, Experiment 2). Cytokine control cultures maintained CFU expansion between 16.3 ± 0.3-fold to 25.6 ± 1.1-fold over 7 ($p < .01$) to 11 ($p < .001$) days compared to non-cultured control which was at least 2.3- to 5.2-fold lower than the treatment group (Fig. 2F, Experiment 2). The addition of C7 to either SFEM (2.68-fold higher that cytokine

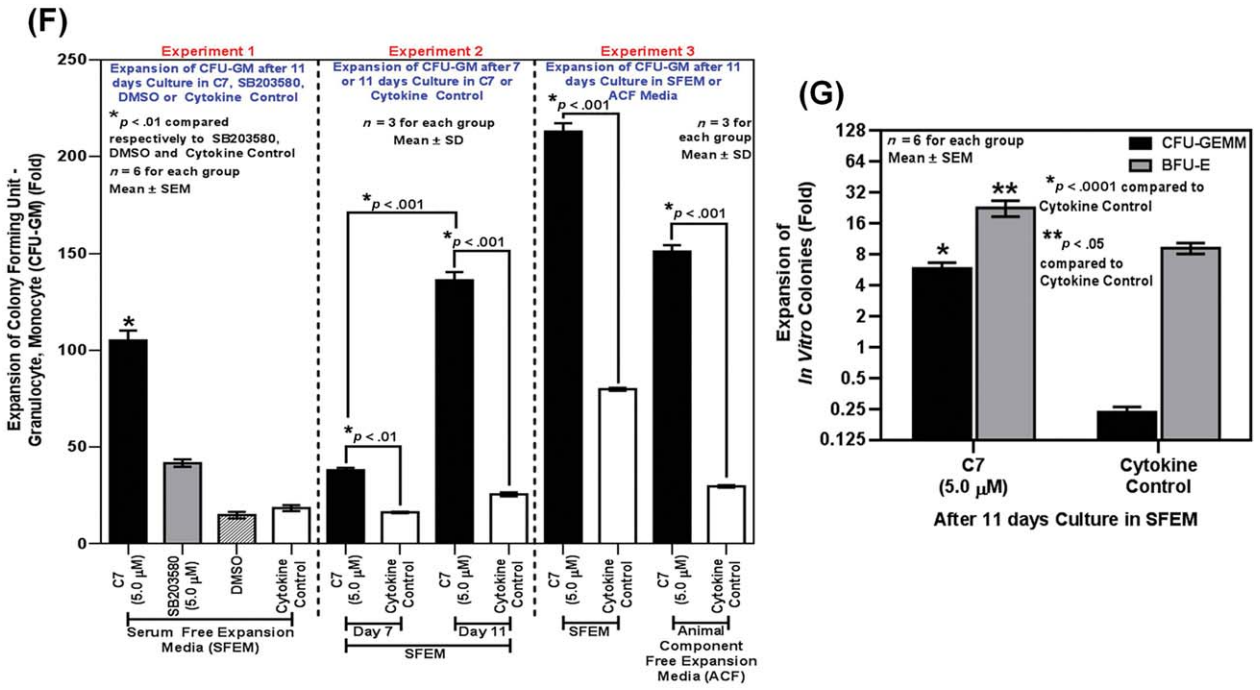


Figure 2. *Continued.*

control; $p < .001$) that contains bovine serum albumin or ACF (5.08-fold higher that cytokine control; $p < .001$) which is chemically defined media allowed significantly better expansion of UCB HPC as measured by CFU formation (Fig. 2F, Experiment 3) and concurrent phenotypic assays (data not shown). UCB MNC expanded in C7 supplemented cultures also produced significantly higher number of CFU–granulocyte-erythrocyte-monocyte-megakaryocyte ($p < .001$) and erythroid burst-forming units ($p < .05$) compared to cytokine control (Fig. 2G).

FISH using various probes relating to hematological malignancies revealed normal results for C7 expanded graft compared to cytokine expanded or non-cultured grafts (data not shown). Cell morphology and leukocyte cytochemistry analysis showed no evidence of leukemic transformation of the C7 expanded grafts (data not shown). Cytogenetic analysis revealed that C7 cultured cells maintained normal karyotype which had no difference with karyotype of non-cultured cells (data not shown).

A head-to-head comparison of C7 protocol (1,021.7 ± 36.6-fold) with BM–MSC coculture system (598.1 ± 30.5-fold) (the only other known expansion protocol that could expand HSPC without prior CD34/CD133 enrichment) exhibited significantly ($p < .001$) higher (by at least 1.71-fold) expansion of HPC (Fig. 2B). Compared to cytokine control both C7 and BM–MSC augmented expansion cultures could expand common myeloid (CMP: $CD45^+CD34^+CD13^+CD33^+$) and lymphoid (CLP: $CD45^+CD34^+CD38^-CD7^+$) progenitor cells ($p < .05$) (Fig. 2B). Preliminary data from C7 and MSC coculture expanded grafts gave comparable early human $CD45^+$ cell engraftment in the PB of transplanted NSG mice at week 4 ($p = .134$) (Supporting Information Fig. 3A).

Comparison of C7 with the clinically relevant HSPC expanding small molecules particularly SR-1 (1.0 μM), UM171 (50.0 nM) and NAM (5.0 mM), showed that C7 (5.0 μM) was extremely potent in augmenting expansion of both $CD34^+$ bright (early HPC) and dim (late HPC) populations when cultures were initiated with non-selected MNC (Fig. 2C). Interestingly, combination of C7 and UM171 (1,197.1 ± 39.9-fold) significantly boosted expansion of $CD34^+$ bright HPCs which was at least six-fold higher than the individual molecules (C7: 183.6 ± 9.4-fold; UM171: 112.3 ± 9.7-fold) ($p < .01$). The representative flow cytometer dot plots depicting the $CD34^+$ bright and dim populations at day 11 in presence of C7, UM171 or in combination are shown in Supporting Information Figure 3B.

A kinase inhibition profiling for C7 at a concentration of 5.0 μM revealed strong inhibition of the MAPK (p38α, p38β, JNK3, JNK2, and JNK1) and Wnt/β catenin (CSNK1ε, CSNK1δ, and CSNK1α1) pathway related kinases (Supporting Information Fig. 3C). HPC expansion cultures using non-selected UCB MNC in presence of commercial small molecules (SB203580, SB202190, Vx702, and Ly2228820 as modulators of p38 MAPK pathway; and IWR-01-endo, IWP-2, CHIR99021, PF-1, and PF-2 as modulators of Wnt/β catenin pathway) at a concentration of 5.0 μM that are known to modulate the activity of the identified kinases failed to recapitulate the HPC expansion effect of C7 ($p < .01$).

Finally, UCB grafts were enriched for CD34 cells using magnetic columns to mimic clinical grade selection methods. Culturing of these $CD34^+$ cells in presence of C7 resulted in 283.7 ± 14.7-fold of viable $CD45^+CD34^+CD38^-CD45RA^-$ HPCs within 11 days which was approximately 1.9-fold higher compared to cytokine control cultures (Fig. 2E).

C7 Supported Culturing of UCB–MNC Boosted Rare HSPC Populations Expressing CD90 and CD49f in the Expanded Graft

Rare hematopoietic stem cells (HSC) obtained from non-manipulated UCB have been shown to express $CD45^+CD34^+CD38^-CD45RA^-CD90^+CD49f^-$ (HSC1) or $CD45^+CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ (HSC2). As shown in Figure 3A, in presence of C7 and cytokines, the percentage expression of both

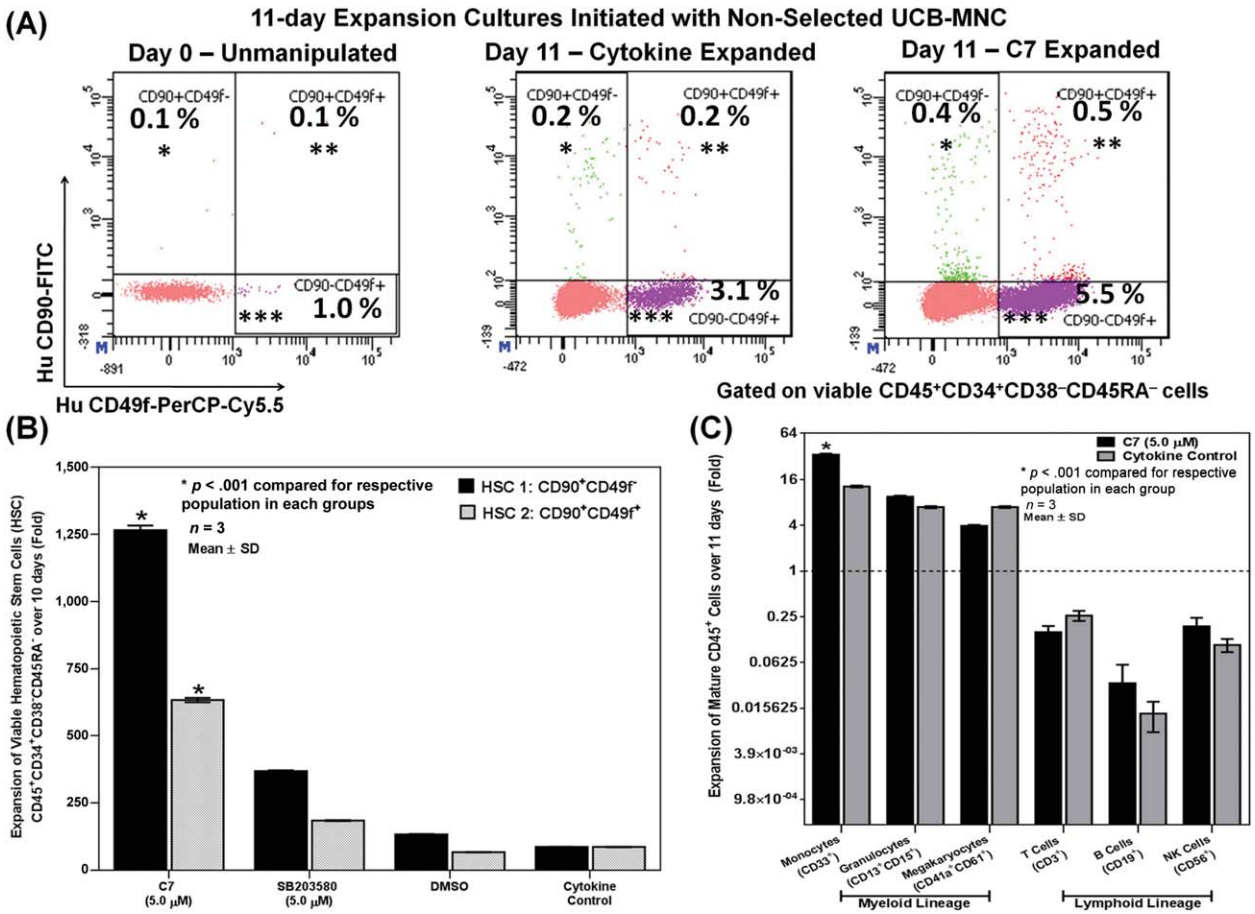


Figure 3. C7 augmented expansion of rare $CD34^+CD90^+CD49^+$ hematopoietic stem and progenitor cells when cultures were initiated with non-enriched umbilical cord blood (UCB)–mononucleated cells (MNC). **(A):** Representative flow cytometer dot plots depicting (a) $CD90^+CD49f^-$ (region depicted with *); (b) $CD90^+CD49f^+$ (region depicted with **) and (c) $CD90^-CD49f^+$ (region depicted with ***) population which are subsets of $CD45^+CD34^+CD38^-CD45RA^-$ cells of (i) thawed UCB MNC at 0 hours followed by culturing for 10 days in (ii) cytokine control and (iii) 5.0 μM of C7 supplemented with cytokines using serum free expansion media (SFEM). Media, cytokines, and C7 were replenished at day 7. **(B):** Expansion of viable ($7AAD^-$) HSC1 and HSC2 with phenotypic expression of $CD45^+CD34^+CD38^-CD45RA^-CD90^+CD49f^-$ and $CD45^+CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ respectively when cultures were initiated with non-selected UCB–MNC. The expansion cultures lasted for 10 days, with SFEM, cytokine, and 5.0 μM of C7 replenishment being done on day 7. Cytokines alone in media served as the blank control. *, $p < .001$ compared to respective population in each group. Data represents mean ± SD for $n = 3$. **(C):** Expansion of mature myeloid and lymphoid lineage cells in C7 and cytokine control cultures over 11 days. These MNC expansion cultures were supplemented with animal component free media, cytokine, and 5.0 μM of C7 at day 7. Myeloid lineage consisted of $CD45^+CD33^+$ monocytes, $CD45^+CD13^+CD15^+$ granulocytes, and $CD45^+CD41a^+CD61^+$ megakaryocytes. Lymphoid lineage consisted of $CD45^+CD3^+$ T cells, $CD45^+CD19^+$ B cells, and $CD45^+CD56^+$ NK cells. *, $p < .001$ compared to respective population in each treatment group. Data represents mean ± SD for $n = 3$. Abbreviations: DMSO, dimethyl sulfoxide; HSC, hematopoietic stem cells.

$CD90^+CD49f^-$ (HSC1) and $CD90^+CD49f^+$ (HSC2) (gated on viable $CD45^+CD34^+CD38^-CD45RA^-$) increased by 4.0- to 5.0-fold compared to non-cultured, non-enriched grafts. In terms of absolute cell numbers, C7 increased the proportion of immunodeficient mice engrafting HSC1 to at least 1,000-fold over 10 days compared to day 0, whereas cytokine control cultures could merely increase the same population by about 80-fold (Fig. 3B) ($p < .001$). In terms of HSC2, C7 could enhance expansion by at least 7.5-fold compared to cytokine control over 10 days (Fig. 3B). As shown in Figure 3C, both C7 and cytokine control culture mainly supported expansion of mature myeloid lineage cells (primarily $CD33^+$ monocytes) with minimal expansion of mature lymphoid cells (T, B, and NK cells). When C7 expansion cultures were initiated with fluorescence-activated cell sorting purified $CD45^+CD34^+CD38^-$ HPC, the phenotypic expansion (Supporting Information Fig. 4A, 4B) of HSC1 and HSC2 were maintained which resulted in both short term human cell engraftment in PB and long-term multilineage human cell reconstitution in BM of transplanted NSG mice (Supporting Information Fig. 4C).

C7 Expanded UCB–MNC Supported Early Human $CD45^+$ Engraftment in PB and Sustained Multilineage Reconstitution in BM of Transplanted NSG Mice

Transplantation of C7 expanded UCB grafts ($n = 11$) at equivalent dosage of 2.5×10^7 cells/kg to sub-lethally irradiated NOD SCID Gamma (NSG) mice resulted in 3.2- and 2.1-fold higher engraftment of human $CD45^+$ cells in the PB by day 21 compared to non-expanded ($p < .05$; $U = 6$; $n = 11$) and cytokine expanded grafts ($p < .05$; $U = 16$; $n = 12$), respectively (Fig. 4A). Moreover, administration of cryopreserved C7 expanded grafts (1.3% ± 0.3%; $n = 13$) maintained equivalent human $CD45^+$ engraftment

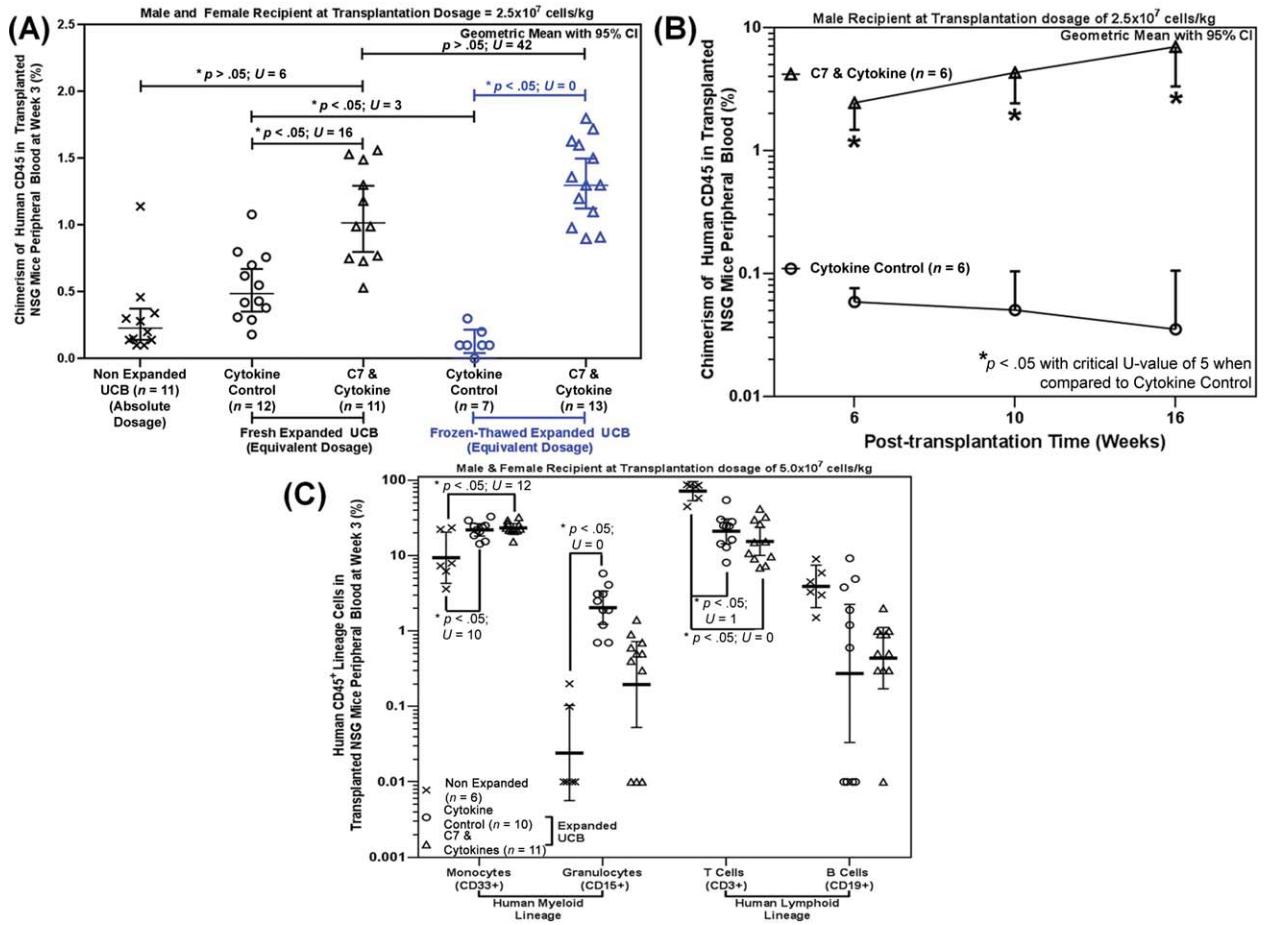


Figure 4. Grafts generated by expanding UCB–MNC in presence of C7 resulted in enhanced engraftment of human cells in the PB and bone marrow (BM) of NSG mice week 2–3 of post-transplantation. **(A):** Human CD45 chimerism in PB of NSG mice at week 3 post-transplantation with non-expanded or expanded UCB. Expansion of the UCB grafts was carried out using the mononuclear fraction (i.e., without CD34 selection) in either serum free expansion media or animal component free media that were supplemented with cytokines. The absolute cell dose of non-expanded graft was 2.5×10^7 cells/kg while the expanded grafts (either fresh or frozen-thawed) were transplanted at equivalent cell dosage of 2.5×10^7 cells/kg. The scatter plot represents the human CD45 chimerism of individual animals and depicts the geometric mean with 95% confidence interval (CI) of respective treatments. *p* values generated from Mann-Whitney *U* test among indicated experimental groups are shown in the graph for the stated *n* values. **(B):** Human CD45 chimerism in PB of male NSG mice transplanted with cytokine control or C7 and cytokine expanded UCB at equivalent cell dosage of 2.5×10^7 cells/kg at week 6, 10, and 16 post-transplantation. Each data point represents the geometric mean with 95% CI of respective treatments. *p* values generated from Mann-Whitney *U* test among indicated experimental groups are shown in the graph for the stated *n* values. **(C):** Lineage commitment of the human CD45 cells those are present in the PB of NSG mice at week 3 post-transplantation. The absolute cell dose of non-expanded graft was 5.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 5.0×10^7 cells/kg. The scatter plot represents the proportion of monocytes ($CD45^+CD33^+$), granulocytes ($CD45^+CD15^+$), T cells ($CD45^+CD3^+$), and B cells ($CD45^+CD19^+$) present among the total human cells in each individual animals and depicts the geometric mean with 95% CI of respective treatments. *p* values generated from Mann-Whitney *U* test among indicated experimental groups are shown in the graph for the stated *n* values. **(D):** Determination of severe combined immunodeficiency repopulating capacity (SRC) frequency by limiting dilution assay using L-Calc software and Extreme Limiting Dilution Assay. A NSG mouse is considered to be positive if human CD45 chimerism is >0.40% in PB at week 3. The data is calculated at two transplantation dosage of 2.5×10^7 and 5.0×10^7 cells/kg for both male and female recipients. **(E):** A scatter plot of human CD45 chimerism in PB of NSG mice at week 2 post-transplantation. The absolute cell dose of non-expanded graft was 10.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 10.0×10^7 cells/kg. The scatter plot represents the human CD45 chimerism of individual animals and depicts the geometric mean with 95% CI of respective treatments. *p* values generated from Mann-Whitney *U* test among indicated experimental groups are shown in the graph for the stated *n* values. **(F):** A scatter plot of human $CD45^+CD3^+$ T cell chimerism in PB of NSG mice at week 2 post-transplantation. The absolute cell dose of non-expanded graft was 10.0×10^7 or 5.0×10^7 cells/kg while the C7 expanded grafts were transplanted at equivalent cell dosage of 10.0×10^7 or 5.0×10^7 cells/kg. The scatter plot represents the human T cell chimerism of individual animals and depicts the geometric mean with 95% CI of respective treatments. *p* values generated from Mann-Whitney *U* test among indicated experimental groups are shown in the graph for the stated *n* values. **(G):** Kaplan-Meier survival curve of the NSG mice transplanted with C7 or cytokine expanded UCB–MNC and non-expanded graft over 60-days observation period. The absolute cell dose of non-expanded graft was 10.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 10.0×10^7 cells/kg. The overall statistical comparisons for the experimental groups are also shown. **(H):** A scatter plot of human $CD45^+$ cells, $CD45^+CD34^+$ progenitors, $CD45^+CD3^+$ T cells, $CD45^+CD19^+$ B cells and $CD45^+CD34^+CD33^+$ myeloid progenitor cells chimerism in BM of female NSG mice at week 2 post-transplantation. The absolute cell dose of non-expanded graft was 10.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 10.0×10^7 cells/kg. The scatter plot represents the human $CD45^+$ cells, $CD45^+CD34^+$ progenitors, $CD45^+CD3^+$ T cells, $CD45^+CD19^+$ B cells, and $CD45^+CD34^+CD33^+$ myeloid progenitor cells chimerism of individual animals and depicts the geometric mean with 95% CI of respective treatments. *p* values generated from Mann-Whitney *U* test among indicated experimental groups are shown in the graph for the stated *n* values. Abbreviations: MNC, mononucleated cells; PB, peripheral blood; UCB, umbilical cord blood.

(D)

Confidence Intervals for 1/(HSPC frequency)

Graft	Frequency of human HSPC in NSG PB at Week 3	(–) 1 SE (Lower)	(+) 1 SE (Upper)
Non Expanded	1/794,495	1/1,649,757	1/382,616
Expanded with Cytokines	1/938,713	1/1,644,241	1/535,938
Expanded with C7 and Cytokines	1/320,642	1/594,803	1/172,849

Overall Differences in HSPC Frequencies between any Group

Chi-square	DF	*p* Value
7.57	2	.0227

Pair-wise Tests for Differences in HSPC Frequencies

Group 1	Group 2	Chi-square	DF	Pr(>Chi-sq)
Non Expanded	Expanded with Cytokines	0.12	1	0.7280
Non Expanded	Expanded with C7 and Cytokines	3.63	1	0.0567
Expanded with Cytokines	Expanded with C7 and Cytokines	7.15	1	0.0075

Goodness of Fit Tests: Estimated Slope is 0.203

Test	Chi-square	DF	*p* Value
Likelihood Ratio Test of Single-Hit model	1.96	1	0.161
Score Test of Heterogeneity	1.89	1	0.170

Note: A NSG mouse is considered to be positive if human CD45 chimerism is >0.40% in PB at week 3. The data is calculated at two transplantation cell dosage of 2.5x10^7 and 5.0x10^7 cells/kg using L-Calc and ELDA software.

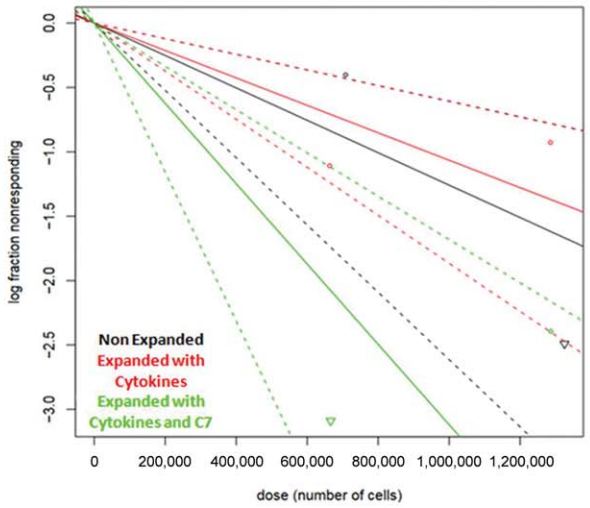


Figure 4. *Continued.*

in the NSG PB compared to fresh product (1.1% ± 0.4%; $n = 11$) ($p > .05$; $U = 42$) whereas performance of the cryopreserved cytokine expanded graft (0.1% ± 0.1%; $n = 7$) dropped significantly ($p < .05$; $U = 3$) compared to its fresh counterpart (0.5% ± 0.1%; $n = 12$) (Fig. 4A). The C7 expanded graft sustained human cell engraftment in the NSG PB for up to at least 19 weeks (Fig. 4B). At week 3 post-transplantation (cell dosage: 5.0 × 10^7 cells/kg), the human cell reconstitution in the NSG mice PB from the expanded grafts maintained multilineage human cells with a greater bias toward myeloid cells (CD33$^+$/CD15$^+$) (Fig. 4C). Moreover, C7 expanded grafts could allow quick engraftment of human donor cells. The frequency of SCID repopulating cells contributing to early peripheral blood engraftment was 2.5- and 2.9-fold higher in C7 expanded graft compared to non-expanded and cytokine expanded graft, respectively (Fig. 4D).

When transplantations in NSG mice were carried out at a higher dosage of 10.0 × 10^7 cells/kg, C7 expanded grafts gave 6.8% ± 2.2% of human CD45$^+$ cells in the NSG PB by week 2 which was at least sixfold higher than cytokine expanded graft (1.0% ± 0.8%) recipients ($p < .00001$; $U = 0$; $n = 14$) (Fig. 4E) further supported by an absolute increase in total human cell number (data not shown). However, at such high cell dose transplants, non-expanded grafts (24.9% ± 9.2%) performed significantly better than C7 expanded grafts (6.8% ± 2.2%) ($p < .00001$; $U = 0$; $n = 14$) (Fig. 4E) based on human CD45$^+$ cell detection in PB. Lineage analysis of the circulating human cells in the NSG PB transplanted with either 5.0 × 10^7 or 10.0 × 10^7 cells/kg showed that non-expanded grafts comprised of primarily CD3$^+$ T cells (>95.0%) while C7 expanded grafts had approximately 40% CD3$^+$ T cells engraftment from similar cell dose grafts (Fig. 4F). The increased amount of human T-cells reconstituted from the non-expanded graft resulted in higher incidence of GVHD (as manifested by weight loss, ruffled fur coat, hair loss and poor skin integrity) in the NSG mice recipients which resulted in poorer survival rate of approximately 25% at day 60 post-transplantation (Fig. 4G). Survival of the NSG mice receiving the expanded grafts (with or without C7) had >70% survival at day 60 post-transplantation due to minimal symptoms of GVHD (Fig. 4G).

NSG BM analysis at week 2 post-transplantation (transplantation dosage = 10.0 × 10^7 cells/kg) showed that both non-expanded (33.9% ± 11.6%) and C7 expanded graft (23.7% ± 6.6%) had similar human CD45$^+$ cell engraftment ($p = .0784$; $U = 6.5$; $n = 6$) (Fig. 4H) which was significantly ($p < .05$; $U = 0$; $n = 6$) higher than cytokine expanded graft (1.0% ± 0.8%). In terms of CD45$^+$CD34$^+$ human progenitors, C7 expanded grafts (13.2% ± 1.9%; $n = 6$) resulted in significantly higher engraftment compared to both non-expanded (0.7% ± 0.2%; $p < .05$; $U = 0$; $n = 6$) and cytokine expanded grafts (3.1% ± 1.3%; $p < .05$; $n = 6$) (Fig. 4H). Similar to PB, non-expanded grafts had >90% of CD45$^+$CD3$^+$ T-cells in the BM, while C7 expanded grafts had >30% of CD45$^+$CD34$^+$CD33$^+$ myeloid progenitor cells or mature CD45$^+$CD33$^+$ myeloid cells (Fig. 4H). Interestingly, C7 expanded

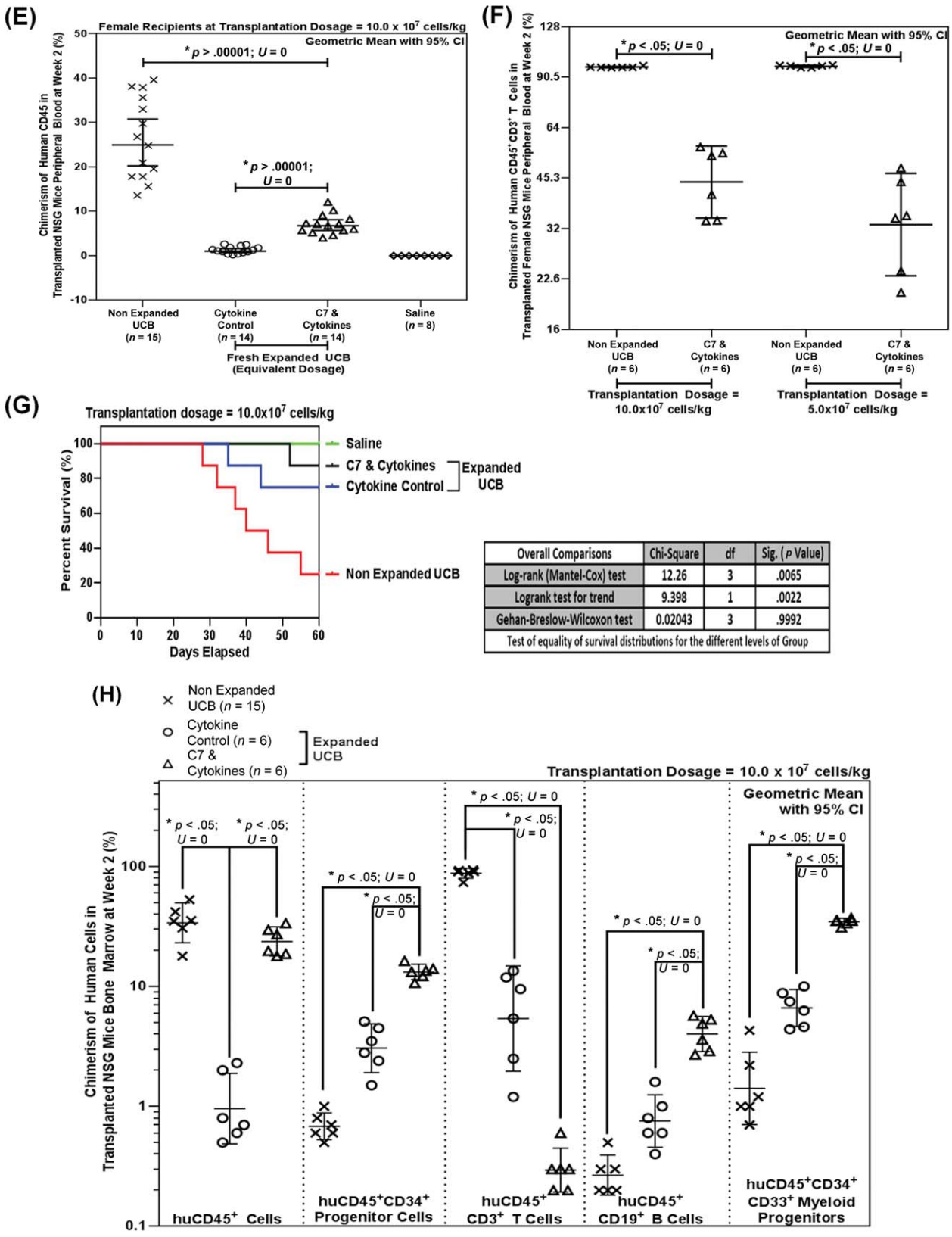


Figure 4. *Continued.*

grafts contributed to a notable 4.1% ± 1.3% ($n = 6$) of $CD45^+CD19^+$ human B-cells by week 2 of transplantation in the NSG BM (Fig. 4H). Representative flow cytometer dot plots showing the various human cell populations in NSG BM (at week 2) is shown in Supporting Information Figure 5A.

C7 expanded grafts at transplantation dosage of 2.5×10^7 or 5.0×10^7 cells/kg retained the ability to impart long-term hematopoiesis as observed by analyzing the BM of recipient NSG mice at 19 weeks post-transplantation (Fig. 5A–5D). In this NSG mouse model irrespective of graft (i.e., expanded or non-expanded), female recipients (>20%; $n = 15$) had significantly higher ($p < .00001$; $U = 5.5$) engraftment rates than their male counterparts (<1.0%; $n = 14$). Despite a difference in absolute geometric means, the C7 expanded grafts gave a statistically comparable level of human $CD45^+$ (Fig. 5A) ($p > .05$) and common ($CD45^+CD34^+$) ($p > .05$), myeloid ($CD45^+CD13^+CD33^+$) ($p > .05$) and lymphoid ($CD45^+CD7^+$) ($p > .05$) progenitor cell engraftment as that of the non-expanded grafts (Fig. 5B) at transplantation dosage of 2.5×10^7 cells/kg and 5.0×10^7 cells/kg in both male and female recipients. Furthermore, similar to early engraftment of human CD45 cells in PB (Fig. 5A), the administration of cryopreserved C7 expanded grafts maintained comparable long-term BM

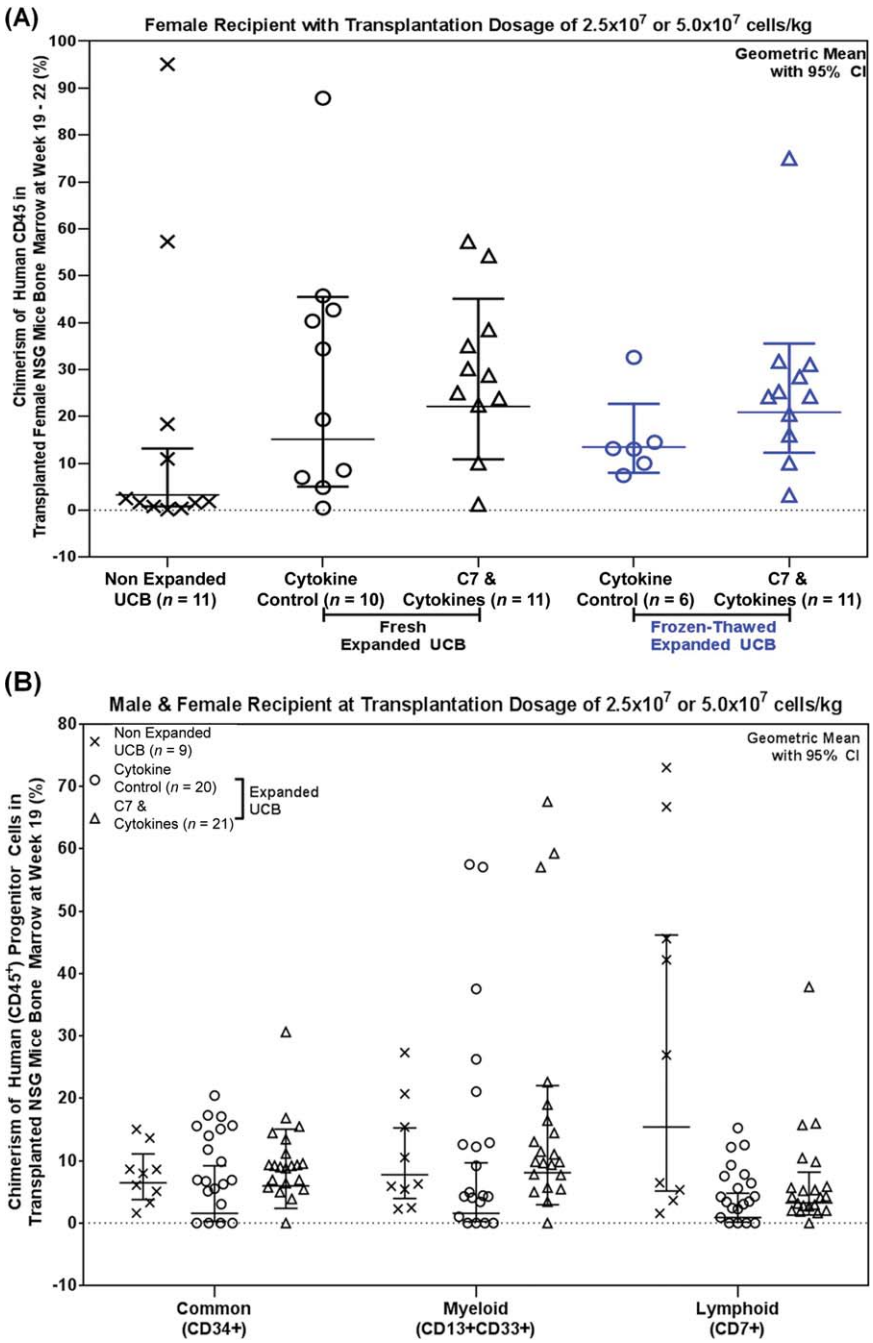


Figure 5.

human cell engraftment ($p = .4902$; $U = 49.5$) between fresh and frozen-thawed C7 expanded graft (Fig. 5A). Multilineage reconstitution of NSG BM comprising both mature myeloid ($CD33^+$ monocytes; $CD13^+/CD15^+/CD66b^+$ granulocytes; and $CD41a^+$ megakaryocytes) (Fig. 5C) and lymphoid ($CD3^+CD4^+$ T helper cells; $CD3^+CD8^+$ cytotoxic T cells; $CD19^+$ B cells; and $CD56^+$ NK cells) (Fig. 5D) human $CD45^+$ cells could be achieved with the C7 expanded graft although initial (week 2–4 post-transplant) PB/BM engraftment was skewed toward the myeloid lineage. Finally, CFU analysis (Fig. 5E) and *secondary* transplantation (Fig. 5F) of purified human $CD45^+$ cells from the BM of the *primary* NSG recipients showed equivalent performance of the non-expanded and C7 expanded grafts ($p > .05$). Absolute TNC dosages administered in the various experimental groups are illustrated in Supporting Information Figure 5B.

Discussion

Modulating pathways important in differentiation, apoptosis and senescence may promote HSPCs expansion by functional maintenance of self-renewing potential during cell proliferation. This has been partly achieved by adding small molecule inhibitors or compounds to the cell culture medium for ex vivo expansion of HSPC from CD34/CD133 enriched grafts. Examples of such small molecules include SR1 [16] that target aryl hydrocarbon receptor; UM171 [17] a pyrimidoindole derivative with unknown cellular targets; SB203580 that inhibits p38 MAP kinase [10, 11]; NR-101 that is an agonist of c-MPL involved in TPO signaling [18]; rapamycin that inhibits mTOR pathway [19]; garcinol that inhibits histone acetyltransferases [20] as well as other epigenetic modulators like valproic acid [21], combination of 5-aza-2′-deoxycytidine and trichostatin A [22]. Anti-apoptotic properties of compounds like zVADfmk (pan caspases inhibitor), zLLYfmk (calpains inhibitor) [23], and serotonin (monoamine neurotransmitter) [24] have been shown to support expansion of $CD34^+$ UCB cells.

To the best of our knowledge, C7, is the first chemical small molecule derived from SAR studies of SB203580, which could expand HSPC including rare and highly self-renewing subpopulations that express CD90 and CD49f from frozen-thawed, non-enriched UCB grafts. Based on the screening data, we identified C4 to be the second best compound in supporting expansion of HPC from non-selected grafts (Fig. 1A, 1D). SAR studies (Fig. 1*C*) showed that certain chemical substituent at specific positions of the imidazole core were essential for retaining the ability of the structural analogs to modulate HPC expansion. For example, the presence of naphthyl substituent at the C-2 position of the azole was critical in achieving >1,000-fold expansion of HPC (Fig. 1C, 1D, Supporting Information Fig. 1A, 1C, 1D). Individual low molecular weight fragments of C7 had sub-optimal effect in increasing both TNC and HPC (Supporting Information Fig. 1E, 1F).

The expansion effect of C7 was optimal at concentration of 5.0 μM with likely cytotoxic effects being observed at higher concentrations (Supporting Information Fig. 2A, 2B). Interestingly, as shown in Figure 2A, the addition of C7 could partly replace the role of essential cytokines/growth factors in supporting the ex vivo expansion of HPC. For example clinically relevant HPC and TNC expansion was observed by adding SCF, TPO and FLT-3L in presence of C7, which could partly indicate that IGFBP-2 may not be essential in cultures supplemented with C7. If expansion could be carried out with lower number of recombinant proteins then that would significantly reduce the clinical cost of implementing such protocols. It was critical to add C7 at start of the expansion cultures with replenishment being done on day 7 followed by harvesting of the expanded graft by day 10–11 (Supporting Information Fig. 2B, 2C) to ensure optimal $CD34^+$ HPC expansion. The use of either serum free or ACF had minimal effect on the enhanced CFU and HPC expansion from C7 augmented cultures compared to cytokine control in respective media (Fig. 2F, 2G). Cell morphology and leukocyte cytochemistry analysis of C7 expanded grafts showed no evidence of leukemic transformation (data not shown).

Figure 5. Long-term multilineage reconstitution of human cells in the bone marrow (BM) of NSG mice transplanted with UCB–MNC grafts expanded in presence of C7. **(A):** Human CD45 chimerism in BM of female NSG mice at week 19 post-transplantation. The absolute cell dose of non-expanded graft was either 2.5×10^7 cells/kg or 5.0×10^7 cells/kg while the expanded grafts (either fresh or frozen-thawed) were transplanted at equivalent cell dosage of 2.5×10^7 cells/kg or 5.0×10^7 cells/kg. The scatter plot represents the human CD45 chimerism of individual animals and depicts the geometric mean with 95% CI of respective treatments. No statistical significance was observed between experimental groups. **(B):** The proportion of progenitor cells present among the total human cells in BM of male and female NSG mice at week 19 post-transplantation. The absolute cell dose of non-expanded graft was either 2.5×10^7 cells/kg or 5.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 2.5×10^7 cells/kg or 5.0×10^7 cells/kg. The scatter plot represents the common progenitors ($CD45^+CD34^+$), myeloid ($CD13^+CD33^+$), and lymphoid ($CD45^+CD7^+$) progenitors of individual animals and depicts the geometric mean with 95% CI of respective treatments. No statistical significance was observed between non-expanded and C7 expanded grafts. **(C):** The proportion of myeloid cells present among the total human cells in BM of male and female NSG mice at week 19 post-transplantation. The absolute cell dose of non-expanded graft was either 2.5×10^7 cells/kg or 5.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 2.5×10^7 cells/kg or 5.0×10^7 cells/kg. The scatter plot represents the monocytes ($CD45^+CD33^+$), granulocytes ($CD45^+CD13^+/CD15^+/CD66b^+$), and megakaryocytes ($CD45^+CD41a^+$) of individual animals and depicts the geometric mean with 95% CI of respective treatments. No statistical significance was observed between non-expanded and C7 expanded grafts. **(D):** The proportion of lymphoid cells present among the total human cells in BM of male and female NSG mice at week 19 post-transplantation. The absolute cell dose of non-expanded graft was either 2.5×10^7 cells/kg or 5.0×10^7 cells/kg while the expanded grafts were transplanted at equivalent cell dosage of 2.5×10^7 cells/kg or 5.0×10^7 cells/kg. The scatter plot represents the T helper cells ($CD45^+CD3^+CD4^+$), cytotoxic T cells ($CD45^+CD3^+CD8^+$), B cells ($CD45^+CD19^+$), and NK cells ($CD45^+CD56^+$) progenitors of individual animals and depicts the geometric mean with 95% CI of respective treatments. No statistical significance was observed between non-expanded and C7 expanded grafts. **(E):** Colony forming unit (GM) assay of purified human $CD45^+$ cells from BM of NSG mice transplanted with (i) non-expanded UCB–MNC; (ii) cytokine expanded UBC–MNC; and (iii) C7 and cytokine expanded UCB–MNC. *, $p < .05$ compared to Non-expanded UCB and C7 and Cytokines group. Data represents mean ± SD for $n = 4$. **(F):** Percentage of NSG female mice with >0.1% human CD45 engraftment in the BM after being transplanted with either 1×10^6 cells or 2×10^6 cells per mouse of purified human $CD45^+$ cells obtained from the BM of primary NSG (after 20 weeks of primary transplant) mice transplanted with (i) non-expanded UCB–MNC; (ii) cytokine expanded UBC–MNC; and (iii) C7 and cytokine expanded UCB–MNC. The average (mean ± SEM) human $CD45^+$ cell engraftment in BM of positively engrafted secondary NSG mice is shown for each group. The secondary mice BM were analyzed after 20 weeks of transplantation of the purified human cells. The number of secondary graft recipient NSG mice in each group is stated in the graph. Abbreviations: GM, granulocyte, monocyte; MNC, mononucleated cells; UCB, umbilical cord blood.

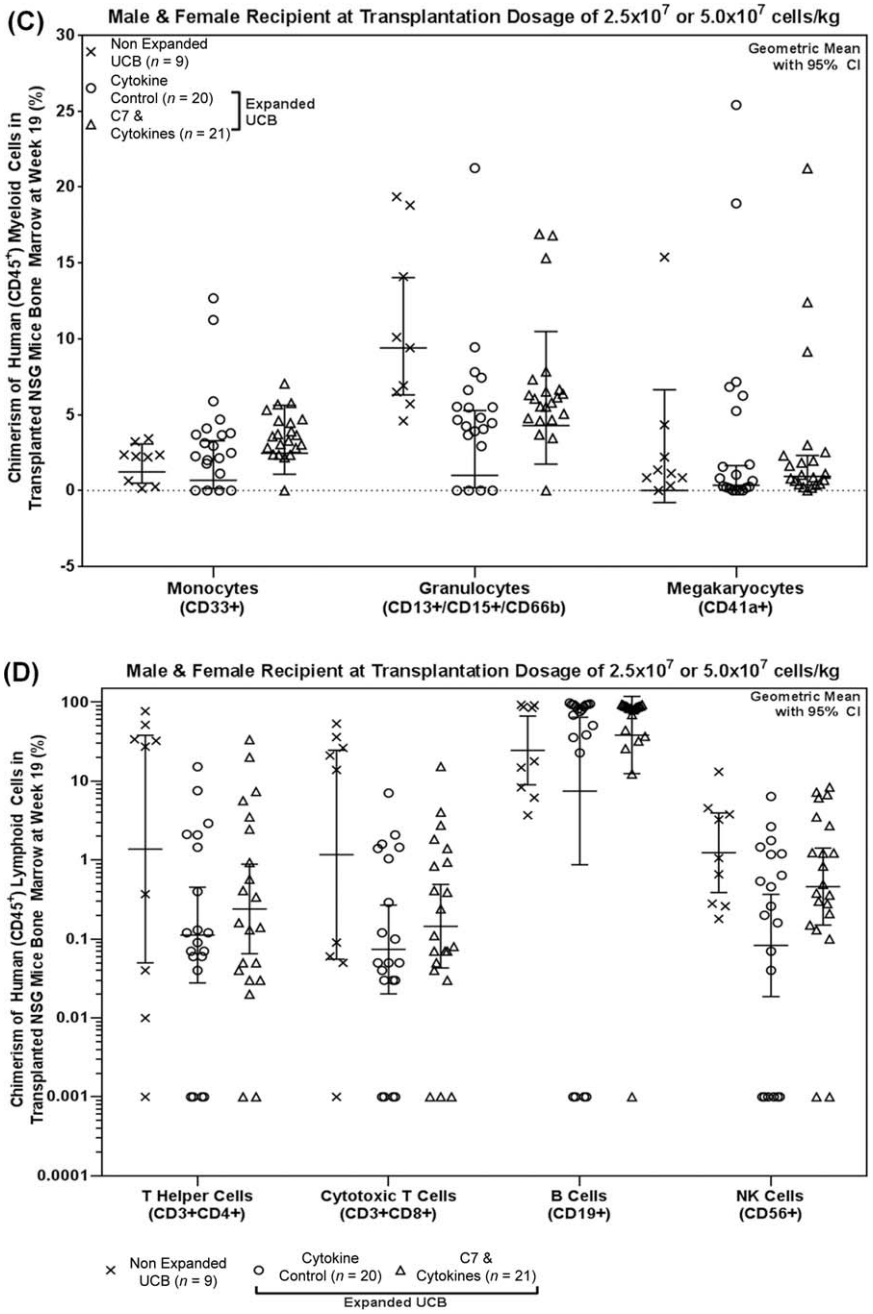
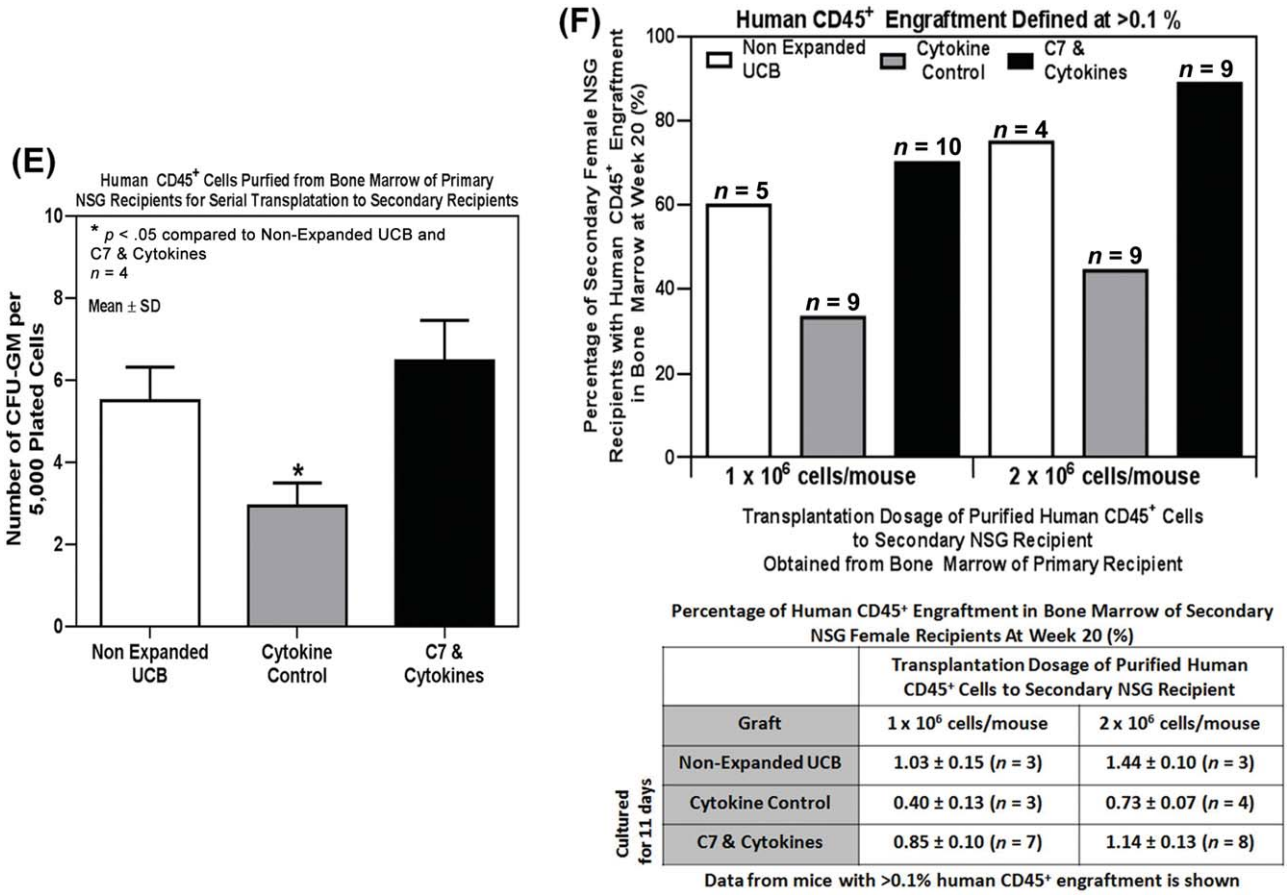


Figure 5. *Continued.*

Based on prior clinical and pre-clinical studies, non-enriched UCB could be successfully expanded only if they were cocultured with BM–MSC that partly mimics the stem cell niche of the BM. As shown in Figure 2B, C7 boosted expansion of HPC by at least twofold when compared to MSC coculture along with significant expansion of lineage committed myeloid and lymphoid progenitors. In preliminary experiments the C7 expanded grafts had similar capacity to repopulate NSG mice PB at week 3 when compared to MSC coculture expanded grafts (Supporting Information Fig. 3A). Similar comparative studies involving non-enriched grafts showed that C7 was significantly superior in expanding both $CD34^+$ bright and $CD34^+$ dim HPC compared to commercially available small molecules such as SR-1[5], NAM[6] and UM171 [17] that are currently being evaluated in clinical trials (Fig. 2C). Combination of C7 (5.0 μM) and UM171 (50 nM) showed synergistic effect on the expansion of $CD34(Bright)^+CD38^-CD45RA^-$ HPC from non-enriched grafts (Fig. 2C, Supporting Information Fig. 3B). Kinase inhibition profiling confirmed that C7 completely inhibited p38-MAPK along with casein kinase-1 (Supporting Information Fig. 3C). However, expansion cultures carried out with specific inhibitors of these top kinase targets, had partial response compared to C7 thus indicating that the HSPC expansion effect of C7 is mediated via novel cellular targets (Fig. 2D). Addition of C7 in presence of only four cytokines



Percentage of Human CD45+ Engraftment in Bone Marrow of Secondary NSG Female Recipients At Week 20 (%)

	Transplantation Dosage of Purified Human CD45+ Cells to Secondary NSG Recipient	
Graft	1 x 10^6 cells/mouse	2 x 10^6 cells/mouse
Non-Expanded UCB	1.03 ± 0.15 (*n* = 3)	1.44 ± 0.10 (*n* = 3)
Cytokine Control	0.40 ± 0.13 (*n* = 3)	0.73 ± 0.07 (*n* = 4)
C7 & Cytokines	0.85 ± 0.10 (*n* = 7)	1.14 ± 0.13 (*n* = 8)

Cultured for 11 days

Data from mice with >0.1% human CD45+ engraftment is shown

Figure 5. *Continued*.

namely, S + T + F + I, to magnetically purified $CD34^+$ cells resulted in at least 280-fold expansion of HPC over 10–11 days (Fig. 2E). Similar HSPC enriched cultures with SR-1 lasting up to 15 days gave a median CD34 expansion of 330-fold whereas NAM could only increase CD34 cells by 72-fold over 21 days [5, 6]. This observation could be inferred as C7 being highly potent at expanding CD34 selected grafts as it attained higher fold expansion in a shorter period of time and lower cost (due to lower amount of media, cytokine, and small molecule replenishment compared to SR1 and NAM).

Prior studies have reported that a single HSPC from non-manipulated UCB that coexpress CD90 and CD49f retain the ability to reconstitute an entire immunodeficient mouse [25]. Expansion of HSC1 expressing CD90 from an MNC population was at least 1,000-fold in presence of C7, whereas HSC2 that coexpress CD90 and CD49f were boosted by at least 500-fold compared to day 0 (Fig. 3A, 3B). Unlike HSPC, the generation of mature myeloid and lymphoid cells were very similar in both C7 and cytokine control cultures (Fig. 3C). When expansion cultures were initiated with highly purified HSPC expressing $CD34^+CD38^-$, C7 further augmented expression of CD90 and CD49f in the manipulated grafts (Supporting Information Fig. 4A, 4B). Expanded grafts generated from exposing purified $CD45^+CD34^+CD38^-$ HPC to C7 and cytokines compared to cytokines alone resulted in significantly higher engraftment of human progenitor and multilineage cells in the PB (by week 3) and BM (at week 31) of transplanted NSG mice (Supporting Information Fig. 4C).

Transplantation of C7 expanded grafts from non-enriched MNC to immunodeficient NSG mice resulted in improved and faster (within 2 weeks of transplantation) human cell engraftment in the PB (Fig. 4A–4E). Furthermore, lineage analysis of the human cells in the NSG PB (Fig. 4*C*) and BM (Fig. 4H, Supporting Information Fig. 5A) at week 2 post-transplant showed that C7 grafts generated more $CD34^+$ progenitors and $CD33^+$ myeloid lineage cells compared to non-expanded grafts, the recipients of which had a high proportion of mature $CD3^+$ T cells (Fig. 4F, 4H) with consequent GVHD associated mortality (Fig. 4G). Grafts expanded with C7 retained the ability to support both long-term (>19 weeks post-transplantation) multilineage human hematopoiesis in the BM (Fig. 5A–5D) of the primary recipient NSG mice along with long-term (week 19) reconstitution of secondary recipients (Fig. 5E, 5F) which were very similar to that of the non-expanded grafts (Fig. 5). The current in vivo data seems to indicate that when non-selected MNCs are cultured in presence of C7 it may boost the absolute number and function of short-term HSPC which is resulting in faster human cell engraftment in NSG PB (Fig. 4A–4E) while overcoming the loss of long-term HSPC seen in the cytokine control cultures (Fig. 5). When compared to non-expanded counterparts, C7 expanded grafts gave comparable engraftment in the BM of primary recipients (Fig. 5) indicating that similar HSPC frequencies were maintained albeit at higher survival rate (Fig. 4G) due to lower incidence of mature T-cell (Fig. 4F) induced GVHD.

When the efficacy of C7 manipulated grafts (UCB1) is to be studied in a phase I clinical trial, it will be necessary to infuse a

second non-manipulated graft (UCB2) as a measure of clinical safety. Based on the in vitro expansion and in vivo repopulation data reported hereby, it is evident that expansion of UCB MNC in the presence of C7 primarily gives rise to $CD45^+CD34^+$ progenitors and mature myeloid cells (similar to BM–MSC coculture system). Such an expanded graft (UCB1) devoid of its lymphoid cells if coinfused with a second immune cell-containing non-manipulated graft (UCB2) would likely face immune-rejection that would result in graft failure. Therefore, in a potential phase I clinical trial it will be necessary to isolate and expand $CD34^+$ cells from the smaller cell dosed UCB graft (UCB1) and infuse the re-cryopreserved $CD34^-$ lymphoid cells at the point of transplantation along with the higher cell dosed non-manipulated UCB unit (UCB2). Such a trial design would likely be applicable in phase I following which single grafts expanded (with or without CD34 selection) with C7 could be evaluated in subsequent clinical studies.

In conclusion, C7 is the first patented small molecule that could expand UCB HSPC without the need to perform prior CD34/CD133 based stem cell enrichment. The C7 expanded grafts retain both phenotypic markers as well as the ability to support early human cell engraftment in NSG PB and contribute to long-term BM hematopoiesis. Further pre-clinical investigations to decipher the putative mechanism of action of C7 along with optimization of expansion and transplantation could eventually allow clinical testing of C7 expanded UCB grafts.

Acknowledgments

We would like to acknowledge the invaluable contributions and support of the following individuals in the stated capacities: (1) B.E.D. Buenaflor and L. Zheng from Singapore Experimental Medicine Center, Singapore for their technical support in performing the murine procedures; (2) S.K. Kee and L.C. Ching from Department of Molecular Pathology, Singapore General Hospital for their technical assistance in performing the karyotyping studies; (3) G.W.P. Chua from National Cancer Center, Singapore for his technical assistance in operating the ^{137}Cs-source Gammacell GC40 Exactor; (4) C.S.F. Foong and P.J.A. Yong from SingHealth Flow Cytometry Core Platform for their technical assistance in operating flow cytometers; (5) S. Venkatesan (from Singapore General Hospital) and J. He (from National University Healthcare System) for their technical and administrative assistance in the laboratory; (6) Dr. A.M.S. Cheung (from Singapore General Hospital), Dr. J.L. Low (from Genome Institute of Singapore, Agency for Science and Technology) and Dr. J.K. Cheong (from Duke-NUS Medical School) for their technical advice on experimental design and data interpretation; and (7) Dr. D.M. Epstein and C. McCaffery from Center of Technology Development (CTeD), Duke-NUS Medical School for their critical evaluation and technical guidance on the intellectual property aspect of the study. Last, we would like to thank the staff especially Dr. P.P.Y. Chu and volunteer donors of Singapore Cord Blood Bank, Singapore. The study was supported by: (1) Innovation (ID: ING1510100-BIO) and Ignition (ID: ING-000252 BIO IGN) Grant from Singapore-MIT Alliance for Research and Technology (SMART); National Medical Research Council (NMRC) Clinician Scientist (CS) – Individual Research Grant (IRG) and Clinician Scientist Salary Support Programme (CISSP) (ID: NMRC/CIRG/1394/2014); Singapore General Hospital (SGH) Research Grant 2014 (ID: SRG/C3/04/2014); and Duke-NUS Signature Research Program Fund (ID: R-913-200-083–263) awarded to Prof. William Y.K. Hwang. (2) National University of Singapore (NUS) Start-up Grant (ID: 148-000-146-133) awarded to Prof. Christina L.L. Chai. (3) NUS Research Scholarship awarded to Dr. Qixing Zhong. (4) President's Graduate Fellowship from NUS awarded to Dr. Sudipto Bari. (5) Laboratory space provided by Duke-NUS Medical School, SGH and NUS.

Author Contributions

S.B.: conception and design, collection and/or assembly of data, data analysis, interpretation and make figures, administrative support, manuscript writing; Q.Z.: provision of study material, collection and/or assembly of data, data analysis and interpretation and make figures, manuscript writing; X.F., Z.P., N.D., and S.L.: data analysis and interpretation, provision of study material, administrative support; A.S.T.L. and T.S.L: collection and/or assembly of data, data analysis and interpretation; C.L.L.C. and G.N.C.C.: conception and provision of study material, financial support, data analysis and interpretation, final approval of manuscript; W.Y.K.H.: conception and design, provision of study material, financial support, data analysis, interpretation and make figures, supervision and planning of experiments, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

S.B., Q.Z., G.N.C.C., C.L.L.C., and W.Y.K.H are named inventors in an international patent application (application number: PCT/SG2017/050409; filing date: 18 August 2017; priority date: 18 August 2016) under the title "Substituted azole derivatives for generation, proliferation and differentiation of hematopoietic stem and progenitor cells." The applicants for this patent application are National University of Singapore and Singapore Health Services Pte. Ltd. Q.Z. and C.L.L.C. are named inventors in an international patent application (application number: PCT/SG2015/050015; publication number: WO2015119579 A1; filing date: 06 February 2015; priority date: 07 February 2014; publication date: 13 August 2015) under the title "2,4,5-Tri-substituted azole-based casein kinase 1 inhibitors as inducers for cardiomyogenesis." The applicants for this patent application are National University of Singapore and Agency for Science Technology and Research.

Note Added in Proof

This article was published online on 2 February 2018. Minor edits have been made that do not affect data. This notice is included in the online version to indicate an updated file was reposted on 19 April 2018.

References

1 Lund TC, Boitano AE, Delaney CS et al. Advances in umbilical cord blood manipulation-from niche to bedside. Nat Rev Clin Oncol 2015;12:163–174.

2 Bari S, Seah KK, Poon Z et al. Expansion and homing of umbilical cord blood hematopoietic stem and progenitor cells for clinical transplantation. Biol Blood Marrow Transplant 2015;21:1008–1119.

3 Norkin M, Lazarus HM, Wingard JR. Umbilical cord blood graft enhancement strategies: Has the time come to move these into the clinic? Bone Marrow Transplant 2013;48: 884–889.

4 Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: The first 25 years and beyond. Blood 2013;122:491–498.

5 Wagner JE, Brunstein CG, Boitano AE et al. Phase I/II trial of StemRegenin-1 expanded umbilical cord blood hematopoietic stem cells supports testing as a stand-alone graft. Cell Stem Cell 2016;18:144–155.

6 Horwitz ME, Chao NJ, Rizzieri DA et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. J Clin Invest 2014;124:3121–3128.

7 de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. N Engl J Med 2012;367:2305–2315.

8 Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med 2010;16:232–236.

9 Pineault N, Abu-Khader A. Advances in umbilical cord blood stem cell expansion and clinical translation. Exp Hematol 2015;43:498–513.

10 Zou J, Zou P, Wang J et al. Inhibition of p38 MAPK activity promotes ex vivo expansion of human cord blood hematopoietic stem cells. Ann Hematol 2012;91:813–823.

11 Wang Y, Kellner J, Liu L et al. Inhibition of p38 mitogen-activated protein kinase promotes ex vivo hematopoietic stem cell expansion. Stem Cells Dev 2011;20:1143–1152.

12 Ito K, Hirao A, Arai F et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med 2006;12:446–451.

13 Baudet A, Karlsson C, Safaee Talkhoncheh M et al. RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion. Blood 2012;119:6255–6628.

14 Navas TA, Mohindru M, Estes M et al. Inhibition of overactivated p38 MAPK can restore hematopoiesis in myelodysplastic syndrome progenitors. Blood 2006;108:4170–4177.

15 Chu PPY, Bari S, Fan X et al. Intercellular cytosolic transfer correlates with mesenchymal stromal cell rescue of umbilical cord blood cell viability during ex vivo expansion. Cytotherapy 2012;14:1064–1079.

16 Boitano AE, Wang J, Romeo R et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 2010;329:1345–1348.

17 Fares I, Chagraoui J, Gareau Y et al. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 2014;345:1509–1512.

18 Nishino T, Miyaji K, Ishiwata N et al. Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL. Exp Hematol 2009;37:1364–1377.

19 Rohrabaugh SL, Campbell TB, Hangoc G et al. Ex vivo rapamycin treatment of human cord blood CD34+ cells enhances their engraftment of NSG mice. Blood Cells Mol Dis 2011;46:318–320.

20 Nishino T, Wang C, Mochizuki-Kashio M et al. Ex vivo expansion of human hematopoietic stem cells by garcinol, a potent inhibitor of histone acetyltransferase. PLoS One 2011;6: e24298.

21 Chaurasia P, Gajzer DC, Schaniel C et al. Epigenetic reprogramming induces the expansion of cord blood stem cells. J Clin Invest 2014;124:2378–2395.

22 Mahmud N, Petro B, Baluchamy S et al. Differential effects of epigenetic modifiers on the expansion and maintenance of human cord blood stem/progenitor cells. Biol Blood Marrow Transplant 2014;20:480–489.

23 Sangeetha VM, Kale VP, Limaye LS. Expansion of cord blood CD34 cells in presence of zVADfmk and zLLYfmk improved their in vitro functionality and in vivo engraftment in NOD/SCID mouse. PLoS One 2010;5: e12221.

24 Yang M, Li K, Ng PC et al. Promoting effects of serotonin on hematopoiesis: Ex vivo expansion of cord blood CD34+ stem/progenitor cells, proliferation of bone marrow stromal cells, and antiapoptosis. Stem Cells 2007; 25:1800–1806.

25 Notta F, Doulatov S, Laurenti E et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 2011;333:218–221.

See www.StemCellsTM.com for supporting information available online.