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

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

**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 25 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\(^+\)/CD49f\(^−\) 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 p38 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−Sca1 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+ CD138− 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 × 10^7 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 (CO2) 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′ST diff Hematology Analyzer, Beckman Coulter Inc., USA) and resuspended 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-Pkdctm1Gmd Ile127<sup>mel</sup>/Szl, better known as non-obese diabetic (NOD)–severe combined immunodeficient (SCID) gamma (NSG) (NSG) mice, purchased from Jackson Laboratory (Bar Harbor, USA), were housed in cages of six of the same gender in...
Figure 1.

(A) Expansion of UCB HSPC from MNC with Azole Compound

(B) 11-day Expansion Cultures Initiated with Non-Selected UCB-MNC

(i) Day 0 – Non-cultured

(ii) Day 11 – Cytokine Expanded

(iii) Day 11 – C7 Expanded

(C) Replacement with pyran-4-yl substituent decreased potency in inducing expansion of HPCs

(D) SB203580

4-[4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-1H-imidazol-5-yl]pyridine
MW: 377.43 g/mol

C7

4-[2-(1-fluoronaphthalen-2-yl)-(4)-(4-fluorophenyl)-1H-imidazol-5-(4)(4)-yl]pyridine
MW: 383.12 g/mol

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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 LSRIFortessa (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]-1H-imidazol-5-yl]pyridine), C4 (4-[2-(1-fluoronaphthalen-2-yl)-4-(m-tolyl)-1H-imidazol-5-yl]pyridine), C7 (4-[2-(1-fluoronaphthalen-2-yl)-4-(5-[4-fluorophenyl]-1H-imidazol-5(4)-yl]pyridine), C22 (4-[4-(5-[4-fluorophenyl]-1H-imidazol-5(4)-yl]pyridine), C37 (4-[4-[4-(4-fluorophenyl)-1H-imidazol-5(4)-yl]pyridine) and C47 (4-[4-(4-(fluorophenyl)-2-(7-methoxynaphthalen-2-yl)-1H-imidazol-5(4)-yl]pyridine) could expand viable HPC (CD45^+ CD34^- CD38^-) 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-[4-(fluorophenyl)-1H-imidazol-5(4)-yl]pyridine), C25 (4-[2-(2-chlorophenyl)-4-[4-(fluorophenyl)-1H-imidazol-5(4)-yl]pyridine) and C28 (4-[4-(5)-(4-fluorophenyl)-2-(2-...
nitrophenyl)-1H-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.
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 < 0.001). 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 < 0.05) 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 < 0.05). Similarly, a twofold augmentation of HPC expansion is observed when C7 is added to a basal cocktail of S + I + T (p < 0.05). 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 < 0.01; 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 < 0.001 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 small molecule are as follows: S represents SR-1 at 1.0 μM, 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 < 0.001 compared to all other conditions. **, p < 0.05 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− 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 < 0.001 compared to respective population of all groups. **, p < 0.05 compared to respective population of all groups. Data represent mean ± SEM for n = 6. (C): Expansion of CD34+ bright (early HPC, CD45− CD34+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; N: represents Nicotinamide at 5.0 mM. *, p < 0.01 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. (E): 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. SB205380, and DMSO, was also included. *, p < 0.01 compared to all other compounds and controls. Data represents mean ± SD for n = 3. (F): 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. (G): 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/AFC media, cytokine, and C7 replenishment being done on day 7. Cytokines alone in SFEM/AFC 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. (H): Ex vivo expansion of CFU–GM 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 < 0.001 and **, p < 0.05 compared to cytokine control for CFU–GM 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.

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from UCB–MNC cultured for 11 days (Fig. 2F, Experiment 2). Cytokine control cultures maintained CFU expansion between $16.3 \pm 0.3$-fold to $25.6 \pm 1.1$-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 than cytokine control cultures maintained CFU expansion between $16.3 \pm 0.3$-fold to $25.6 \pm 1.1$-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 than cytokine control cultures maintained CFU expansion between $16.3 \pm 0.3$-fold to $25.6 \pm 1.1$-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).
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–granuloctye-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 culture should better expansion of CD34\(^+\) CD45\(^-\) CD38\(^-\) CD45RA\(^-\) HPCs within 11 days which was approximately 1.9-fold higher compared to cytokine controls (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\(^-\) 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 2. Continued.
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...
Groups are shown in the graph for the stated dosage of 2.5 of non-expanded graft was 2.5 geometric mean with 95% CI of respective treatments.

Expanded graft was 10.0 of 2.5

Geometric mean with 95% CI of respective treatments. Abbreviations: MNC, mononucleated cells; PB, peripheral blood; UCB, umbilical cord blood.

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 chimeraism 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^-CD3^- 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^-CD3^- 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.
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 × 10⁷ 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 × 10⁷ 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 × 10⁷ or 10.0 × 10⁷ 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 × 10⁷ 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⁺CD3⁺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 engraftment.
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\(^+\) 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 S8.

**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 HSPCs 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]; ganciclovir 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-epitopic 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. 1C) 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 \( \mu \)M 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 recombiant 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 leucocyte cytchemistry 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 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 10\(^7\) cells/kg while the expanded grafts (either fresh or frozen-thawed) were transfected at equivalent cell dosage of 2.5 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 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 non-expanded and C7 expanded grafts. (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 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 10\(^7\) cells/kg while the expanded grafts were transfected at equivalent cell dosage of 2.5 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 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 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 10\(^7\) cells/kg while the expanded grafts were transfected at equivalent cell dosage of 2.5 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 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 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 10\(^7\) cells/kg while the expanded grafts were transfected at equivalent cell dosage of 2.5 \( \times \) 10\(^7\) cells/kg or 5.0 \( \times \) 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\(^+\)) 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 \( \times \) 10\(^8\) cells or 2 \( \times \) 10\(^8\) 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.
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<sup>+</sup> HPC compared to commercially available small molecules such as SR-1<sup>â€”</sup>, NAM<sup>â€”</sup> 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)<sup>−</sup>CD38<sup>−</sup>CD45RA<sup>+</sup> 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

Figure 5. Continued.
namely, S + T+F+I, to magnetically purified CD34<sup>+</sup> 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<sup>+</sup>CD38<sup>-</sup>, C7 further augmented expression of CD90 and CD49f in the manipulated grafts (Supporting Information Fig. 4A, 4B). Expanded grafts generated from exposing purified CD45<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup> 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. 4C) and BM (Fig. 4H, Supporting Information Fig. 5A) at week 2 post-transplant showed that C7 grafts generated more CD34<sup>+</sup> progenitors and CD33<sup>+</sup> myeloid lineage cells compared to non-expanded grafts, the recipients of which had a high proportion of mature CD3<sup>+</sup> 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 confused 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.

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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

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