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Original Article

Pharmacological preconditioning for short-term ex vivo expansion of human umbilical cord blood hematopoietic stem cells by filgrastim

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Abstract: Although umbilical cord blood (UCB) hematopoietic stem cell transplantation (UCBT) has emerged as a promising haematological reconstitution therapy for leukemias and other related disorders, the insufficient UCB stem cell dosage still hinders better clinical outcomes. Previous research efforts, by focusing on ex vivo UCB expansion capabilities have sought to benefit from well-known mechanisms of self-renewal characteristics of UCB stem cells. However, the long-term (> 21 days) in vitro culture period and the low neutrophil recovery significantly reduce the transplantability of such ex vivo expanded UCB stem cells. To overcome the latter hurdles in this study, a post-thaw, short-term ex vivo expansion methodology of UCB mononuclear (UCB-MN) and $CD34^+$ cells has been established. Notably, such effort was achieved through pharmacological preconditioned of UCB cultures by filgrastim agent already used in the clinical setting. In crucial cell populations implicated in the promotion of functional engraftment, the progression of free survival rates (PFS), a marked increase of 6.65 to 9.34 fold for UCB-MN and 35 to 49 fold for $CD34^+$ cells has been noticed. Overall, these results indicate that transplantation of pharmacologically-preconditioned ex vivo expansion of UCB stem and progenitor cells keep high promise upon transplantation to enhance therapeutic potential in everyday clinical practice.

Keywords: Post-thaw, ex vivo expansion, pharmacological preconditioning, hematopoietic stem cell transplantation, filgrastim

Introduction

Umbilical cord blood hematopoietic stem cell transplantation (UCBT) has become a main therapeutic option for paediatric patients with hematologic disorders undergoing high dose of chemotherapy [1-4]. Indeed, UCBT is offering substantial clinical advantages, such as: a) significantly faster availability UCBT units from cryopreserved samples in specialized cell banks, with patients receiving UCBTs *ca.* 25 to 36 days earlier than those required to receive bone marrow (BM) aliquots for clinical application; b) expansion of the donor cell pool, because sufficiently large UCBT units mismatched for one or two HLA-A, -B, and -DR antigens seem to be tolerated for patients survival; c) a lower incidence and severity of acute graft-versus-host disease (GVHD) negative outcome; d) a significantly lower risk of transmitting infections by latent viruses, such as through cytomegalovirus and Epstein-Barr, since the UCBT units are routinely tested for any contamination burden of such viruses; e) reduced donor attrition rates, because cord blood samples can be stored and remain available in a registry; and f) easier targeting of ethnic group minorities and an increased pool of rare patient haplotypes to be served [5].

However still today, the crucial hindering clinical factor broadly affecting UCBT units is the limited number of HSCs included, as well as the fact that they can be collected only once from the

umbilical cord and placenta after birth. The latter, leads to delayed hematopoietic reconstitution, higher risk of graft failure, and transplantation-related mortality after therapeutic application of UCBT cells, as compared with BM and peripheral blood (PB) transplantation [6]. As a matter of fact, several research and clinical groups worldwide have been aiming to evaluate approaches to increase the HSCs dose by *ex vivo* expansion and test the potential of such *ex vivo* expanded UCBT units to enhance engraftment. However, the results to date have shown limited success in demonstrating significant improvement over time upon neutrophil engraftment. In this process, a main disadvantage that remains is the long-term *ex vivo* expansion culture period (> 21 days) in order to obtain increased fold number of HSCs [7-9]. The majority of *ex vivo* UCBT expansion systems have exploited cytokine mixtures, including stem cell factor (SCF), thrombopoietin (TPO), Fms-like tyrosine kinase-3 ligand (FL3 ligand), a complex of interleukin 6 (IL-6) and soluble IL-6 receptor (IL-6/sIL-6R), the Notch ligand Delta1, angiopoietin-like proteins, and pleiotrophin [10, 11]. Recently, pharmacological preconditioning treatments applied to hematopoietic stem cells have been shown to enhance ex vivo expansion potential by increasing survival signals [12, 13]. Many survival and protective molecules including hypoxia-inducible factor-1 (HIF-1), trophic/growth factors, Akt, extracellular signal-regulated kinase (ERK), glycogen synthase kinase-3β (GSK-3β), matrix metalloproteinase-2 (MMP-2), and Bcl-2 are involved in response to pharmacological preconditioning stimuli [14, 15]. Up to now, a number of preconditioning triggers have been tested in stem cells and stem cell-derived progenitor cells [16-18]. Alternatively, preconditioning can be achieved using suitable mediators such as erythropoietin (EPO) [19], stromal-derived factor-1 (SDF-1), insulin-like growth factor-1 (IGF-1), heat shock proteins (HSPs) or pharmacological agents such as diazoxide, apelin, isoflurane, lipopolysaccharide (LPS), cobalt protoporphyrin (COPP), and apelin-13 [20-31].

In this study, based upon existing data we have sought to establish a novel *ex vivo* expansion clinical strategy of post-thawed UCB mono nuclear cells (UCB-MNC). That ex vivo expansion protocol has been achieved by applying 24 hours pharmacologically preconditioning culture conditions on UCB samples by early acting cytokine filgrastim agent. In particular, a substantial 6.65 to 9.34 fold increase for UCB-MNC and 35 to 49 fold for $CD34^+$ cells has been achieved through this methodology. By reducing the needed time for expansion to *ca.* one third (to 8 from 21 days in the previously used *ex vivo* expansion protocols), these data add new insights on how the HSCs pharmacological preconditioning approach can be improved towards better benefiting transplantation therapy for hematopoietic malignancies.

Materials and methods

Collection of UCB

UCB units (N=4) were collected by trained obstetricians of University Gynaecology Clinic of Ioannina with the placenta in utero immediately after clamping the cord and separating the infant. All mothers and infants were in good general condition. An informed consent document was signed from all the donors but were deemed unsuitable for banking due to low collected UCB quantity (< 60 mL). The collection bags (MDE 6500 LN; Macopharma-Rue Lorthiois; France anticoagulant volume CPDA-1 63 mL) were placed in a low position, which facilitates blood flow by gravity. After collection and labelling of the bags with consecutive numbers and date and time of delivery, the samples were stored at room temperature and transported to the clinical laboratory.

Isolation of UCB-MNCs

Fresh UCB units (N=4) centrifuged under a two steps modified Rubinstein's protocol to obtain a clear Total Nucleated Cells (TNC) fraction ("buffy-coat"). The TNC fraction (2 mL from each UCB unit) transferred aseptically with a Pasteur Pipette in a sterile 15 mL falcon tube (CellStar Tubes, greiner bio-one) and diluted 1:1 by sterile Dulbecco's Phosphate Buffered Saline (1X) (Gibco by Life Technologies) in a 4 mL final volume. Red Blood Cells (RBCs)-depleted UCB-MNCs fraction isolated by Ficoll density gradient centrifugation according to manufacturer guidelines (Ficoll-Paque PREMIUM, GE healthcare Bio Sciences AB, Uppsala) and transferred in 2 mL sterile cryotubes (Code: 430659, Corning Corporation) for further cryoprocessing.

Cryopreservation of UCB-MNCs

Each UCB unit (N=4) represented by a 2 mL cryotube containing a concentrate of 1 × 10^6

MNCs per mL suitably coded by bar-code system. The cryotubes filled in an ice pack gradually by a cryoprotective agent's mixture concentrations DMEM 80% (Gibco by Life Technologies), Dimethyl Sulfoxide 5% (Hybri-Max, Sigma Aldrich, St. Louis, MO) and autologous UCB Serum 15% on final MNCs samples. The cryotubes transferred immediately in a clinical grade rate controlled freezer (Nicool Plus PC, Air Liquide) and under a certified protocol by manufacturer reach the crucial temperature of -120°C in 40 minutes time period. Finally, the cryotubes placed carefully in cryoboxes and transferred to Liquid Nitrogen Cryotank -196°C (Arpege 170, Air Liquide) for 14 days storage.

Thawing of UCB-MNCs

The cryotubes containing the UCB-MNCs removed from Liquid Nitrogen Cryotank, sterilized by 70% alcohol solution and transferred immediately in a prewarmed sterile water bath to 38°C for rapid thawing process. After thawing MNCs centrifuged at 400 g for 5 minutes, remove supernatant and resuspend cell pellet in 10 mLs fresh 37°C warm stem cell culture media (Cat# 09605, StemSpan™ SFEM II, 100 ml, Stemcell Technologies) and prepared for flow cytometry viability assays.

Pharmacologically preconditioned ex vivo expansion of UCB-MNCs

Post-thawing UCB-MNCs transferred aseptically to 96-well flat bottom plates (3628, Corning Costar™, Sigma Aldrich, St Louis, MO) for further pharmacological preconditioning with filgrastim agent. For each UCB unit, 200 µl of UCB-MNCs triplicates containing 40.000 MNCs in serum free culture media plus antibiotics (1:100 diluted, PenStrep™) transferred per well plate, pharmacologically preconditioned with 100 ng/ml early acting cytokine filgrastim agent (Nivestim™ 30 MU/0.5 ml, Hospira UK Limited) for 24 hours exposure period. After the 24 hours exposure to pharmacological agent each well plate washed and stimulated by 1:100 diluted StemSpan™ CC100 (x100) (Stemcell Technologies) recombinant early and late acting human cytokines formulated to support the proliferation of human hematopoietic progenitors. StemSpan™ CC100 (x100) contains rhFlt-3 Ligand, rhStem Cell Factor, rhIL-3, rhIL-6 recommended for high cell yields and large numbers of $CD34^+$ cells. The cultures were incubated for 7 days in a 5% CO_2 incubator at 37°C with media and growth factors replacement every 72 hours. After 8 days the ex vivo expanded cells were removed from the well plates and transferred to appropriate aliquots for qualitative and quantitative cell analysis.

Quality and quantity control of ex vivo expanded UCB-MNCs

Flow cytometry analysis: UCB-MNCs were analyzed for $CD45^+CD34^+$ immunophenotyping post thawing and post-ex vivo expansion procedure and stained with anti-CD34-PE and anti-CD45-FITC (Cytometer FC500, Stem Kit™ Reagents, Beckman Coulter, Immunotech, France) Aliquots of cells were also stained with isotype control antibodies.

Viability analysis: UCB-MNCs were analyzed for $CD45^+CD34^+$ 7-AAD +/- viability post-thawing and post-ex vivo expansion procedure and stained with 7-aminoactinomycin D (Cytometer FC500, Stem Kit™ Reagents, Beckman Coulter, Immunotech, France) Aliquots of cells were also stained with isotype control antibodies.

Bacteriology analysis: UCB-MNCs were analyzed for $CD45^+CD34^+$ anaerobic and aerobic bacterial sterility pre- and post-ex vivo expansion procedure in the automated bacteriology analyser BacTalert (BacTalert®, Biomerieux S.A).

Functionality analysis: UCB-MNCs were analyzed for $CD45^+CD34^+$-CFU GEMM multipotentiality pre- and post-ex vivo expansion procedure in Methylcellulose based media-MethoCult (MethoCult®, StemCell Technologies Inc, Vancouver, Canada). Methylcellulose is resistant to changes in pH, the action of cellular enzymes, and does not bind to essential components of the culture medium or exogenously added factors such as cytokines or drugs.

Statistical analysis: UCB-MNCs*, UCB-HSCs** and UCB-HSCs 7-AAD viability*** post-thawing and post-expansion values are expressed as mean ± standard deviation (SD) of four independent experiments from four donors. Statistical significance was determined by two-tailed parametric-tests using GraphPad Prism 5 software (Version 5.01). Differences were considered to be significant for post-thawing

Table 1. Post-thaw ex vivo expansion of pharmacologically preconditioned UCB-MNCs*

UCB-MNCs ($CD45^+$-FITC)/μl					
Exp#	1	2	3	4	Mean +/- SD, p* < 0.005
Post-Thawing Day 0	140	165	229	216	187.50 +/- 42.01
Post-Expansion Day 8	932	1542	1738	1797	1502.25 +/- 395.48
Fold Expansion	6, 65	9, 34	7, 58	8, 32	7.97 +/- 1.13

UCB-MNCs*: Umbilical Cord Blood Mononuclear Cells, SD: Standard Deviation, p*: *p*-value.

Table 2. Post-thaw ex vivo expansion of pharmacologically preconditioned UCB-HSCs

UCB-HSCs ($CD34^+$-PE)/μl					
Exp#	1	2	3	4	Mean +/- SD
Post-Thawing Day 0	5	1	1	9	04 +/- 3.82
Post-Expansion Day 8	205	35	49	382	167.75 +/- 162.29
Fold Expansion	41	35	49	42, 44	41.86 +/- 5.74

Table 3. Post-thaw absolute number of ex vivo expanded pharmacologically preconditioned UCB-HSCs**

UCB-HSCs ($CD34^+$-PE absolute number/cells)					
Exp#	1	2	3	4	Mean +/- SD, p** < 0.05
Post-Thawing Day 0	51	5	10	82	37 +/- 36.39
Post-Expansion Day 8	1417	466	627	1959	1117.25 +/- 698.29
Fold Expansion	27, 78	93, 2	62, 7	23, 89	51.89 +/- 32.60

UCB-HSCs**: Umbilical Cord Blood Hematopoietic Stem Cells, SD: Standard Deviation, p**: *p*-value.

Table 4. Post-thaw $CD34^+$ and 7-AAD viability*** percentage of ex vivo expanded pharmacologically preconditioned UCB-HSCs

$CD34^+$-PE %					
Exp#	1	2	3	4	Mean +/- SD
Post-Thawing Day 0	3, 66	0, 23	0, 32	4, 27	2.12 +/- 2.14
Post-Expansion Day 8	21, 95	2, 24	2, 81	21, 79	12.19 +/- 11.17
Fold Expansion	5, 99	9, 73	8, 78	5, 1	7.4 +/- 2.20
VIABILITY 7-AAD-/%					
Exp#	1	2	3	4	Mean +/- SD, p*** < 0.05
Post-Thawing Day 0	70, 21	63, 91	88, 8	87, 35	77.56 +/- 12.41
Post-Expansion Day 8	92, 12	95, 63	94, 88	92, 83	93.86 +/- 1.65

7-AAD viability***: 7-Aminoactinomycin viability, SD: Standard Deviation, p***: *p*-value.

and post-expansion absolute cell values with degrees of significance p* < 0.005 (**Table 1**), p** < 0.05 (**Table 3**), p*** < 0.05 (**Table 4**).

Results

Pharmacologically preconditioned ex vivo expansion of UCB-MNCs

Each UCB sample was pharmacologically preconditioned for 24 hours on 96-well plates and then ex vivo expanded by cultivating the UCB-MNCs for 7 days stimulated by StemSpan CC100 early and late acting cytokines. By systematically working to improve a pharmacologically preconditioned ex vivo expansion protocol for UCB post-thawed samples, the agent of filgrastim have been identified to possess such behaviour. Through evaluating that capacity of filgrastim, it has been finally a success to define ex vivo expansion effects in a concentration- and time-dependent manner. In particular, the concentration range for filgrastim was 10-100

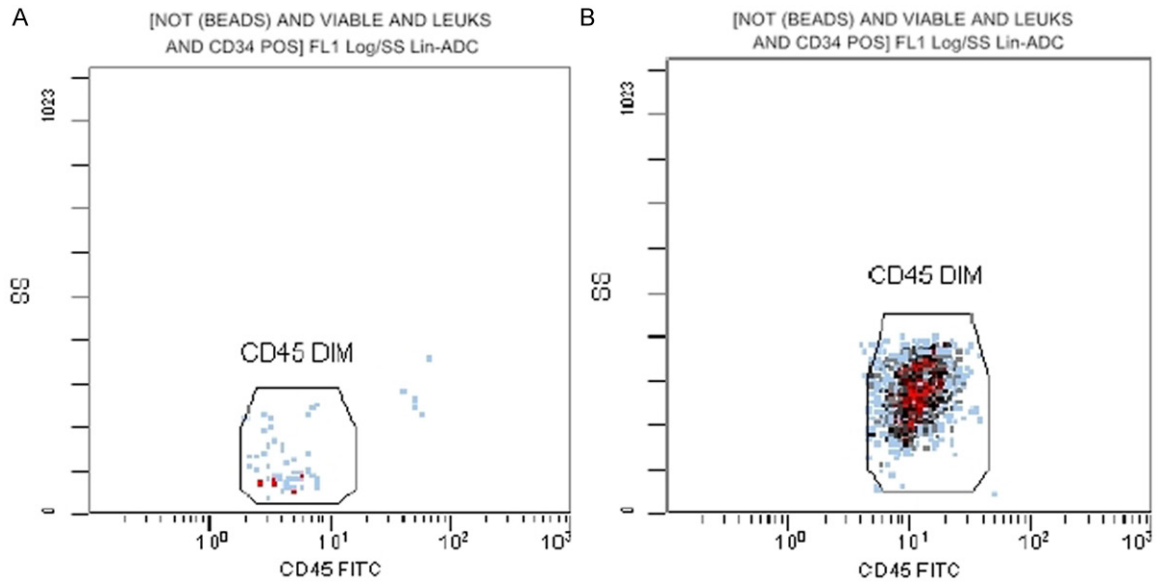

Figure 1. Role of 24 hours filgrastim pharmacological preconditioning in 8 days hematopoietic stem cell ex vivo expansion culture. Representative sample experiment #1: Flow cytometry comparison analysis of post-thawing $CD45^+$ FITC/Side Scatter (A) and post-expansion $CD45^+$ FITC/Side Scatter (B).

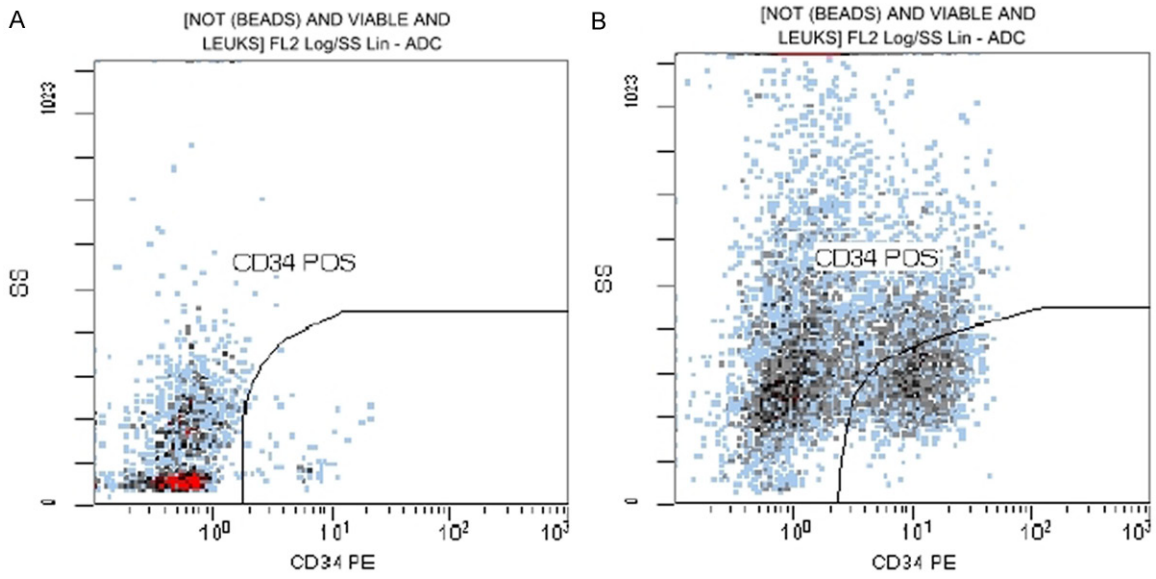

Figure 2. Role of 24 hours filgrastim pharmacological preconditioning in 8 days hematopoietic stem cell ex vivo expansion culture. Representative sample experiment #1: Flow cytometry comparison analysis of post-thawing $CD34^+$ PE/Side Scatter (A) and post-expansion $CD34^+$ PE/Side Scatter (B).

ng/ml (data not shown). The maximum preconditioned ex vivo expansion effect from the use of filgrastim has been found at 24 hours with 100 ng/ml of filgrastim; a fact that has been afterwards applied throughout the experiments described in this work. The total ex vivo expansion culture time period including the exposure time to filgrastim agent was 8 days. After 8 days ex vivo cultivation a median of 7.97 fold expansion of UCB-MNCs was obtained with a range of 6.65 to 9.34 fold (**Table 1**). The median UCB-MNCs post-thawing was 188.50 cells/μl with a range of 140 cells/μl to 229 cells/μl. The median UCB-MNCs post-expansion was 1502.25 cells/μl with a range of 932 cells/μl to 1797 cells/μl. The UCB-MNCs quantification

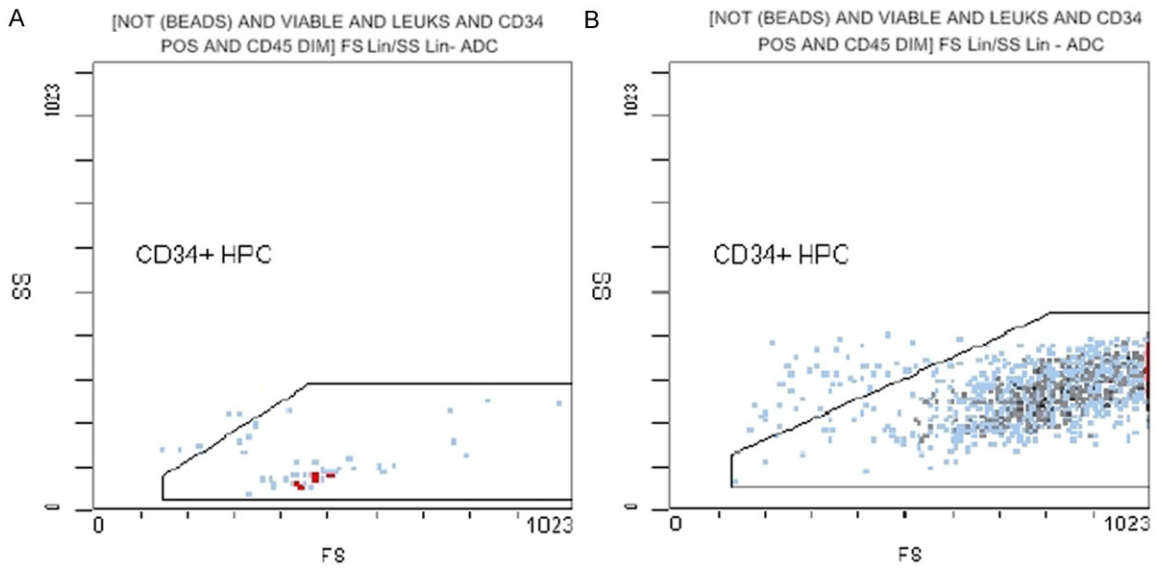

Figure 3. Role of 24 hours filgrastim pharmacological preconditioning in 8 days hematopoietic stem cell ex vivo expansion culture. Representative sample experiment #1: Flow cytometry comparison analysis of post-thawing CD34$^+$ Forward/Side Scatter (A) and post-expansion CD34$^+$ Forward/Side Scatter (B).

was issued by anti-CD45$^+$ monoclonal antibody fixed by fluorescein isothiocyanate (FITC) compared with or only to forward (FS) and side scattering (SS) physical parameters (**Figure 1**).

Pharmacologically preconditioned ex vivo expansion of UCB-HSCs

The UCB-HSCs quantification was issued by anti-CD34$^+$ monoclonal antibody fixed by Phycoerythrin (PE) compared with side scattering (SS) (**Figure 2**) or only to forward (FS) and side scattering (SS) physical parameters (**Figure 3**). Surprisingly after 1 day of pharmacological preconditioning and 7 days of cytokines stimulation a median of 41.86 fold expansion of CD34$^+$ PE per µl was obtained with a range of 35 to 49 fold (**Table 2**). The median absolute number of CD34$^+$ PE cells post-thawing was 37 cells/µl with a range of 05 cells/µl to 82 cells/µl. The median absolute number of CD34$^+$ PE cells post-expansion was 1117.25 cells/µl with a range of 466 cells/µl to 1959 cells/µl (**Table 3**). A median of 5.75 fold expansion percentage of CD34$^+$ PE cells post-expansion with a range of 5.10% to 9.73% was observed (**Table 4**). Finally, in comparison to control group (median 5 fold increase, data according to manufacturer [32]) that stimulated only to CC100 early and late acting cytokines, the pharmacologically preconditioned experimental group obtain a median of 41.86 fold increase in CD34$^+$ PE per µl.

Viability and quality control of ex vivo expanded UCB-HSCs

The UCB-HSCs viability assay was issued to CD34$^+$ cells fixed by 7-aminoactinomycin D (7-AAD). 7-AAD is a fluorescent intercalator that undergoes a spectral shift upon association with DNA. 7-AAD/DNA complexes can be excited by the 488 nm laser and has an emission maxima of 647 nm, making this nucleic acid stain useful for multicolor fluorescence microscopy and flow cytometry. 7-AAD appears to be generally excluded from live cells, but can be used with cells that have been fixed and permeabilized (**Figure 4**). The median viability of CD34$^+$ PE cells post-thawing was 77.56% with a range of 63.91% to 88.80%. The median viability of CD34$^+$ PE cells post-expansion was 93.86% with a range of 92.12% to 95.63% (**Table 4**). The UCB-HSCs bacteriology assay was performed by automated analyser BacTalert and was negative to aerobic and anaerobic blood cultures. The in vitro functionality demonstrates the efficiency of hematopoietic stem/progenitor cells to develop into myeloid and erythroid colonies in the presence of various combinations of cytokine factors. In this regard, this capacity was analyzed in the expanded pharmacologically preconditioned CD34$^+$ cells. After expanded CD34$^+$ cells for 8 days, cells were isolated and seeded in methylcellulose media as described in Methods. The

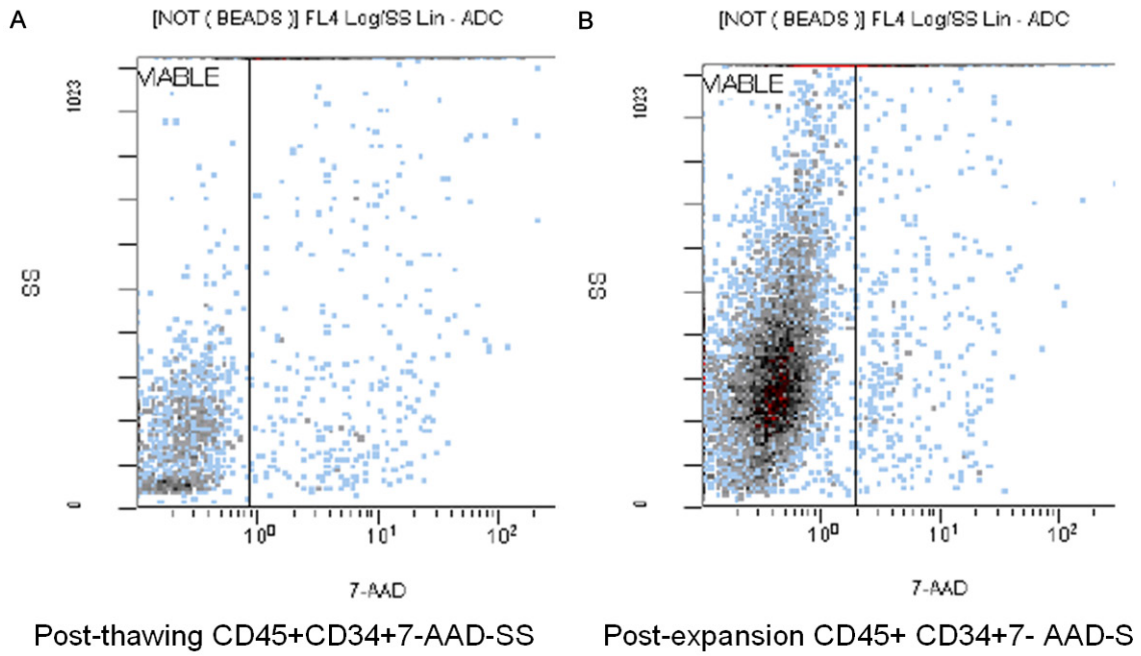

Figure 4. Role of 24 hours filgrastim pharmacological preconditioning in 8 days hematopoietic stem cell ex vivo expansion culture. Representative sample experiment #1: Flow cytometry comparison analysis of post-thawing viability $CD45^+CD34^+$ 7-AAD-/Side Scatter (A) and post-expansion viability $CD45^+CD34^+$ 7-AAD-/Side Scatter (B).

results showed that pharmacologically preconditioned expanded cells contained the ability to produce clonogenic progenitor cells: CFU-GEMM, CFU-GM, BFU-E, and CFU-M similar to the fresh isolated $CD34^+$-cell. These findings indicated the achievement of repopulating capacity of preconditioned expanded UCB-HSCs population in vitro (see Supplementary information data for total experiments).

Discussion

Hematopoietic stem and progenitor cells can be obtained from several sources. The three main sources are bone marrow (BM), mobilised peripheral blood (mPB), and UCB. Among these, UCB-HSCs exhibit crucial transplant advantages compared to BM and mPB, such as low Graft Versus Host Disease (GVHD) phenomena, high proliferation capacity *in vitro* and safe noninvasive collection procedure. Although the transplantability of UCB-HSCs is enhanced, the main disadvantage remains the cell concentration of $CD34^+$ cells per single UCB unit. Therefore, administration of ex vivo expanded $CD34^+$ cells is a keystone for the improvement of progression free survival (PFS) rates, reduction of the relative mortality outcomes and promotion of functional engraftment of malignant patients after UCBT. In this present study, we demonstrated an efficient, clinical grade ex vivo expansion of post-thawed UCB-HSCs based on UCB-MNCs pharmacologically preconditioned by filgrastim agent in serum-free medium supplemented with cytokine cocktail.

Regarding *ex vivo* expansion signalling pathways, many research groups have demonstrated that classical hematopoietic cytokines promote HSC expansion with less differentiation by manipulating newly discovered signalling pathways (Notch, Wnt, bone morphogenetic protein 4 and Tie2/angiopoietin-1) and intracellular mediators (phosphatase and tensin homolog and glycogen synthase kinase-3). Moreover, the pharmacological preconditioning of peripheral blood stem cells by filgrastim (Nivestim®) has been shown to decrease IFN-g and increase IL-4 production, a finding that is observed under specific stimulatory condition *in* vitro and related to immunomodulatory effects of on Th1 and Th2 cytokine profiles (IFN-g and IL-4 respectively), Th-specific transcriptional regulators, and T cell proliferation and survival [33]. IL-4 has been already evaluated successfully in ex vivo expansion of $CD34^+$ cells and immunocytes from umbilical cord blood with T cells, NK cells and DCs as well as stem/progenitor cells could be expanded in the

same medium from CB MNCs with the combinations of cytokines. The combination of SCF, IL-2, IL-3, IL-6 and IL-4 showed a balanceable expansion result of both $CD34^+$ cells and immunocytes at 7th culture days [34]. Further research studies of pharmacologically preconditioned UCB-MNCs by filgrastim may provide the necessary evidence for immune-mediated ex vivo expansion of $CD34^+$ and prove the molecular stimulatory effects of immunocytes to hematopoietic progenitor and stem cells.

In conclusion, this work demonstrates that early acting pharmacology preconditioning by filgrastim in the presence of IL-3, IL-6, SCF, Flt-3 cytokine cocktail, is a superior option than utilizing serum-free medium for higher expansion efficiency of the UCB-HSCs on UCB-MNCs fraction. The UCB-MNCs main advantage is that simultaneously can synergize and enhance the capacity of UCB-HSCs reconstitution compared to immunomagnetically separated $CD34^+$ cells. These data demonstrate that the ex vivo expanded UCB-HSCs fulfill the minimal transplant cell requirements (30×10^6 Nucleated Cells/kg, 1.7×10^5 $CD34^+$/kg) for repeated therapeutic applications to hematological or non hematological clinical indications with no limit to small weight (< 40 kg) pediatric patients. Moreover, the utilization of human early and late acting cytokines and filgrastim agent in the culture media is feasible, safe, and not complicated or at risk by the use of animal product system. Furthermore, the present study may be of high interest to private and public stem cell bank authorities demonstrating the guidelines of post-thawing standard operation procedures prior to ex vivo expansion methodologies that increase significantly the therapeutic value of cryopreserved UCB grafts. Finally, design and development of a clinical trial will evaluate the engraftment potential of previous post-thawed ex vivo expanded UCB-HSCs.

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Disclosure of conflict of interest

None.

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Supplementary information

Flow cytometry analysis for sample (N=4) experiments (exp.)

Post-Thawing: A: exp#1, C: exp#2, E: exp#3, G: exp#4. Post-Expansion: B: exp#1, D: exp#2, F: exp#3, H: exp#4.

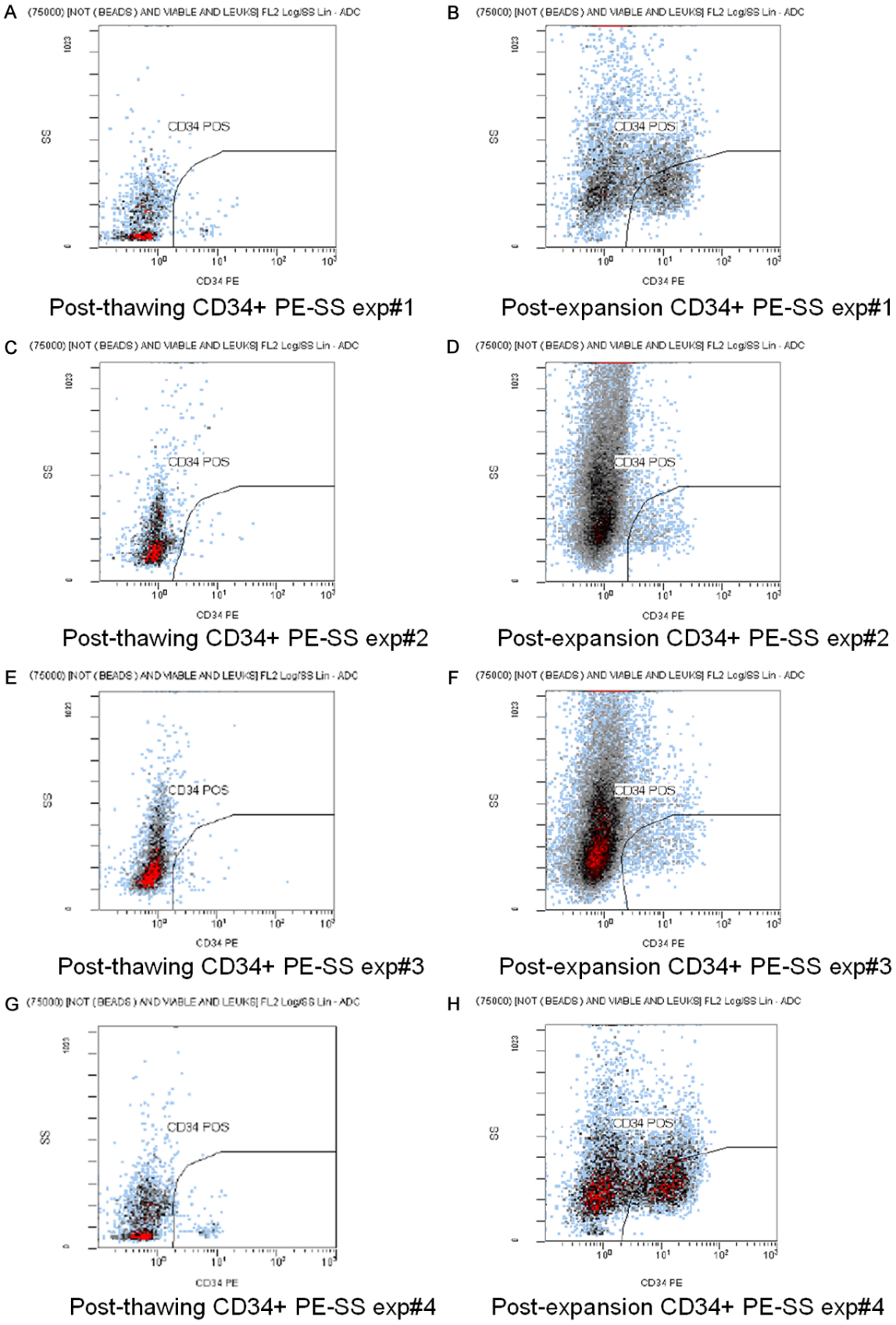

Figure S1. Flow cytometry comparison analysis of post-thawing CD34$^+$ PE/SS (A: exp#1, C: exp#2, E: exp#3, G: exp#4) and post-expansion CD34$^+$ PE-SS (B: exp#1, D: exp#2, F: exp#3, H: exp#4).

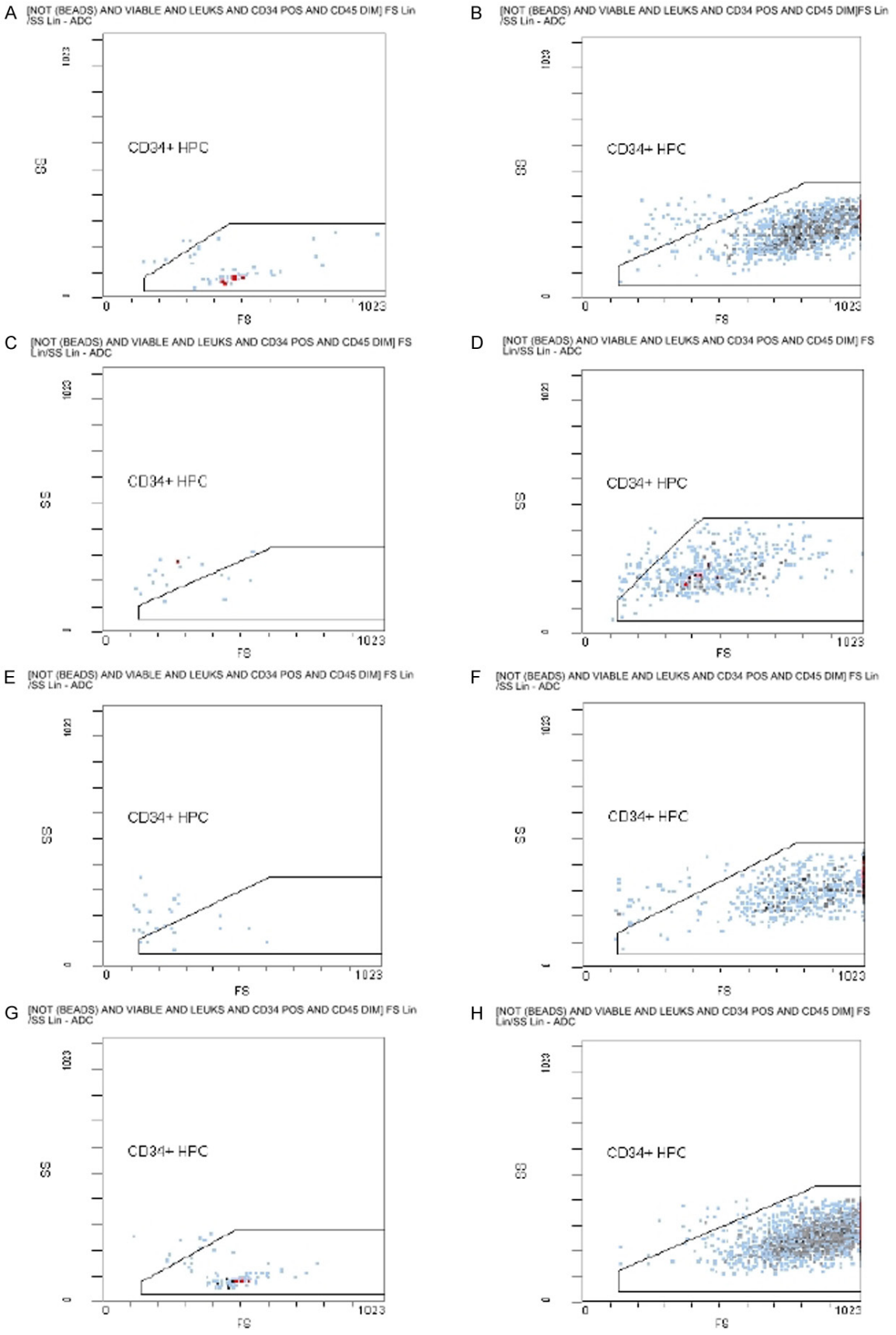

Figure S2. Flow cytometry comparison analysis of post-thawing $CD34^+$ FS/SS (A: exp#1, C: exp#2, E: exp#3, G: exp#4) and post-expansion $CD34^+$ FS/SS (B: exp#1, D: exp#2, F: exp#3, H: exp#4).

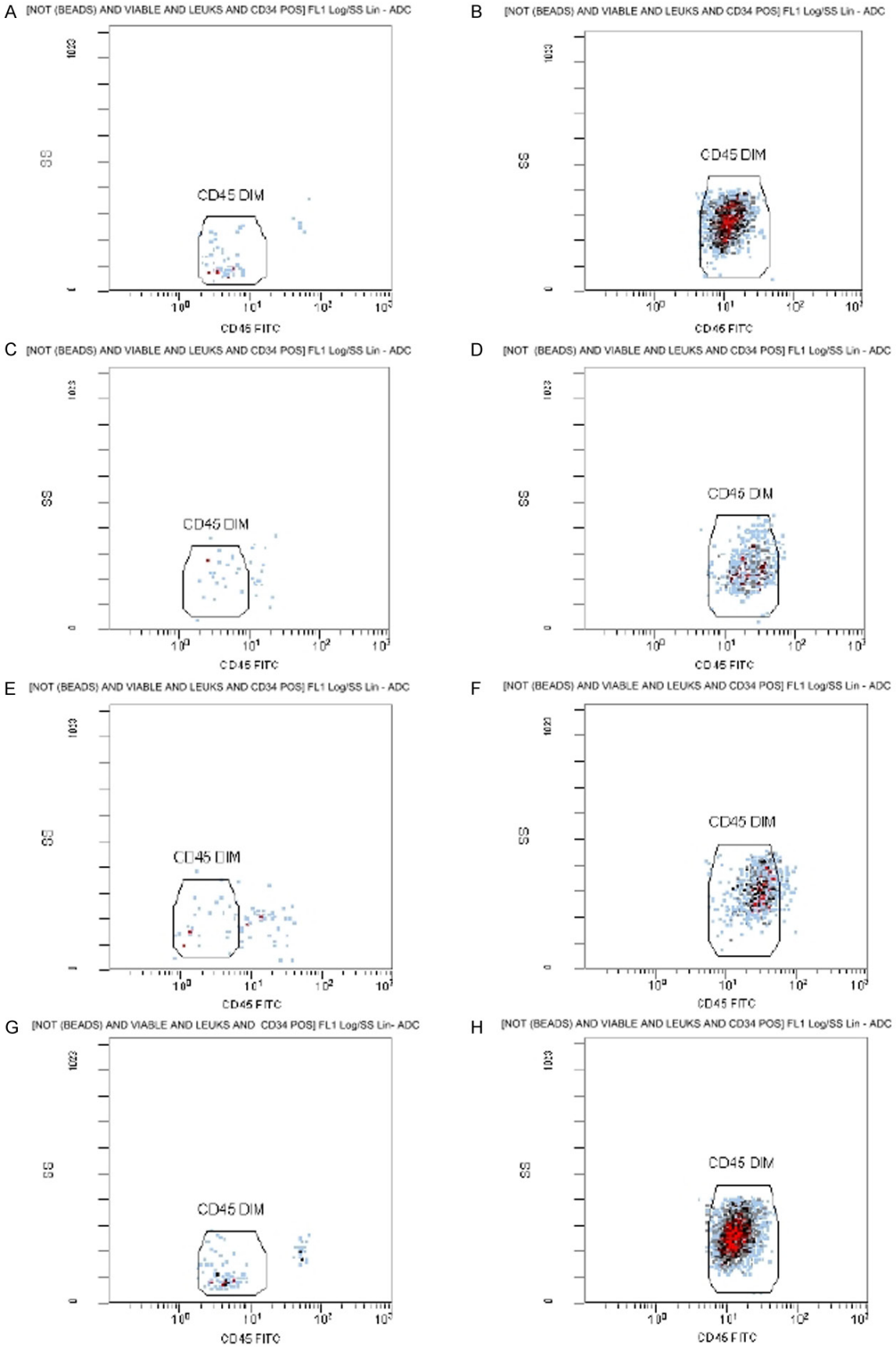

Figure S3. Flow cytometry comparison analysis of post-thawing CD45⁺ FITC/SS (A: exp#1, C: exp#2, E: exp#3, G: exp#4) and post-expansion CD45⁺ FITC/SS (B: exp#1, D: exp#2, F: exp#3, H: exp#4.)

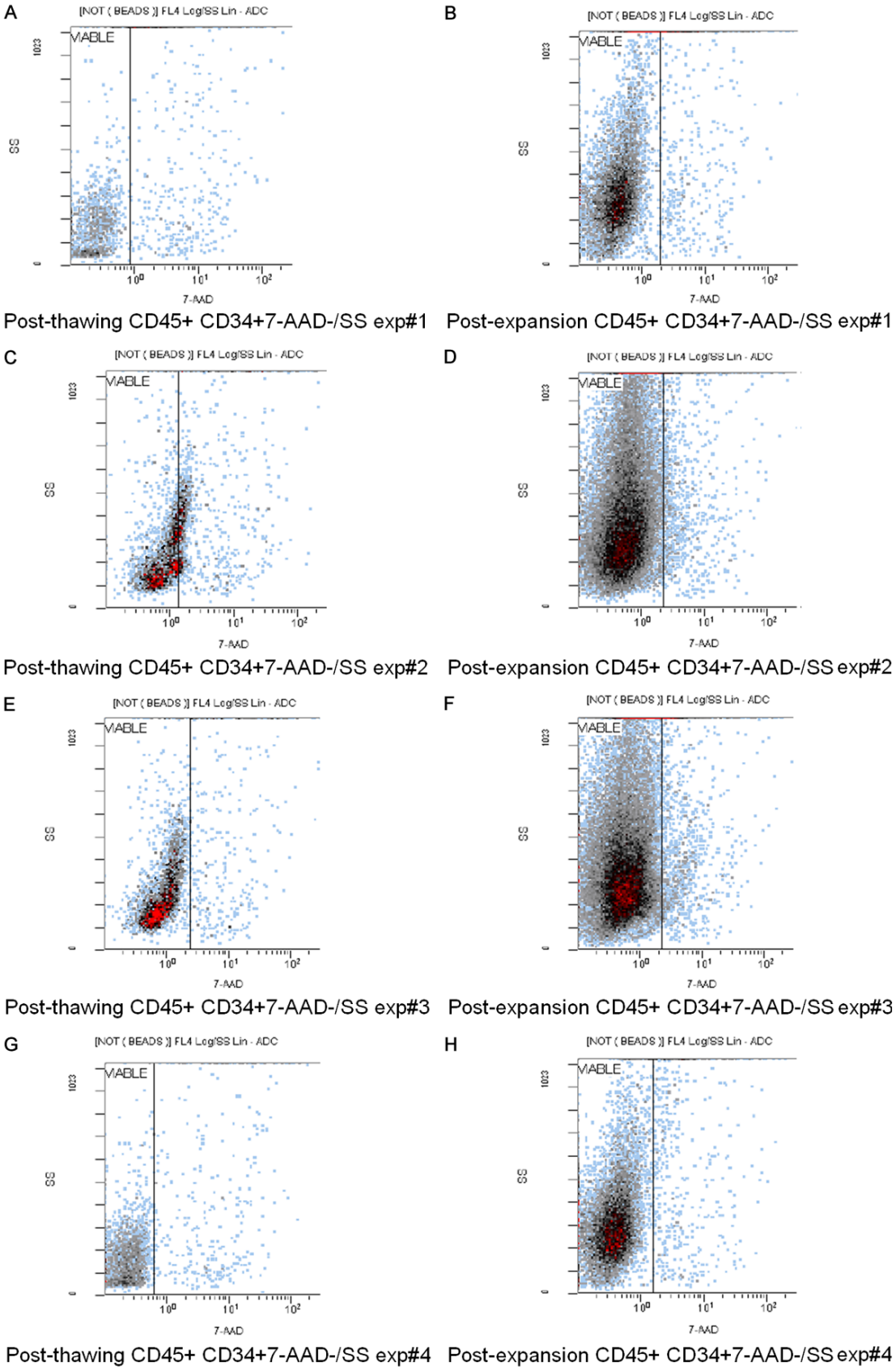

Figure S4. Flow cytometry viability comparison analysis of post-thawing $CD45^{+}CD34^{+}$ 7-AAD-/SS (A: exp#1, C: exp#2, E: exp#3, G: exp#4) and post-expansion $CD45^{+}CD34^{+}$ 7-AAD-/SS (B: exp#1, D: exp#2, F: exp#3, H: exp#4).