Additional file 1: Cytokine and SR1 optimisation

Five independent UCB samples were collected to determine the optimal cytokine combination for the expansion (increased cell number) of CD34+ HSPCs. FACS-isolated CD34+ HSPCs (1 x 10^4 cells/well) were cultured in 24-well plates using 1 mL serum-free StemSpan ACF medium supplemented with 2% penicillin-streptomycin. Four different combinations of growth factors (Life Technologies, ThermoFisher Scientific, Waltham, Massachusetts, USA), each at a 100 ng/mL, were used for these experiments (**Table S1**). All experimental conditions were performed in triplicate. Cells were maintained at 37^{o}C and 5% CO_2 in a humidified atmosphere for seven days.

Table S1 Cytokine combinations. The four cytokine combinations used during the optimisation (each at 100 ng/mL).

Combinations	Cytokines
Combination 1	FLT3L, SCF, TPO and IL-3
Combination 2	FLT3L, SCF, TPO, IL-3 and G-CSF
Combination 3	FLT3L, SCF, TPO and IL-6
Combination 4	FLT3L, SCF, TPO, IL-6 and G-CSF

FLT3L, FMS-like tyrosine kinase 3 ligand; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; SCF, stem cell factor; TPO, thrombopoietin

After 7 days in culture, HSPCs were stained with CD45 FITC, CD34 PE and the viability dye, 7AAD (Beckman Coulter). HSPC expansion was determined by measuring the total cell numbers and percentages of viable CD34+ cells (**Fig. S1 and S2**) using a 3-laser, 10-colour Gallios flow cytometer (Beckman Coulter).

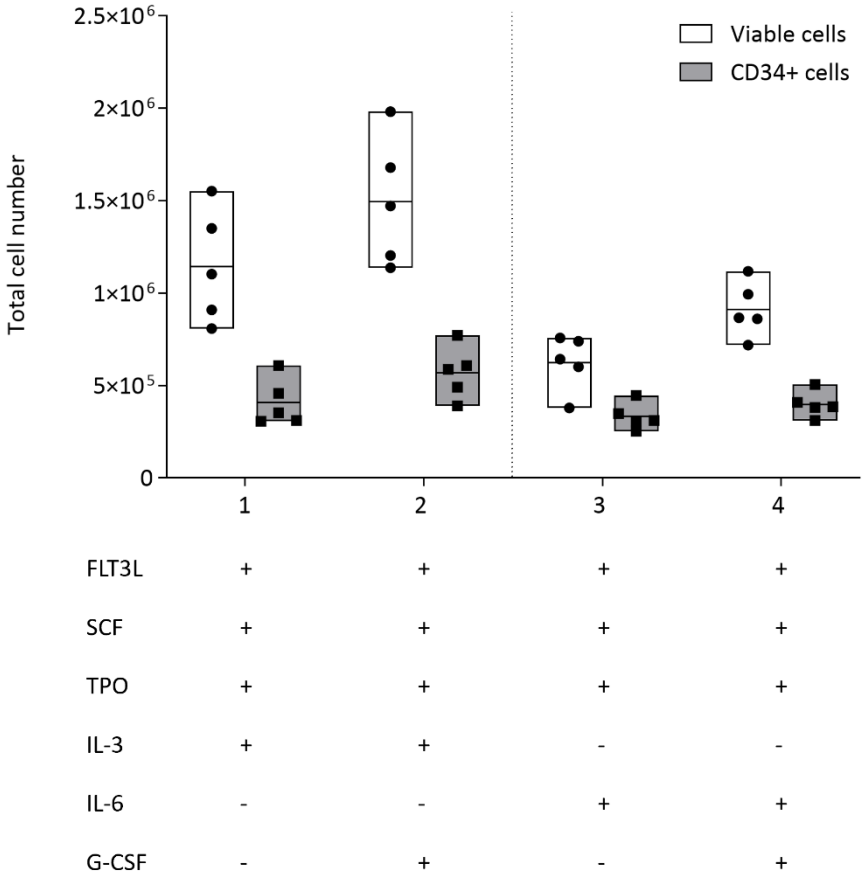

Fig. S1 Proliferation of viable and CD34+ cells. Total number of viable (white) and CD34+ HSPCs (grey) after seven-day expansion with four different cytokine combinations (n=5). 1 x 10^4 CD34+ cells were seeded on the day of isolation (Day 0) for expansion. All cytokine combinations showed an overall increase in proliferation (total cell number) after 7 days in culture. Increased total proliferation (mean total cell number) was observed when cultures were supplemented with G-CSF [Combination 2, 1.5 x 10^6 (± 3.5 x 10^5) and Combination 4, 9.1 x 10^5 (± 1.5 x 10^5)] compared to their respective controls [Combination 1, 1.1 x 10^6 (± 3.1 x 10^5) and Combination 3, 6.2 x 10^5 (± 1.5 x 10^5)] although not statistically significant. The total number of CD34+ HSPCs was higher in cultures supplemented with G-CSF, Combinations 2 (5.7 x 10^5 ± 1.4 x 10^5) and 4 (3.3 x 10^5 ± 7.2 x 10^4), when compared to cultures without G-CSF, Combinations 1 (4.1 x 10^5 ± 1.3 x 10^5) and 3 (3.3 x 10^5 ± 7.2 x 10^4), although not statistically significant.

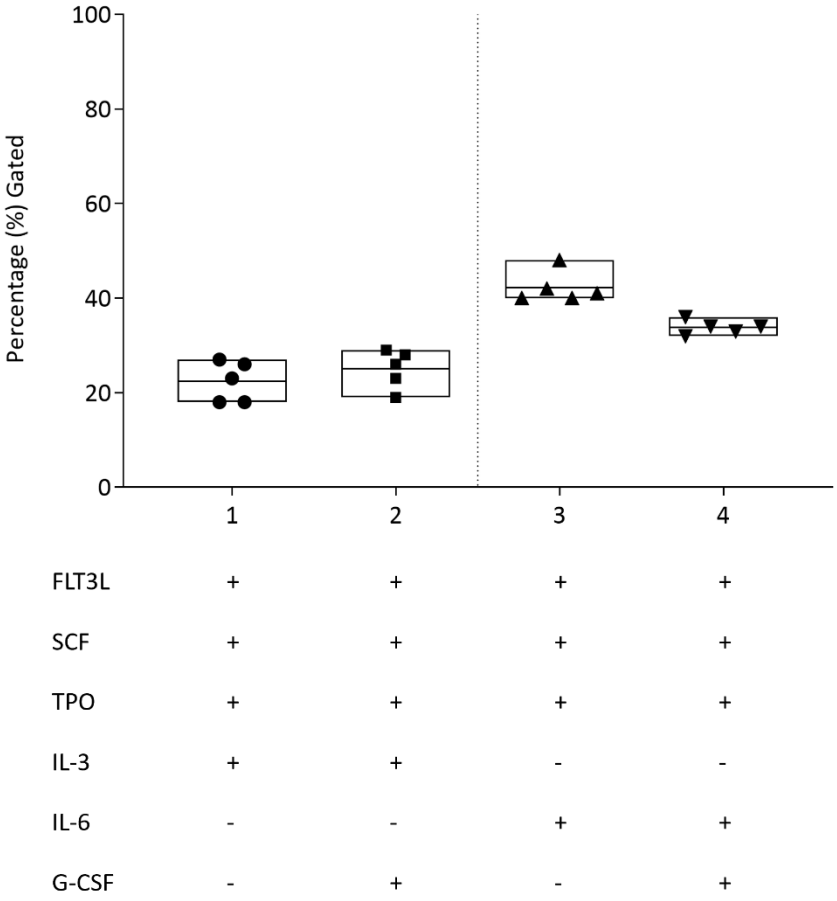

Fig. S2 Percentage CD34+ cells. The percentage CD34+ cells after seven-day expansion with four different cytokine combinations (n=5). Although the CD34+ HSPC absolute numbers increased (Figure S1), the mean proportion of CD34+ cells decreased in all cytokine combinations during the 7-day expansion period: Combination 1, 22.4% (± 4.3%); Combination 2, 25% (± 4.1%); Combination 3, 42.2% (± 3.3%); and Combination 4, 33.8% (± 1.5%). Combination 3 showed the highest proportion of CD34+ cells after expansion, which decreased with the addition of G-CSF, although not statistically significant. Combinations 1 and 2, containing IL-3, had similar proportions of CD34+ cells. The decrease in proportions is due to the fact that CD34+ cells were sorted on Day 0 (prior to expansion) with a purity above 95%.

Side population (SP) analysis was performed on Day 8 (D8) and the results are representative of pooled triplicate wells. Vybrant DyeCycle (VDC) Violet was used in this study to identify the SP. VDC Violet was excited with a UV laser on a FACS Aria Fusion cell sorter. The SP fraction (displayed as percentage) was identified as cells with higher dye efflux ability and therefore cells with low/negative VDC blue (450/50-A) and red (710/50 and 690LP-A) fluorescence compared to the rest of the population (**Fig. S3**). A verapamil control was used to confirm the specificity of the SP and to set the regions of interest. The mean SP percentages (subtracted SP %) were calculated by subtracting the SP with verapamil percentages from the SP with VDC only (**Tables S2 and S3**). The SP has previously been identified in CD34+ and CD34- HSPCs, and for that reason its presence was investigated in both CD34+CD38- and CD34-CD38- HSPCs.

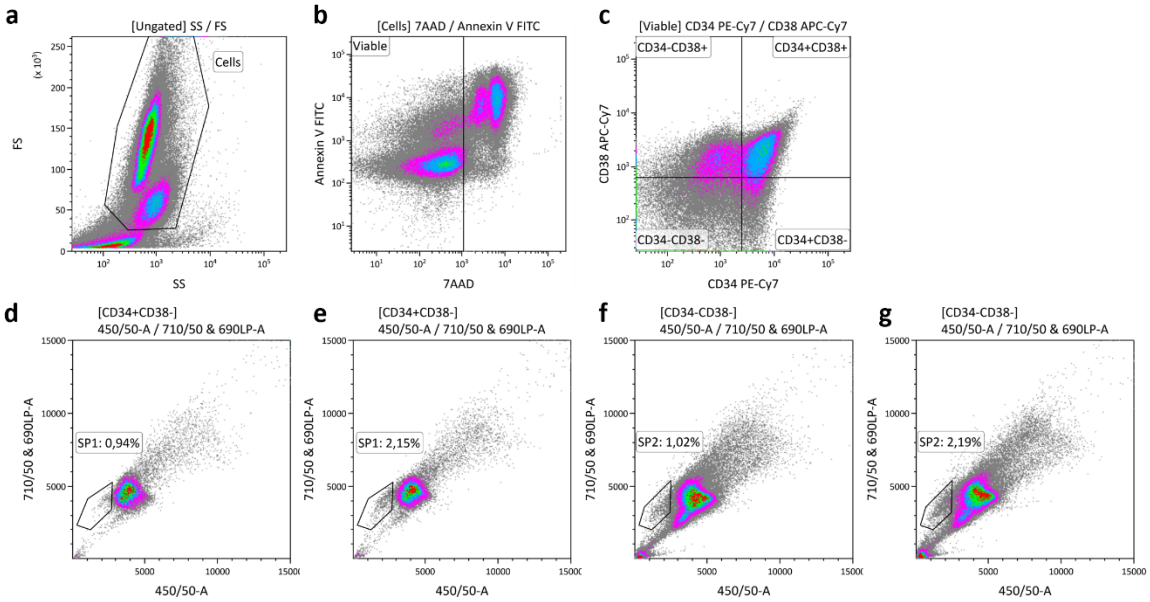

Fig. S3 Side population gating strategy. A schematic illustration of the sequential gating strategy used to identify the SP in CD34+CD38- and CD34-CD38- cells. Density plots showing (a) intact cells and (b) viable (7AAD-negative) cells. (c) Density plot showing cells stained with CD38 APC-Cy7 and CD34 PE-Cy7 monoclonal antibodies. (d–g) Density plots showing cells stained with VDC Violet, with (d and f) and without (e and g) Verapamil. Plots were used to detect SP in (d–e) CD34+CD38- and (f–g) CD34-CD38- cells. The SP fraction (%) was identified as cells with higher dye efflux ability and therefore cells with low/negative VDC blue (450/50-A) and red (710/50 and 690LP-A) fluorescence compared to the rest of the population.

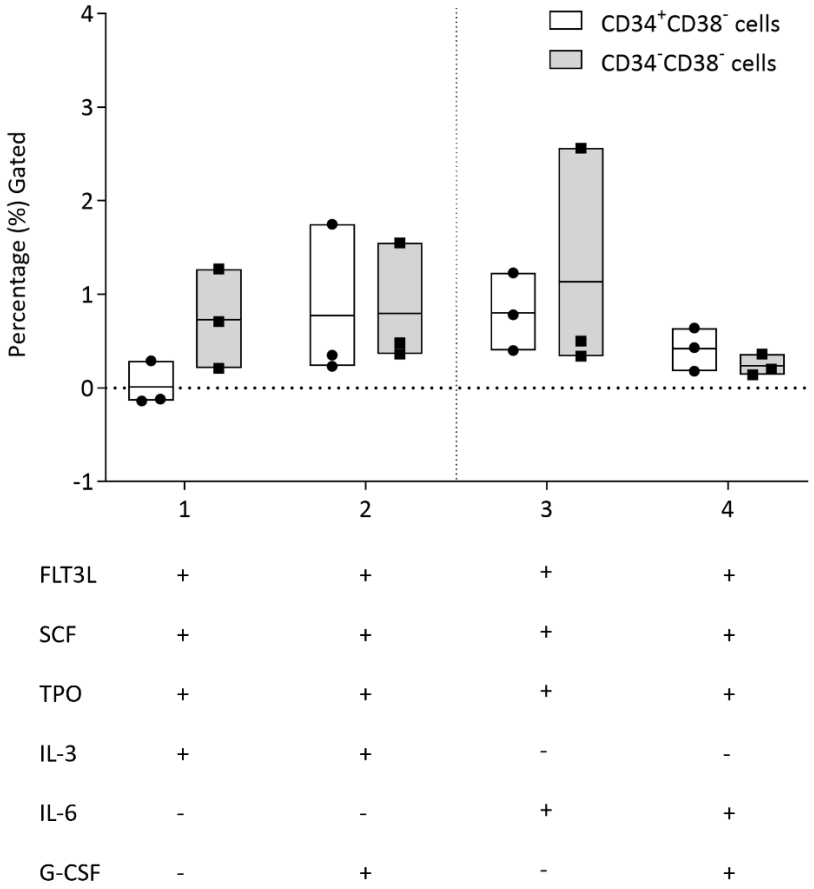

Fig. S4 Side population percentages with different cytokine combinations. The mean percentage SP observed in the CD34+CD38- and CD34-CD38- HSPC sub-populations on D8 in culture with different cytokine combinations (n=3). The differences in SP observed between the four cytokine combinations were not statistically significant, but there seems to be a tendency of increased SP in Combinations 2 and 3 for both CD34+CD38- and CD34-CD38- cells. There also seems to be a tendency of increased SP in CD34-CD38- cells in cultures without G-CSF, Combinations 1, 0.73% (± 0.53%) and 3, 1.13% (± 1.24%), when compared to the SP in CD34+CD38- cells in Combinations 1 (0.01 ± 0.24%) and 3 (0.80 ± 0.41%). The lack of significance may be ascribed to the variability in the SP observed between different donors and the limited number of samples.

Table S2 Percentage side population with different cytokine combinations. The mean percentage (±SD) SP observed in CD34+CD38- and CD34-CD38- populations after eight days in culture with different cytokine combinations (n=3). The subtracted SP percentages were calculated by subtracting the SP with verapamil percentages from the SP with VDC only.

Condition	SP with VDC only (%)	SP with Verapamil (%)	Subtracted SP (%)
	CD34+CD38- cells		
Combination 1	0.36 ± 0.19	0.35 ± 0.10	0.01 ± 0.24
Combination 2	1.43 ± 0.93	0.65 ± 0.31	0.78 ± 0.85
Combination 3	1.05 ± 0.47	0.25 ± 0.13	0.80 ± 0.42
Combination 4	1.14 ± 0.38	0.72 ± 0.53	0.42 ± 0.23
	CD34-CD38- cells		
Combination 1	1.20 ± 0.71	0.47 ± 0.20	0.73 ± 0.53
Combination 2	1.26 ± 0.70	0.46 ± 0.14	0.80 ± 0.66
Combination 3	1.45 ± 1.05	0.48 ± 0.26	1.13 ± 1.24
Combination 4	1.25 ± 0.53	1.01 ± 0.52	0.23 ± 0.11

CD, Cluster of differentiation; SP, Side population; VDC, Vybrant DyeCycle

Since the greatest CD34 expansion, both in total number and percentages, was observed with Combinations 2 and 3 (**Fig. S1 and S2**), respectively, these cytokine combinations were subsequently tested with different SR1 concentrations (0.25, 0.5, 0.75, and 1 µM) to determine the optimal combination. Once again, the percentage of CD34+ cells (**Fig. S5**) and the total and CD34+ cell numbers (**Fig. S6**) were determined following a 7-day expansion.

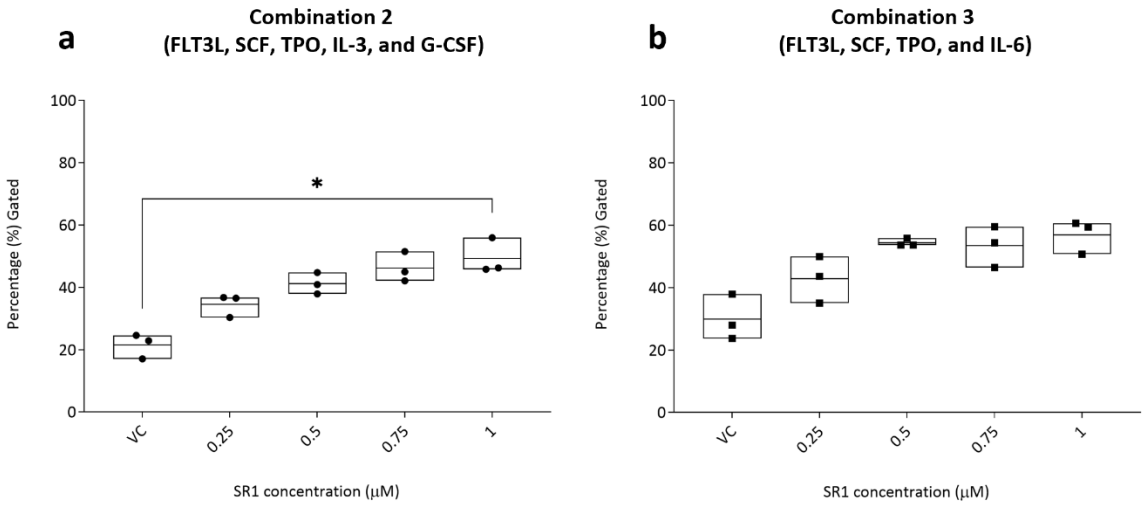

Fig. S5 Percentage CD34+ cells. The percentage CD34+ cells after seven-day expansion with (a) cytokine combination 2 (FLT3L, SCF, TPO, IL-3, and G-CSF) (n=3) and (b) cytokine combination 3 (FLT3L, SCF, TPO, and IL-6) (n=3). The proportions of CD34+ cells increased with higher SR1 concentrations, reaching the highest percentage with 1 µM in both cytokine combinations: Combination 2 (49.30 ± 5.72%) and Combination 3 (56.91 ± 5.40%). Combination 3 showed the highest proportion of CD34+ cells after expansion.

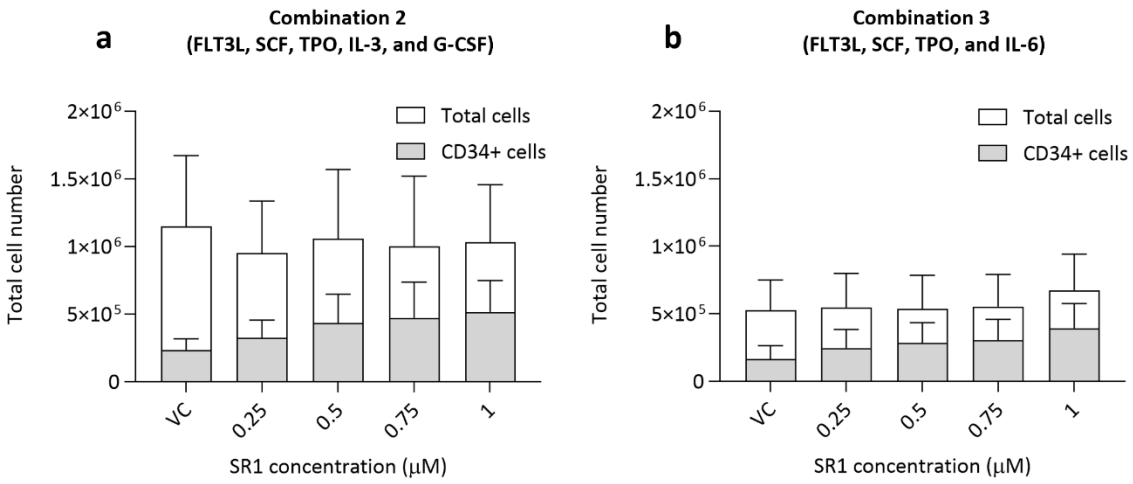

Fig. S6 Proliferation of total and CD34+ cells. Total number of cells (white) and CD34+ HSPCs (grey) after seven-day expansion with (a) cytokine combination 2 (FLT3L, SCF, TPO, IL-3, and G-CSF) (n=3) and (b) cytokine combination 3 (FLT3L, SCF, TPO, and IL-6) (n=3). On Day 0, 1 x 10^4 CD34+ cells were seeded for expansion. The total number of cells remained constant within each cytokine combination across all concentrations of SR1 but was higher in Combination 2 compared to Combination 3. However, the number of CD34+ cells (expressed as cell number x 10^5) increased with higher concentrations of SR1 and reached the highest count with 1 µM, Combination 2 (5.1 x 10^5 ± 2.3 x 10^5) and 3 (3.9 x 10^5 ± 1.8 x 10^5). Notably, the total number of CD34+ cells was highest in cultures supplemented with cytokine combination 2 and 1 µM SR1, compared to combination 3 and 1 µM SR1. Therefore, cytokine combination 2 was selected for downstream experiments.

Table S3 Percentage side population after expansion with different SR1 concentrations. The percentage SP (±SD) observed in CD34+CD38- and CD34-CD38- cells after expansion with different SR1 concentrations for eight days in culture (n=2). The mean SP percentages (subtracted SP %) were calculated by subtracting the SP with verapamil percentages from the SP with VDC only.

Condition	SP with VDC only (%)	SP with Verapamil (%)	Subtracted SP (%)
	CD34+CD38- cells		
VC	2.06 ± 0.33	1.03 ± 0.44	1.03 ± 0.11
0.25 µM	1.88 ± 1.23	0.48 ± 0.11	1.40 ± 1.34
0.5 µM	1.18 ± 0.89	0.93 ± 0.08	0.26 ± 0.97
0.75 µM	0.54 ± 0.02	0.32 ± 0.01	0.22 ± 0.01
1 µM	0.79 ± 0.43	0.54 ± 0.16	0.25 ± 0.59
	CD34-CD38- cells		
VC	1.71 ± 0.43	0.67 ± 0.23	1.04 ± 0.20
0.25 µM	2.49 ± 1.19	0.58 ± 0.24	1.91 ± 0.95
0.5 µM	1.85 ± 0.78	0.72 ± 0.45	1.13 ± 0.33
0.75 µM	1.51 ± 0.54	0.47 ± 0.23	1.04 ± 0.31
1 µM	1.72 ± 0.19	1.02 ± 0.01	0.70 ± 0.20

CD, Cluster of differentiation; SP, Side population; VC, Vehicle control; VDC, VybrantDye Cycle Violet .