HIV-exposed uninfected umbilical cord blood haematopoietic stem/progenitor cells differ immunophenotypically from those from HIV unexposed umbilical cord blood but have similar expansion and colony-forming properties in vitro

Running title: **HIV-exposed uninfected UCB for transplantation**

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To the editor:

South Africa (SA) is a human immunodeficiency virus (HIV) endemic country in which allogeneic haematopoietic stem cell transplantation (HSCT) is performed at a low rate of approximately 2,5/million population per annum. A deficit in donors constitutes a major obstacle, and efforts advocating for an umbilical cord blood (UCB) bank(1), while concurrently improving donor numbers from African ethnolinguistic groups, are underway. Although haploidentical HSCT is gaining favour globally, UCB has particular advantages in ethnically diverse populations(2) due to human leukocyte antigen (HLA) typing being at ≥4/6 or ≥4/8

loci(3) instead of 8/8-10/10; *ex vivo* expansion of UCB haematopoietic stem/progenitor cells (HSPCs) is an additional advantage.(4) In South Africa, the prevalence of pregnant women living with HIV (LWH) is reported to be 30-40%.(5) However, <2% of these mothers will give birth to HIV-infected infants(5) as a consequence of successful preventive antiretroviral (ARV) strategies. Haematological abnormalities have been reported in HIV exposed uninfected (HEU) children, with zidovudine (AZT) being implicated.(6) We have recently advocated for the inclusion of HIV exposed or infected donors in the repertoire of donors for HSCT in South Africa, given that it is an HIV endemic country.(7) In addition to adult donors LWH, we proposed that HEU UCB should be considered once sufficient evidence for the optimal functioning of these HSPCs had been obtained. The first step in determining the potential of using HEU units for HSCT was to perform extensive in vitro analysis as a basis for future in vivo analysis. This study thus provides information on the characteristics of HEU HSPCs by determining their expansion capacity, in the event that HSPC expansion is considered prior to infusion in HSCT in the future. Immunophenotypic analysis of cells expanded with StemRegenin 1 (StemCell™ Technologies, Canada, at a final in-well concentration of 1 µM)(8) was then compared to a vehicle control (VC; 0.01% DMSO and cytokines only) to determine if expansion changes the potential Lin-CD34+ sub-groups in HIV exposed HSPCs. Finally, gene expression and colony-forming ability *in vitro*, were assessed to determine if potential gene pathways or colony formation are affected by HIV exposure. We collected UCB from HIV negative and HIV exposed infants post elective caesarean section at term. All samples tested negative for HIV-1 by GeneXpert analysis using HIV-1 Qual XC cartridges (Cepheid, USA). For HEU samples, mothers undergoing elective caesarean section were on ARVs and were virologically suppressed (at least one viral load [VL] <100 copies/mL within 3 months prior to delivery), and infant HIV polymerase chain reaction (PCR) (within the first few days of birth) was negative (Fig. S1D). Eleven mothers living with HIV consented to UCB collection with five samples meeting the above inclusion criteria. Of these, 4 had sufficient cell numbers to allow for a complete set of experiments. All mothers were on fixed drug combination ARVs, four on

TLD (Tenofovir disoproxil fumarate [TDF], Lamivudine, Dolutegravir) and one on TEE (TDF, Emtricitabine, Efavirenz) (Fig. S1D).

Mononuclear cells (MNCs) were isolated using a CD34+ magnetic kit (Miltenyi, Germany) (>95% purity obtained, Fig. S1A) and plated at $1x10^4$ cells/well in a 24-well plate in triplicate in Stemspan animal origin free medium (StemCell™ Technologies, Canada) for each condition (StemRegenin 1 [SR1] and vehicle control [control]). Recombinant human growth factors were added, each at 100 ng/mL: Stem cell factor (SCF), thrombopoietin (TPO), FMS-like tyrosine kinase 3 ligand (FLT3L), granulocyte colony-stimulating-factor (G-CSF) and interleukin-3 (IL-3) (all from Life Technologies, Thermo Fisher Scientific, USA). Cells were cultured for 7 days in 5% CO_2 at 37°C. Between $5x10^5$-$1x10^6$ MNCs were stained for immunophenotypic analysis using Lin-FITC (clones:CD3:UCHT1; CD14:HCD14; CD16:3G8; CD19:HIB19; CD20:2H7; CD56:HCD56), CD117-PE (clone:104D2), CD90-BV510 (clone:5E10) and CD49f-SB780 (clone:G0H3) from BioLegend, San Diego, USA; CD34-APC AF700 (clone:581), CD38-ECD (clone:LS198-4-3) and CD45RA-APC (clone:2H4) from Beckman Coulter, Miami, USA; and CD133-PE-Violet 770 (clone:REA753) from Miltenyi, Germany. Data was acquired on a CytoFLEX flow cytometer (Beckman Coulter, Miami, USA). Compensated, logicle-transformed, pre-gated data (see Fig. S1B for D0 and Fig. S1C for D7 gating strategy) was exported to the Cytobank platform using the Kaluza Cytobank plugin (http://www.cytobank.org; Beckman Coulter, Miami, USA). After dimensionality reduction, the FlowSOM clustering algorithm (self-organizing map) was applied. Statistically significant differences (p-value <0.05) in FlowSOM metaclusters (MCs) were identified using the Kruskal Wallis test in the statistical inference tool in the Cytobank platform. For CFUs, 200 cells in 4 µL were added in 500 µL Methocult medium per well (StemCell™ Technologies, Canada) in three wells of a scored 24-well plate. Colonies were identified on Day 14 (D14) using a Zeiss Axio-microscope with an Axiocam 1Cc-5 (Zeiss, Germany) and a CFU atlas as a guide (R&D systems, USA). Staining was then performed using CD235a-PE (clone:11E4B-7-6), CD71-APC-AF750 (clone:YDJ1.2.2), CD41-ECD (clone:P2), CD15-KO (clone:80H5), CD14-APC-

AF700 (clone:RM052), CD19-PC5.5 (clone:J3-119), CD20-PC5.5 (clone:B9E9), CD3-PC5.5 (clone:UCHt1), CD56-PC5.5 (clone:NP01) and CD33-APC (clone:D3HL60.251) [All antibodies, Beckman Coulter, Miami, USA]. A second tube was stained with 7-AAD (Beckman Coulter, Miami, USA).

RNA extraction was performed using the Qiagen RNeasy Micro Plus kit (Qiagen, Germany). The Human HT Clariom S Pico Assay was used for transcriptomic analysis on the human Affymetrix Clariom S array at the Centre for Proteomic and Genomic Research, Cape Town, South Africa. Microarray data has been deposited in NCBI GEO (Gene Expression Omnibus) with accession number GSE253897**.** Functional annotation of differentially expressed genes was performed for Gene Ontology (GO) biological processes and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses using g:Profiler (version:e110_eg57_p18_4b54a898) (https://biit.cs.ut.ee/gprofiler/gost). The g:SCS algorithm was used to compute multiple testing correction (p-value 0.05).

We found that HEU samples had a higher (albeit not statistically significant) average fold-increase in total viable cells in the control (54±43 HEU vs 43±26 HUU) and StemRegenin1 conditions (62±45 HEU vs 49±30 HUU). For viable CD34+ cells, average fold-increases of 10 and 20 were observed in HUU samples and 18 and 31 in HEU samples (VC and SR1, respectively) (Fig. 1A). On colony forming unit analysis, manual colony counting revealed no statistically significant differences between HUU and HEU sub-groups (Fig. 1B) and this was confirmed on Flow self-organising map (FlowSOM) metacluster analysis (Figs. S9A, B, C).

Dimensionality reduction on the Lin-CD34+ population using viSNE showed substantial differences between D0 and D7 (N=4: HEU; N=5: HUU, Figs. S2A-D), as has previously been shown by our group using fluorescence activated cell sorting (FACS).(9) The Flow self-organising map metaclusters (Fig. S2E) guided manual gating of viSNE plots (Figs. S2F, G). Sub-populations were named according to established nomenclature,(10,11) and “Extended”

(Ext) was added to accommodate our panel. Sub-populations were overlaid onto the viSNE plot (Fig. 2A), and frequencies are shown in Table 1. Statistically significant population frequencies were identified using two methods: FlowSOM box plots with heat maps (Figs. S3A-F) and CITRUS (cluster identification, characterization, and regression, Figs. S4, S5, S6) analysis. The significantly different cluster seen on D0 using both methods corresponded to Ext- common myeloid progenitors (ExtCMP) which were more frequent in HUU (metacluster 13, Figs. S3A, B; CITRUS cluster 17962, Figs. S5A, B). D0 HEU HSPCs had higher proportions of Ext-multipotent progenitors (Table 1) as shown previously(12), although this was only found to be statistically significant using CITRUS. In the cited study, the authors suggested that increased haematopoietic progenitors might represent a possible compensatory response to ARV toxicity. On D7 in the presence of Stemregenin1, Ext-lymphoid primed myeloid progenitors 2 (ExtLMPP2 [CD49f-]; FlowSOM Metacluster 3, CITRUS cluster 269996) were significantly greater in number in HUU while ExtLMPP3 ([CD49f+]; FlowSOM Metacluster 12, CITRUS cluster 269995) was greater in HEU using FlowSOM and CITRUS (Fig. 2A, Table 1, Fig. S2E, Fig, S3E, F; Fig. S6C, D). On CITRUS alone, D7 VC showed Ext-lymphoid primed myeloid progenitors 1 and 3 (CITRUS clusters 62995 and 62997, both CD49f+) to be higher in number in HEU samples (Figs. S6A, B). Other notable features were the presence of a newly described haematopoietic stem/progenitor cell (HSPC) population which was Lin-CD34+CD38-CD133-/dimCD45RA+/v. HSPC1 and 3 were present at a higher frequency in HEU samples (Table 1) and differed from HSPC2 as they had bright CD45RA expression and variable/dim expression of CD49f.

For the Lin-CD34- population, Uniform Manifold Approximation and Projection (UMAP) analysis was used (Fig. 2B, Figs. S7A-G). No known nomenclature exists for the Lin-CD34- population, thus sub-populations were denoted as POP. D0 Lin-CD34- POP1, enriched for primitive long-term repopulating (LTR)-HSCs (Lin-CD34-CD117-[FLT-3-])(13), was higher in HEU samples, although this was not statistically significant (Fig. S8A). There was a statistically significant increase in POP5 in D7 HEU samples (SR1 and VC) compared to HUU samples,

characterised as CD38-CD133vCD45RA+CD90vCD49f+ (FlowSOM Metacluster 13, see Fig. S7H, Figs. S8B, C).

A total of 271 differentially expressed genes (108 upregulated; 163 downregulated) were found (Fig. 2C, Fig. S10); the full gene list is in Fig. S11. Functional analysis by GO revealed that upregulated genes play a role in response to defense and normal metabolic processes (Fig. S12); however, KEGG pathway analysis revealed that no genes were significantly enriched for any signalling pathway. Ours is the first report, to our knowledge, to describe the impact of newer (AZT-free) drug combinations on gene expression signatures of HEU UCB HSPCs. CFU colonies observed in both HUU and HEU groups were similar (Fig. 1B) and FlowSOM box plots confirmed that there were no statistically significant differences (Fig. S9C).

We show that HEU HSPCs expand efficiently and make colony forming units that are similar to those seen with HUU HSPCs. The propensity of HEU samples to express CD90 and CD49f, known HSC markers, and CD45RA, a known lymphoid marker, requires further investigation including pre-clinical *in vivo* studies, to determine the potential feasibility of using HEU HSPCs for HSCT in HIV endemic areas.

Disclosure of Conflicts of interest

MSP has received research grants from the South African Medical Research Council, the Bill and Melinda Gates Foundation, the South African National Health Laboratory Service Research Trust, The National Research Foundation (South Africa) and the Wellcome Trust. MSP is a co-founder and non-remunerated founder and board member of Antion Biosciences and Altera Biosciences and is also CSO of the latter. CLH is a non-remunerated board member of DKMS Africa, a not-for-profit organisation.

Data sharing statement

Microarray data are available at GEO under accession number **GSE253897.**

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE253897

For original flow cytometry data files, please contact michael.pepper@up.ac.za

Author contributions

Conceptualisation: CLH, JM, MSP; Methodology: CLH, JM, MSP; Investigation: CLH, JM; Writing- Original draft: CLH; Writing- Review and Editing: CLH, JM, VS, MA, MSP; Formal analysis: CLH, VS, MA; Data curation: CLH, VS, MA; Resources and funding acquisition: MSP; Supervision: JM and MSP.

Funding

This research was supported by the South African Medical Research Council (SAMRC) Extramural Unit for Stem Cell Research and Therapy, and the University of Pretoria through the Institute for Cellular and Molecular Medicine (to MSP).

Ethics statement

Ethics approval for this work was obtained from the University of Pretoria Faculty of Health Sciences Research Ethics Committee, approval number 102/2020. All enrolled mothers gave written informed consent, for collection of umbilical cord blood after delivery of the infant, for the purposes of the study. All methods were performed in accordance with the relevant guidelines and regulations.

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Figure legend

Figure 1: Comparison between HEU and HUU samples in HSPC number after 7 days in culture, and colony forming units after 14 days in culture

A. Difference in viable total and viable CD34+ cells between HIV exposed (HEU, red and pink bars, N=4) and unexposed (HUU, light and dark green bars, N=5) groups after 7 days of expansion. Total cell numbers are on the y-axis. There was no statistically significant difference between the groups. Total viable cells (HUU VC vs HEU VC p=0.9219; HUU SR1 vs HEU SR1 p=0.8941); Viable CD34+ cells (HUU VC vs HEU VC p=0.9735; HUU SR1 vs HEU SR1 p=0.9354).

B. CFU colony types were identified by manual colony counting in HEU and HUU samples. The colour scale is on the right, the sub-group on the x-axis and average colony numbers shown on the left. N=4 for both HEU and HUU groups. There was no statistically significant difference between the two groups. CFU-GEMM (p=0.2870); CFU-GM (p=0.9429); CFU-G (p=0.5204); CFU-M (p=0.5675); B/CFU-E (p=0.3550).

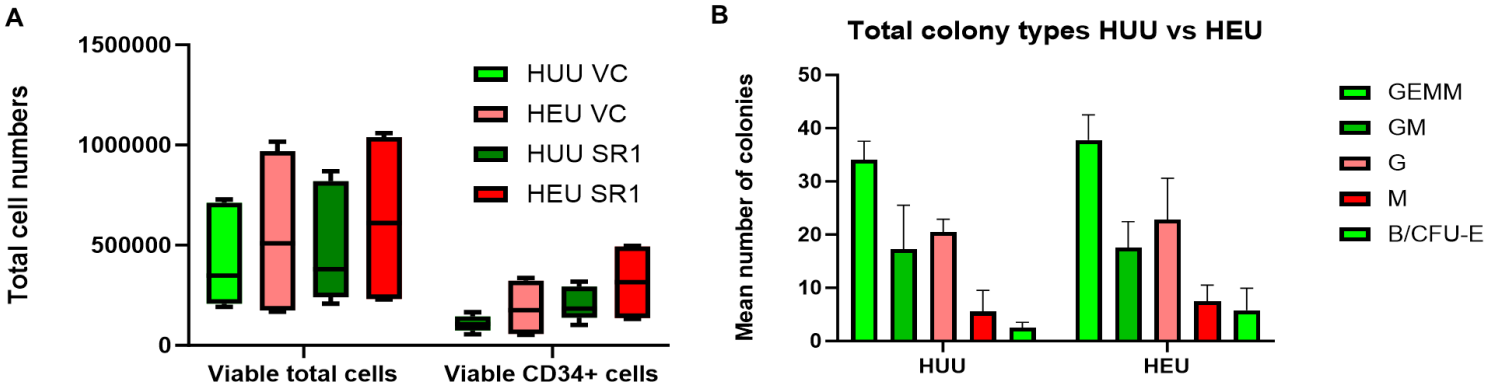
Figure 2: Differences in immunophenotype and gene expression between HEU and HUU samples

A. After FlowSOM MCs were identified, manual gating was performed on the viSNE plots (see Figures S2F and G) to elucidate the immunophenotype of each MC. The backgated viSNE maps for the HUU and HEU groups respectively on D0, D7 control, and D7 StemRegenin 1 are shown, coloured according to the map on the left of the image and named according to phenotype in Table 1. ExtCMP correlated with MC13, ExtLMPP2 with MC3, and ExtLMPP1 and 3 with MC12. Only one sample is shown for ease of visualisation. N=4 for HEU and HUU.

B. Backgated UMAP plots generated for the Lin-CD34- population. The map is coloured by immunophenotypic sub-populations identified by manual gating and described in Figure S8A, guided by FlowSOM MCs of the UMAP (Figure S7H). All events were included in the analysis (D7 VC and D7 SR1, one sample shown). POP5 (CD38-

CD133vCD45RA+CD90vCD49f+) is statistically significantly higher (p=<0.05) in HEU D7 VC and SR1 samples compared to HUU samples (Figure S8, B, C). N=4 for HEU and HUU.

C. Hierarchical clustering showed that HEU samples differ from HUU samples; however, these differences do not translate to significant changes in gene expression patterns as illustrated in the heatmap. N=4 for HEU and HUU.


A
Total cell numbers
1500000
1000000
500000
0
HUU VC
HEU VC
HUU SR1
HEU SR1
Viable total cells
Viable CD34+ cells
B
Total colony types HUU vs HEU
Mean number of colonies
50
40
30
20
10
0
GEMM
GM
G
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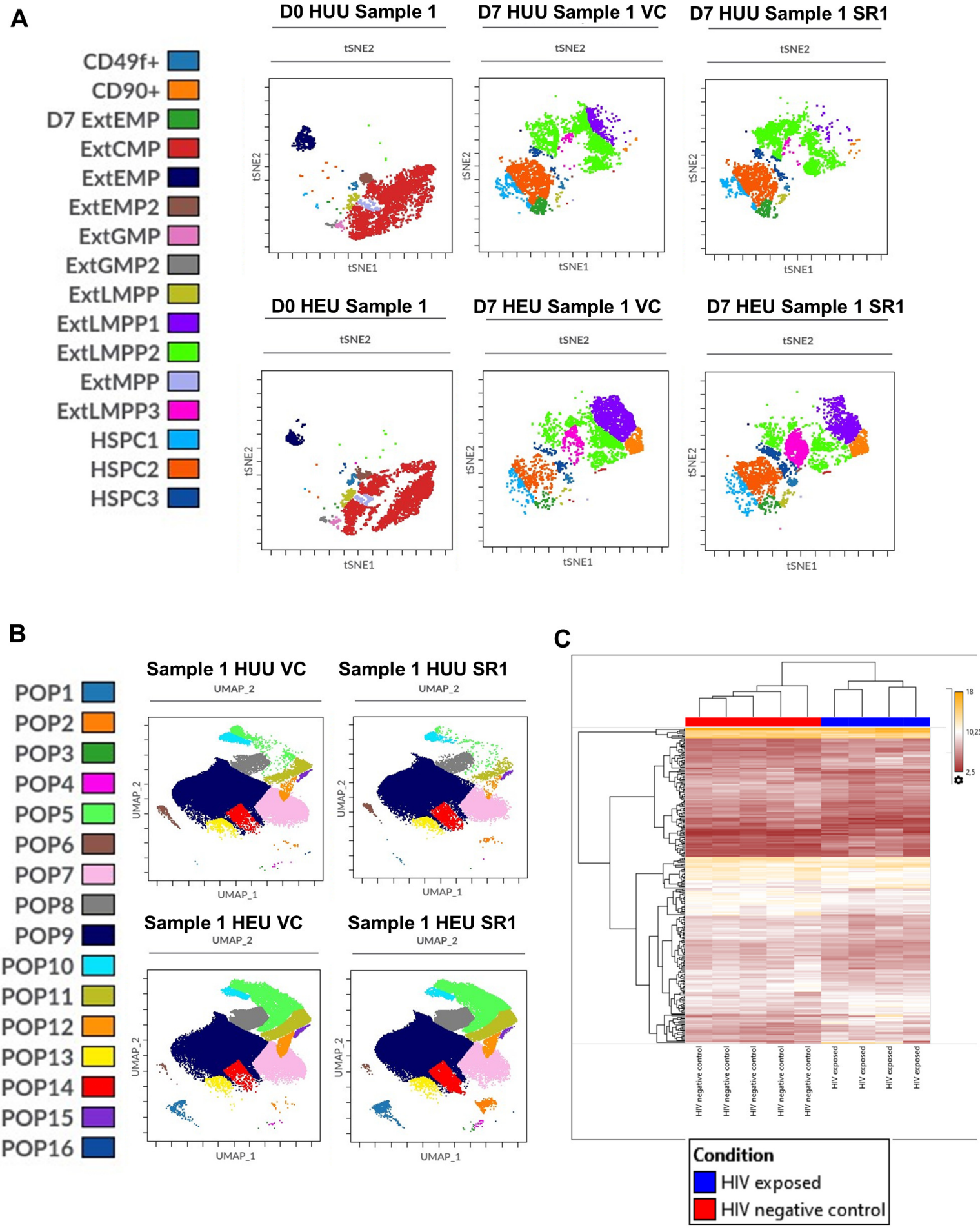
B/CFU-E
HUU
HEU


A
CD49f+
CD90+
D7 ExtEMP
ExtCMP
ExtEMP
ExtEMP2
ExtGMP
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ExtLMPP
ExtLMPP1
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ExtMPP
ExtLMPP3
HSPC1
HSPC2
HSPC3
D0 HUU Sample 1
D7 HUU Sample 1 VC
D7 HUU Sample 1 SR1
D0 HEU Sample 1
D7 HEU Sample 1 VC
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Sample 1 HEU VC
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UMAP_1
UMAP_2
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18
10,25
2,5
HIV negative control
HIV exposed
Condition
HIV exposed
HIV negative control

Table 1: Average percentage of immunophenotypic sub-populations present in the Lin-CD34+ population on D0 and D7 between HIV Unexposed Uninfected and Uninfected samples

Population name	Phenotype	% D0 HUU	% D0 HEU	% D7 VC HUU	% D7 VC HEU	% D7 SR1 HUU
ExtMPP	Lin-CD34+CD38-CD133+CD45RA-CD117+CD90dimCD49f+	2.19	3.77	0.01	0.01	0
ExtCMP	Lin-CD34+CD38+CD133+CD45RAvCD117+CD90dimCD49f+	80.02	71.76	0.05	0.12	0.01
ExtGMP	Lin-CD34+CD38+CD133dimCD45RA+CD117+CD90-CD49f-	1.02	1.25	0.01	0.01	0.01
ExtGMP2	Lin-CD34+CD38+CD133dimCD45RA+CD117-CD90-CD49f-	0.59	1.22	0.02	0.01	0.02
ExtEMP	Lin-CD34+CD38+CD133dimCD45RA-CD117+CD90-CD49f-	9.14	8.03	0.39	0.01	1.07
ExtEMP2*	Lin-CD34+CD38+CD133-/dimCD45RA-CD117+CD90-CD49f-	3.48	3.14	0.02	0.07	0.03
ExtLMPP	Lin-CD34+CD38-CD133+CD45RA+CD117-CD90-CD49f-	0.93	3.77	0.67	0.69	1.63
ExtLMPP1	Lin-CD34+CD38-CD133+CD45RA+CD117vCD90-CD49f+	0.04	0.03	10.08	27.21	3.88
ExtLMPP2	Lin-CD34+CD38-CD133+CD45RAvCD117dim/-CD90vCD49fdim	0.12	0.19	58.12	19.04	59.03
ExtLMPP3	Lin-CD34+CD38-CD133+CD45RA+CD117-CD90-CD49f+	0	0.02	2.04	9.98	0.98
CD90+	Lin-CD34+CD38-CD133+CD45RAvCD90+CD49fv	0	0.01	0.91	5.61	0.46
CD49f+	Lin-CD34+CD38-CD133-CD45RA-CD117-CD90dim/-CD49f+	0.24	0.94	0.6	1.29	0.7
HSPC1	Lin-CD34+CD38-CD133-CD45RA+CD117-CD90dim/-CD49fv	0.06	0.04	3.13	7.9	3.2
HSPC2	Lin-CD34+CD38-CD133-CD45RAvCD117vCD90dim/-CD49f-	0.08	0.12	14.98	13.26	18.57
HSPC3*	Lin-CD34+CD38-CD133dimCD45RA+CD117-CD90dim/-CD49fdim/-	0.02	0.08	1.48	5.45	1.97
D7ExtGMP**	Lin-CD34+CD38+CD133-/dimCD45RAvCD117+CD90vCD49fv					
CD90+(2)**	Lin-CD34+CD38+CD133-CD45RA-CD117dimCD90+CD49fv					
D7ExtEMP**	Lin-CD34+CD38+CD133-CD45RA-CD117+CD90dimCD49f-					

*Populations found in MACS and not FACS **Populations found in FACS and not MACS; HUU, HIV unexposed uninfected, HEU, HIV exposed uninfected, CD, cluster of differentiation; CMP, common myeloid progenit day 7; EMP, erythro-myeloid progenitor; Ext, extended; GMP, granulocyte-macrophage progenitor; HSC, haematopoietic stem cell; LMPP, lympho-myeloid primed progenitor; MPP, multipotent progenitor; SR1, stemre VC, vehicle control; N=4 for HEU and N=5 or HUU

HIV Exposed

% D7 SR1 HEU
0.01
0.13
0.01
0
0.03
0.2
1.46
12.77
10.7
22.36
5.59
1.24
14.06
13.59
9.57

or; D0, day 0; D7,
genin1; v, variable;