UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

The haematological profile of a treatment-naïve HIV-positive cohort: A pilot study

Submitted in fulfilment of the requirements for the degree

Master of Scicence (Medical Immunology)

In the Faculty of Health Sciences

Department of Immunology

University of Pretoria

2024

Tsungai Vesta Mashingaidze

Student number: 16306890

Supervisor: Dr Chrisna Durandt

Co-Supervisors: Dr Juanita Mellet and Prof Vanessa Moodley (SMU)

Declaration of Authenticity

I, TV Mashingaidze, declare that the dissertation, which I hereby submit for the degree of MSc Medical Immunology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution. Furthermore, I declare that all source material used has been appropriately cited, and to my knowledge, neither I nor any of the declared contributors are aware of any plagiarism in the following work.

Ethics statement

I, TV Mashingaidze, have obtained the required research ethics approval for the research described in this work. Furthermore, I declare that I have observed the ethical standards required in terms of the University of Pretoria's Code of ethics for scholarly activities.

Contributor	**Contribution**
Ms Tsungai Vesta Mashingaidze	Laboratory work, data analysis and write up of dissertation.
Dr Chrisna Durandt (supervisor)	Conceptualization of project, project direction and overview, laboratory assistance, editing and revisions of dissertation.
Dr Juanita Mellet (co-supervisor)	Assistance in laboratory, project overview, editing and revisions of dissertation.
Prof Vanessa Moodley (co-supervisor)	Project overview, editing and revisions of dissertation.
Dr Letha Mafisa	Coordination with patient recruitment clinics.
Dr TJ Mashamba	Assistance with patient recruitment.

I hereby submit the following title as my own work and confirm that all contributors are listed in the above table.

Title of work:

The haematological profile of a treatment-naïve HIV-positive cohort: A pilot study

(MSc dissertation)

Date: 20/05/2024

Name: Tsungai Vesta Mashingaidze

Signature: 

Dedication

I would like to dedicate this dissertation to my loving parents. To my father Tapuwa Mashingaidze, *In Omnia Paratus* - with the hope of living a life half as meaningful as yours. To my mother Constance Mashingaidze, thank you for everything you have sacrificed to get me here. You are, have always been, and will always be the wind beneath my wings.

Acknowledgments

I would like to thank my supervisor Dr Chrisna Durandt for her endless support and willingness to teach me. For her countless hours spent in the laboratory with me, kindness, her detail orientated nature that challenged me to consistently strive for excellence and her encouragement to look deeper into the science, to understand why things are.

I would like to thank my co-supervisor Dr Juanita Mellet for her willingness to provide assistance both outside and inside the laboratory. Prof Vanessa Moodley, my co-supervisor for her support throughout my studies and Prof Michael Pepper for his leadership and inspiration.

I would like to thank the nursing staff at the Ante-Natal clinic at the Dr George Mukhari Academic Hospital for their crucial role in identifying and recruiting study participants as well as their keen interest in my studies. Additionally, all the individuals who so kindly agreed to participate in this study for making it possible. Dr Letha Mafisa for her logistical assistance and the staff at the National Health Laboratory Services at Dr George Mukhari Academic Laboratory for performing all routine diagnostic tests reported in this study.

I would like to thank my funders, the Poliomyelitis Research Foundation, National Research Foundation, University of Pretoria, South African Medical Research Council and the Institute of Cellular and Molecular Medicine for their financial assistance throughout my studies.

I would like to thank my friends at the ICMM for the great chats and laughs. To my dearest siblings Tsitsi, Chido, and Fungai, thank you for your listening ears and ever supportive words. Tete Wendy and Uncle Robb for being my home. To my very best friends Kundai, Lorraine, Tariro, and Rumbi - thank you for being good men in the storm, for being the warm sunshine on my skin constantly reminding me that abundance is my portion.

I would like to thank my husband, Tinashe for being my steadfast. Your ever-supportive, calm, and positive demeanour has replaced all my fears with hope.

Lastly, I would like to thank God Almighty, the one who sustains me. Thank you for giving me the opportunity to do this, and the courage to see it through. As I stand on this mountain top, I bow my life to you- for you have set me here. Thank you for remaining faithful and true to your word.

Executive Summary

Human immunodeficiency virus (HIV) infection has been observed to significantly impact both the immune system and haematopoiesis. Haematopoiesis is the regulated process of producing the cellular components of blood with the haematopoietic stem and progenitor cells (HSPCs) as the cells of origin. Haematopoiesis occurs mainly in the bone marrow (BM). Haematopoietic stem and progenitor cells are necessary for the maintenance of steady-state haematopoiesis and a fully functional immune system. In HIV-negative individuals, it has been noted that a small proportion of HSPCs consistently migrate from the BM into the peripheral circulation. Under stress conditions such as infection with HIV, steady-state haematopoiesis is disrupted, and downstream HSPC differentiation activity becomes dysregulated. Literature reports that dysregulated HSPC differentiation leads to increased levels of the immunosuppressive myeloid-derived suppressor cells (MDSCs) in HIV infection, with no evidence of MDSCs in healthy individuals. To better understand this disruption, we investigated whether a link exists between the immature circulating HSPCs, heterogeneous MDSCs, and the haematological profile of treatment-naïve HIV-positive and HIV-negative (control) groups, respectively. This was performed using peripheral blood donated by consenting treatment-naïve HIV-positive and HIV-negative (control) participants. The frequency and phenotype of the circulating HSPCs in a treatment-naïve HIV-positive cohort was determined and compared to an HIV-negative (control) cohort using flow cytometry. This study found a significant decrease in circulating HSPCs in the treatment-naïve HIV-positive group in comparison to the HIV-negative (control) group with no difference in the phenotype between the two groups. Furthermore, HIV-mediated disruption of haematopoiesis often results in haematological abnormalities, such as cytopenias. Cytopenias arising from HIV infection are associated with increased morbidity and mortality. A full blood count with a white cell differential was performed to investigate the extent of the disruption. We observed anaemia and thrombocytopenia within the HIV-positive group. Neither leukopenia nor neutropenia were observed in this study. Additionally, significant reductions were observed in the white and red cell counts, haemoglobin, and absolute neutrophil counts among the HIV-positive group, in comparison to the HIV-negative (control) group. The exact mechanism of HIV-mediated cytopenias is not clear, but scientific evidence strongly suggests that HIV disrupts the BM milieu which impacts on the function of HSPCs. Therefore, the functionality

of circulating HSPCs were also investigated with the colony forming unit (CFU) assay. The HSPCs differentiated to produce CFU-granulocyte, erythrocytes, monocyte, megakaryocyte (GEMM), CFU-granulocyte, monocyte (GM), and burst forming units-erythrocyte (BFU-E). No phenotypic differences were observed between HIV-positive and HIV-negative (control) cohorts. However, when investigating the impact of HIV on the immune system using flow cytometry, various immune cell populations, including the cluster of differentiation (CD)4^+ T cells were significantly decreased in the HIV-positive group when compared to the HIV-negative (control) group. Whereas the $CD8^+$ T cells were significantly increased in the HIV-positive group compared to the HIV-negative (control) group. Furthermore, this study did not observe increased levels of MDSCs in the HIV-positive group as hypothesised. In summary, the study observed decreased frequencies of circulating HSPCs, which is indicative of abnormal haematopoiesis and subsequent BM disruption. The BM disruption trickles down into haematological indices and supports the onset of cytopenias.

Keywords: HIV, haematopoiesis, haematological abnormalities, treatment-naïve, circulating HSPCs, differentiation, immune dysfunction, flow cytometry.

List of Abbreviations

7-AAD	7-aminoactinomycin D
A	Area
ADAMTS-13	A-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13
AIDS	Acquired immunodeficiency syndrome
ANC	Antenatal clinic
APC	Allophycocyanin
APCs	Antigen presenting cells
APC-AF	Allophycocyanin-Alexa fluor
ARG	Arginase
ART	Antiretroviral therapy
ARV	Antiretroviral
BFU	Burst -forming unit
BM	Bone marrow
BV	Brilliant violet
cART	Combination antiretroviral therapy
CCR5	C-C chemokine receptor type-5
CD	Cluster of differentiation
CD4/CD8	$CD4^+$ T helper/$CD8^+$ T cytotoxic cell ratio
CDC	Centres of disease control and prevention
CFA	Colony forming abilities
CFC	Colony forming cells
CFU	Colony forming unit
CHI	Chronic stages of HIV infection
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CO_2	Carbon dioxide
CRP	C-reactive protein
CSF	Colony stimulating factor
CXCR4	C-X-C Motif Chemokine Receptor type-4

CXCL12	C-X-C Motif Chemokine Ligand 12
DAMPs	Damage-associated molecular patterns
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
DGMAH	Dr George Mukhari Academic Hospital
DGMTL	Dr George Mukhari Tertiary Laboratory
E	Erythroid
EDTA	Ethylenediamine tetra-acetic acid
ECD	R- Phycoerythrin-Texas red®-X
EPO	Erythropoietin
FACS	Fluorescence activated cell sorting
FBC	Full blood count
FBCD	Full blood count with white cell differential
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
Flt3L	Fms related receptor tyrosine kinase 3 ligand
FS	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GEMM	Granulocyte, erythroid, macrophage, megakaryocyte
GMP	Granulocyte-macrophage progenitors
GM	Granulocyte/macrophage
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCT	Haematocrit
HCV	Hepatitis C virus
HGB	Haemoglobin
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-DR
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
HSPC	Haematopoietic stem and progenitor cell
i	Immature

IMC	Immature myeloid cell
iNOS	inducible nitric oxide synthase
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
IP	Inducible protein
ISG	Interferon-stimulated genes
ITP	Immune thrombocytopenic purpura
iTreg	induced regulatory T cell
IQR	Interquartile range.
KO	Krome orange
LRR	Leucine-rich repeats
Lin	Lineage
LTRC	Long-term repopulating cells
Lymphs	Lymphocytes
M	Myeloid
M-CSF	Macrophage colony-stimulating factor
MACS	Magnetic-activated cell sorting
mAB	Monoclonal antibodies
MCV	Mean corpuscular volume
MEP	Megakaryocyte-erythrocyte progenitor
MDSC	Myeloid derived suppressor cells
MFI	Median fluorescence intensity
Mk	Megakaryocyte
MNC	Mononuclear cells
MPP	Multipotent progenitors
Neuts	Neutrophils
NHLS	National health laboratory service
NK	Natural killer cell
NKT	Natural killer T cells
NLR	NOD-like receptors

NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-L	Programmed cell death ligand
PE	Phycoerythrin
PC	Phycoerythrin-cyanine
PLTs	Platelets
PLWH	People living with HIV
PHI	Primary HIV infection
PMN	Polymorphonuclear
PRRs	Pattern recognition receptors
pSTAT	Phosphorylated signal transducer and activator of transcription
RBC	Red blood cell
RPMI	Roswell park memorial institute-1640 medium
RT	Room temperature
Tfh	Follicular helper T cell
Th	T helper
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TPO	Thrombopoietin
SB	Super bright
SCF	Stem cell factor
SCID-SRC	Severe combined immunodeficiency -repopulating cell
SD	Standard deviation
SOP	Standard of procedure
SSC	Side scatter
STRC	Short-term repopulating cells
WBC	White blood cell
Vio	Violet

VL	Viral load
ZV	Zombie violet

List of Symbols and Units

α	Alpha
β	Beta
dL	decilitre
°C	degree Celsius
fL	Femtolitre
γ	Gamma
g	Gram
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
L	Litre
µL	Microlitre
mL	Millilitre
µm	Micrometre
mM	Millimolar
%	Percentage
x g	Times gravitational force

Table of Contents

Declaration of Authenticity ii
Ethics statement ii
Dedication iv
Acknowledgments v
Executive Summary vi
List of Abbreviations viii
List of Symbols and Units xii
Table of Figures xviii
List of Tables xxi
Chapter 1: Introduction, Aim and Objectives 23
1.1. Introduction 23
1.1.1. Aim 25
1.1.2. Objectives 25
1.2. Dissertation outline 26
1.3. References 27
Chapter 2: Literature review 30
2.1. Introduction to the immune system 30
2.1.1. Communication within the immune system 30
2.1.2. Introduction to the innate immune system 32
2.1.3. Introduction to the adaptive immune system 33
2.2. Haematopoiesis 35
2.2.1. Differentiation pathways 37
2.2.2. Characterisation of haematopoietic stem and progenitor cells phenotype 37
2.2.3. Colony forming abilities of haematopoietic stem and progenitor cells 41
2.3. Human immunodeficiency virus 42
2.3.1. Human immunodeficiency virus origin and distribution 44
2.3.2. The impact of human immunodeficiency virus on the innate immune system 45
2.3.3. The impact of human immunodeficiency virus on the adaptive immune system 46
2.3.4. The impact of human immunodeficiency virus on haematopoiesis 48
2.3.4.1. Anaemia 50

2.3.4.2. Thrombocytopenia 50
2.3.4.3. Neutropenia 51
2.4. Conclusion 51
2.5 References 53
Chapter 3: Functionality of circulating CD34⁺ haematopoietic stem and progenitor cells 70
3.1. Introduction 70
3.2. Methodology 71
3.2.1 Study design 71
3.2.2. Ethics approval 72
3.2.3. Study participant enrolment 73
3.2.3.1. Consent 73
3.2.3.2. HIV counselling and confirmation 73
3.2.3.3. Study participant demographics 73
3.2.4. Experiments 74
3.2.4.1. Distribution of peripheral blood samples for research purposes 74
3.2.4.2. Isolation of peripheral blood mononuclear cells 74
3.2.4.3. Enumeration of peripheral blood mononuclear population 75
3.2.4.4. Circulating CD34⁺ haematopoietic stem and progenitor cell frequency and absolute counts 76
3.2.4.4.1. Colour compensation for the haematopoietic stem and progenitor cell panel 76
3.2.4.4.2. Standardisation setup for the haematopoietic stem and progenitor cell panel 77
3.2.4.4.3. Sample preparation and acquisition for the haematopoietic stem and progenitor cell panel 78
3.2.4.4.4. Gating strategy to enumerate circulating CD34⁺ HSPCs 79
3.2.4.4.5. Gating strategy to phenotypically characterise circulating CD34⁺ haematopoietic stem and progenitor cells 80
3.2.4.5. Magnetic bead isolation 81
3.2.4.5.1. Sample preparation for magnetic bead isolation 82
3.2.4.5.1.1. Sample enumeration 82
3.2.4.5.1.2. Magnetic labelling 82
3.2.4.5.1.3. Magnetic separation 83

3.2.4.6. Colony-forming unit assay....83
3.2.4.6.1. Assessing colony formation of Lin^{-}CD34^{++} haematopoietic stem and progenitor cells....83
3.2.4.6.2. Phenotypic characterisation of differentiated colonies....85
3.2.4.6.2.1. Colour compensation and standardisation setup for phenotypic characterisation of colonies....85
3.2.4.6.2.2. Staining procedure and sample acquisition....86
3.2.4.6.2.3. Gating strategy for the phenotypic characterisation of progenitor cells....87
3.2.5. Statistical considerations....93
3.4. Results....93
3.4.1. Quantification and phenotypic assessment of peripheral blood CD34^{+} haematopoietic stem and progenitor cell sub-populations....93
3.4.2. Functionality of circulating Lin^{-}CD34^{++} haematopoietic stem and progenitor cells....95
3.5. Discussion....102
3.6. References....106
Chapter 4: Enumeration of immune cell and myeloid derived suppressor cell populations 112
4.1. Introduction....112
4.2. Methodology....115
4.2.1 Study design....115
4.2.2. Study participant enrolment....116
4.2.2. Experiments....116
4.2.3. Basic immune profile....116
4.2.3.1. Colour compensation setup for basic immune profile....116
4.2.3.3. Sample preparation to obtain a basic immune profile....117
4.2.4. Isolation and enumeration of peripheral blood mononuclear cells....118
4.2.4.1. Myeloid-derived suppressor cell frequency and absolute counts....119
4.2.4.1.1. Colour compensation setup for myeloid derived suppressor cells panel....119
4.2.4.1.2. Standardisation setup for myeloid derived suppressor cell panel....120
4.2.4.1.3. Sample preparation for the MDSC panel....120
4.2.4.1.4. Gating strategy for the MDSC panel....122
4.2.5. Statistical analysis....124
4.3. Results....124

4.3.1. Enumeration of immune cell populations 124
4.3.2. Enumeration of myeloid derived suppressor cells populations 129
4.3.3. Myeloid derived suppressor cells and human immunodeficiency virus disease progression 130
4.4. Discussion 131
4.5. References 136
Chapter 5: Associations of circulating haematopoietic stem and progenitor cells with the haematological and immunological parameters in treatment-naïve, HIV-positive pregnant women 141
5.1. Introduction 141
5.2. Methodology 143
5.2.1. Study design 143
5.2.2. Ethics approval 144
5.2.3. Full blood count and differential 144
5.2.4. Enumeration of $CD4^+$ T helper and $CD8^+$ cytotoxic cells 145
5.2.5. Isolation of peripheral blood mononuclear cells 145
5.2.6. Circulating haematopoietic stem and progenitor cell frequency 145
5.3. Statistical analysis 146
5.4. Results 146
5.4.1. General characteristics of study participants 146
5.4.2. Haematological profiles of study participants 147
5.4.3. Circulating haematopoietic stem and progenitor cell frequencies 152
5.5. Discussion 154
5.6. Conclusion 160
5.7. References 161
Chapter 6: General discussion and concluding remarks 169
6.1. Discussion 169
6.2. Concluding remarks 173
6.3. Limitations of this study 174
6.4. Future work 174
6.5. References 176
Appendix I: Normal cell surface expression of CD markers used in this study 178

Appendix II: Ethics Approval 183
Appendix III: Informed consent documents.....184
Appendix IV: Standard Operating Procedure (SOP 194
Appendix V: Supplementary figures.....205

Table of Figures

Figure 2.1: Schematic illustration of the differentiation process of haematopoietic stem and progenitor cells into mature blood cell types (haematopoiesis). Image adapted from Konieczny and Arranz (2018)[78]. Image created by Tsungai Mashingaidze using BioRender. 38

Figure 3.1: A schematic illustration of the various layers that form during density-gradient centrifugation of peripheral blood. Image created by Tsungai Mashingaidze using Microsoft PowerPoint. MNC, Mononuclear cells; x g, Times gravitational force. 75

Figure 3.2: Representative flow cytometry plots to illustrate the gating strategy used to identify and enumerate circulating $CD34^{+}$ haematopoietic stem and progenitor cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; FITC, Fluorescein isothiocyanate; FSC, Forward scatter; Lin, Lineage; SSC, Side scatter; ZV, Zombie violet. 80

Figure 3.3: Representative flow cytometry plots [(A-C) density plots and (D) tree plot] to illustrate the gating strategy used to outline and phenotypically characterise circulating Lin^{-} $CD34^{++}$ haematopoietic stem and progenitor cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FITC, Fluorescein isothiocyanate; FSC, Forward scatter; Lin, Lineage; PE, Phycoerythrin; SSC, Side scatter; Vio, Violet, ZV, Zombie violet. 81

Figure 3.4: A schematic illustration of the experimental layout used for the colony forming units assay. Wells C3-C5 (red) indicate the wells containing the MethoCult™ medium and Lin^{-} $CD34^{++}$ haematopoietic stem and progenitor cell. The remaining wells contained PBS. Image created by T Mashingaidze using Microsoft PowerPoint. HSPCs, Haematopoietic stem and progenitor cells. 84

Table 3.3: General descriptions and representative micrographs, adapted from STEMCELL™ Technologies[25, 27] used for colony identification in colony forming unit-assays. Representative micrographs [4X objective (left) and 10X objective (right)] were extracted from STEMCELL™ Technologies- Wallchart: Identification of Colonies Derived from Human Haematopoietic Progenitor Cells[27]. 88

Figure 3.5: Representative flow cytometry plots to illustrate the gating strategy used to determine cell viability of the $Lin^{-}CD34^{++}$ HSPCs isolated from PBMNCs using the CFU assay. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. 7-AAD, 7-Aminoactinomycin-D; A, Area; FSC, Forward scatter; Inter, Intermediate; SSC, Side scatter. 91

Figure 3.6: Representative flow cytometry plots to illustrate the sequential gating strategy used to identify and enumerate erythroid lineage cells. Image created by T Mashingaidze using

Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; FSC, Forward scatter; KO, Krome orange; RBC; Red blood cells; PE, Phycoerythrin; SSC, Side scatter. 91

Figure 3.7: Representative flow cytometry plots to illustrate the sequential gating strategy used to identify and enumerate myeloid lineage cells. A Boolean gate "Total CD33" was created using the Boolean string of '(Viable AND $CD15^{+}CD33^{+}$) OR (Viable AND $CD15^{-}CD33^{+}$)'. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FSC, Forward scatter; KO, Krome orange; PE, Phycoerythrin; SSC, Side scatter. 92

Figure 3.8: Representative flow cytometry plots to illustrate the sequential gating strategy used to identify and enumerate lymphoid lineage cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa Fluor; CD, Cluster of differentiation; FSC, Forward scatter; Lymph, Lymphocytes; PC, Phycoerythrin-cyanine; SSC, Side Scatter.....93

Figure 3.9: Enumeration of circulating $Lin^{-}/CD34^{++}$ haematopoietic stem and progenitor cells present in peripheral blood of treatment-naïve, HIV-positive (n=13), and HIV-negative (control) groups (n=25). Control refers to the HIV-negative (control) group. Bar graphs were used to demonstrate the (A) mean percentage of viable peripheral blood mononuclear cells, and (B) mean absolute cell count (cells/µL), with the error bars indicating the standard deviation (SD). Significance was reported as * and **, indicating p-values of <0.05 and <0.01, respectively. 94

Figure 3.10: Assessment of the functionality of the isolated $Lin^{-}CD34^{++}$ haematopoietic stem and progenitors, plated on semi-solid MethoCult™ medium after 14-day incubation. (A) Identification and enumeration of colonies observed. Bar graphs were used to denote the average (mean) number of the different colony types observed, with the error bars indicating the standard deviation between samples. (B-D) Representative images of the different types of colonies observed. Images were captured using a 5X (left) and 10X objective (right). Control refers to the HIV-negative (control) group. B, burst; CFU, colony forming unit; E, erythroid; GEMM, granulocyte erythroid macrophage megakaryocyte; GM, granulocyte macrophage.97

Figure 3.11: Quantification of the erythroid, myeloid, and lymphoid precursors and progeny compared between treatment-naïve, HIV-positive, and HIV-negative (control) groups. Bar graphs were used to demonstrate the mean percentage of either viable cells or as indicated by the y-axis title. Control refers to HIV-negative (control) group. The error bars indicate the standard deviation for both groups. (A-B) Erythroid progeny. (C-I) Myeloid progeny and their respective proliferating cells. (J-M) Lymphoid precursors and their proliferating lymphoid progenitors.CD, cluster of differentiation; HIV, human immunodeficiency virus; Lymph, lymphocyte; ns, non-significant. 101

Figure 4.1: Phenotypic characterisation of myeloid derived suppressor cells sub-populations. Density plots were generated using Kaluza analysis version 2.1 software and compiled using Microsoft PowerPoint by T Mashingaidze. 7-AAD, 7-Aminoactinomycin-D; APC, Allophycocyanin; APC-A, Allophycocyanin-Alexa fluor; A, Area; CD, Cluster of differentiation; FSC, Forward scatter; H; Height; HLA-DR, Human Leukocyte Antigen – DR; KO, Krome orange; M-MDSC, Myeloid derived suppressor cells; PC, Phycoerythrin-cyanine; SSC, Side scatter..124

Figure 4.2: Scatter plot displaying the distribution of the CD4/CD8 ratios in HIV-positive and HIV-negative (control) groups. The median value is represented by the horizontal line. The CD4/CD8 ratio was calculated for each patient enrolled and is represented by a singular dot within the respective group. Control refers to the HIV-negative (control) group. CD, Cluster of differentiation; HIV, Human immunodeficiency virus.....129

Figure 4.3: Correlation analysis between the frequency of myeloid derived suppressor cell sub-populations and the frequency (left) and absolute count (right) of the $CD4^+$ T cells present within the Treatment-naïve, HIV-positive group. Correlations between M-MDSC (A-B), PMN-MDSC (C-D), and iMDSC (E-F) and CD4 count, and frequency are shown. CD, Cluster of differentiation; i, Immature; IQR, Interquartile range; MDSC, Myeloid derived suppressor cells; M, Myeloid; PMN, Polymorphonuclear. 131

Figure 5.2: Comparative graph of circulating Lin^-CD34^{++} haematopoietic stem and progenitor cells in HIV-positive and -negative (control) individuals. (A) Lin^-CD34^{++} haematopoietic stem and progenitor cells as a percentage of viable cells. (B) Absolute Lin^-CD34^{++} haematopoietic stem and progenitor cells cell counts (cells/µL). Control refers to the HIV-negative (control) group. Bar graphs represent mean ± SD values. **p<0.01. CD, Cluster of differentiation; HIV; Human immunodeficiency virus; HSPC, Haematopoietic stem and progenitor cells; Lin, Lineage. 153

Figure 5.3: Correlation analysis between the frequency (left) and absolute counts (right) of circulating Lin^-CD34^{++} HSPCs and absolute $CD4^+$ T cell counts. Correlations between (A-B) HIV-positive (n=13) and (C-D) HIV-negative (control) (n=53) groups are shown. CD; Cluster of differentiation; HIV, Human immunodeficiency virus; HSPC, Haematopoietic stem and progenitor cells; Lin, Lineage. 153

List of Tables

Table 2.1: Examples of type 1 and 2 cytokines[8, 10-13]. 31

Table 2.2: Cytokines produced by the various major immune populations[14-21].....31

Table 2.3: Haematopoietic stem and progenitor cell sub-populations and their associated cell surface markers[52, 89, 90, 94]. 40

Table 2.4: Myeloid derived suppressor cell subsets and their associated cell surface markers[176, 179]. 49

Table 3.1: The panel of fluorochrome-conjugated monoclonal antibodies and the volume used for the circulating $CD34^{+}$ haematopoietic stem and progenitor cell panel. 78

Table 3.2: Panel of fluorochrome-conjugated monoclonal antibodies and the volume used for the isotypic control tube for the circulating $CD34^{+}$ haematopoietic stem and progenitor cell panel. 79

Table 3.4: Panel of fluorochrome-conjugated monoclonal antibodies used to assess viability and phenotypically characterise the differentiated progeny of $Lin^{-}CD34^{++}$ haematopoietic stem and progenitor cells. 90

Table 3.5: The cell lineages identified by the respective fluorochrome-conjugated monoclonal antibodies used in the colony forming units immunophenotypic panel. 90

Table 3.6: CD38, CD117, and CD133 co-expression profiles observed in the treatment-naïve, HIV-positive and HIV-negative (control) groups. Results are expressed as the mean percentage with standard deviation of viable $Lin^{-}CD34^{++}$ haematopoietic stem and progenitor cells. 95

Table 3.7A: Mean percentages of total viable and dead cells obtained from the colony forming unit viability analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group. 98

Table 3.7B: Mean percentages of viable and dead cells obtained from colony forming unit viability analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group. 99

Table 4.2: Reference range percentages for immune cell populations in peripheral blood[33]. 119

Table 4.3: Panel of fluorochrome-conjugated monoclonal antibodies used to set up the colour compensation matrix for the myeloid derived suppressor cell panel. 120

Table 4.4: The panel of fluorochrome-conjugated monoclonal antibodies and their volume used to enumerate the myeloid derived suppressor cells sub-populations.....121

Table 4.5A: Median (Interquartile range) of frequency of various immune cell populations present in peripheral blood, obtained using flow cytometric analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group. 126

Table 4.5B: Median (Interquartile range) of absolute cell counts of various immune cell populations present in peripheral blood, obtained using flow cytometric analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group. 127

Table 4.6: Median (interquartile range) of the frequency of myeloid derived suppressor cells sub-populations, expressed as percentage of peripheral blood mononuclear cells present within peripheral blood mononuclear cells isolated from the treatment-naïve, HIV-positive, group and the HIV-negative (control) group, respectively. 130

Table 5.1: Defining criteria for various cytopenias. 145

Table 5.2: Comparison of haematological parameters of the respective study groups. 148

Table 5.3: Distribution of CD4 counts of study groups. 149

Table 5.4: Prevalence of cytopenia observed in the HIV-positive and HIV-negative (control) groups. 150

Table 5.5A: Associations between varying degrees of CD4 counts and cytopenias within the HIV-positive group.....151

Table 5.5B: Associations between varying degrees of CD4 counts and cytopenias within the HIV-negative (control) group.....152

List of Equations

Equation 3.1: Volume of cell suspension to be aliquoted into MethoCult™ media. 84

Equation 4.1: Determination of the true frequency of myeloid derived suppressor cell sub-populations present within peripheral blood mononuclear cells. 123

Chapter 1: Introduction, Aim and Objectives

1.1. Introduction

Since the declaration of the Human Immunodeficiency Virus (HIV) pandemic by the World Health Organisation (WHO), significant strides have been made in the race against HIV infection. The most remarkable advancement to date has been the introduction of antiretroviral therapy (ART). Initially, ART treatment involved a combination of three or more antiretroviral (ARV) drugs, termed as highly active antiretroviral therapy (HAART)[1]. However, the development of complications with HAART such as increased drug toxicities and resistance associated with high pill burdens led to a decline in treatment adherence and subsequent effectiveness[2]. This resulted in the introduction of more effective (increased potency, long-term and safer) HIV medicine, termed combination ART (cART)[2]. Due to the significant advances in cART, effective suppression of viral replication to an undetectable and non-transmissible level is now possible. However, cART is incapable of curing HIV infection. If HIV infection is left untreated, the virus will severely compromise the immune system, resulting in acquired immunodeficiency syndrome (AIDS)[3].

The hallmarks of HIV infection include the accelerated depletion of cluster of differentiation (CD)4^+ helper T cells, which are the primary target of HIV. This depletion is accompanied by chronic inflammation and immune activation which leads to the rapid expansion of $CD8^+$ cytotoxic T cells[4]. However, the detrimental effects of HIV infection are not limited to the immune system but extend to the haematopoietic system. Haematopoiesis, the production of all blood cell lineages throughout an individual's entire lifespan, is particularly affected[5]. This process is sustained by haematopoietic stem and progenitor cells (HSPCs) residing in the bone marrow (BM)[6].

The BM is an optimal niche for HSPCs, as it contains all the required components to support self-renewal and survival[7]. Within the BM microenvironment, stromal cells play an important role in the production of haematopoiesis-associated cytokines[8]. These cytokines, along with chemokines, cell-to-cell, and cell-to-matrix interactions, collectively regulate HSPC function, including fate determination, proliferation, and differentiation[9, 10]. However, HIV infection

impacts the balance of the BM stroma which in turn disrupts HSPC regulation, resulting in defective haematopoiesis[8, 11, 12].

Haematopoietic stem and progenitor cells are predominantly quiescent, and their differentiation and self-renewal activity are tightly regulated by the BM niche[7, 13]. However, under steady-state haematopoiesis, a small proportion of HSPCs move from the BM into the peripheral circulation whilst retaining their BM homing abilities[14, 15]. These circulating HSPCs play an important role in repopulating the blood cell lineages in niches outside the BM that are depleted due to stress or cell-cycling[16]. An example of this is the seeding of the thymus by HSPCs to ensure the maintenance of leukocyte populations in the event of infection[17, 18]. Circulating HSPCs were investigated in this study, and typically express the CD34 marker ($CD34^+$) and lack expression of lineage markers (Lin^-).

Notably, HSPC migration from the BM into peripheral circulation is not limited to normal physiological conditions but may also be triggered by stress conditions, such as HIV infection, to replenish blood cell lineages that have been depleted by the virus[15]. Human immunodeficiency virus infection disrupts steady-state haematopoiesis, consequently leading to an imbalance in haematopoiesis[19]. Importantly, key physiological processes of oxygen transportation, haemostasis, and host defence are all mediated by mature blood cells [red blood cells (RBCs), platelets (PLTs), and leukocytes (myeloid and lymphoid), respectively] which are produced by the haematopoietic system[20]. These mature blood cells are generated by lineage-committed precursors, differentiated from common lymphoid- and myeloid-progenitor cells (CLP and CMP, respectively)[21].

In healthy individuals, CMPs differentiate to produce immature myeloid cells (IMCs), which further differentiate into functional dendritic cells, macrophages, and granulocytes[22]. However, HIV-induced chronic inflammation impacts CMP activity, thereby stunting IMC maturation[23, 24], resulting in the occurrence and subsequent elevation of myeloid-derived suppressor cells (MDSCs)[25-27]. Myeloid derived suppressor cells have potent innate and adaptive immunosuppressive abilities[28]. They primarily exert their negative effects on T cells by inhibiting their activity and expansion[29]. The occurrence of MDSCs in HIV infection is commonly associated with disease progression[26, 30].

Overtime, the negative effect of HIV extends into the downstream processes such as erythropoiesis, thrombopoiesis, leukopoiesis and subsequently, T cell expansion[31, 32]. The distortion of these haematological processes results in peripheral blood cytopenias as a manifestation of ineffective haematopoiesis caused by HIV infection[33]. Human immunodeficiency virus-mediated cytopenias have been linked to increased mortality and are associated with HIV disease progression[34, 35]. These cytopenias are characterised by abnormally low levels of RBCs, PLTs, white blood cells (WBCs) and absolute neutrophil count, which give rise to anaemia, thrombocytopenia, leukopenia, and neutropenia, respectively[36].

The vast majority of haematopoietic abnormalities that manifest in the presence of HIV have been attributed to viral replication in the BM[37, 38]. Consequently, the majority of HIV-associated cytopenias are reversed when HIV patients receive cART[35]. The frequency of cytopenias in HIV-infected persons and the observation that most patients recover from these cytopenias when receiving cART[36] suggests that HIV directly impacts on haematopoiesis. The exact mechanism by which HIV affects haematopoiesis is yet to be elucidated. It is important to note that all individuals enrolled into this study, regardless of HIV status were pregnant and pregnancy itself can result in the occurrence of peripheral blood cytopenias. However, the primary focus of this study is to explore the impact of HIV-infection on the haematopoietic system, in the absence of cART.

1.1.1. Aim

The aim of the project was to determine the relationship of immature circulating haematopoietic cells, namely HSPCs and MDSCs, with the haematological profile in HIV-positive patients not currently receiving combination antiretroviral (cART) treatment.

1.1.2. Objectives

i. To determine and compare the frequency of circulating HSPCs present in the peripheral blood of treatment-naïve, HIV-positive and HIV-negative (control) patients.
ii. To determine if the circulating HSPCs are functional, i.e., able to differentiate into the various blood cell types using the colony-forming unit (CFU) assay.

iii. To phenotypically characterise the differentiated colonies observed using the CFU assay to determine if there are any differences in the phenotypic distribution of these cells in HIV-negative (control) and treatment-naïve, HIV-positive patients.

iv. To determine and compare the basic immune profiles (T cells, B cells, NK cells, monocytes, and neutrophils) of treatment-naïve HIV-positive and HIV-negative (control) patients.

v. To determine and compare the frequency of MDSCs in the peripheral blood of treatment-naïve HIV-positive and HIV-negative (control) patients.

vi. To report on and compare the haematological profiles of treatment-naïve, HIV-positive and HIV-negative (control) patients.

1.2. Dissertation outline

This dissertation explored the impact of HIV infection on haematopoiesis in a pregnant, treatment-naïve, HIV-positive cohort. The results obtained are detailed across four chapters, prefaced by an overview of the relevant literature regarding haematopoiesis and HIV in Chapter 2. Chapter 3 explores the frequency, phenotypic differences, and functionality of circulating $Lin^{-}CD34^{++}$ HSPCs in a treatment-naïve, HIV-positive cohort. Chapter 4 investigates the frequency of various immune cells and MDSC sub-populations. In Chapter 5, the haematological profiles of the two cohorts (treatment-naïve, HIV-positive and (control) cohort) are assessed. Finally, Chapter 6 presents an overarching discussion and conclusion, summarising the main findings from each chapter.

1.3. References

1. Eggleton JS, Nagalli S. Highly active antiretroviral therapy (HAART) [Internet]. In: StatPearls, editor. Treasure Island, Florida: StatPearls Publishing; 2023. [cited: 2024/03/25]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK554533/

2. Weichseldorfer M, Reitz M, Latinovic OS. Past HIV-1 Medications and the Current Status of Combined Antiretroviral Therapy Options for HIV-1 Patients. Pharmaceutics. 2021; 13(11):1798.

3. Balasubramaniam M, Pandhare J, Dash C. Immune Control of HIV. J Life Sci (Westlake Village). 2019; 1(1):4-37.

4. Vidya Vijayan KK, Karthigeyan KP, Tripathi SP, Hanna LE. Pathophysiology of CD4+ T-Cell Depletion in HIV-1 and HIV-2 Infections. Front. Immunol. 2017; 8(580):1-8.

5. Omatsu Y. Cellular niches for hematopoietic stem cells in bone marrow under normal and malignant conditions. Inflamm. Regen. 2023; 43(15):1-7.

6. Ho NP-Y, Takizawa H. Inflammation Regulates Haematopoietic Stem Cells and Their Niche. Int. J. Mol. Sci. 2022; 23(3):1125.

7. Fröbel J, Landspersky T, Percin G, Schreck C, Rahmig S, Ori A*, et al.* The hematopoietic bone marrow niche ecosystem. Front Cell Dev Biol. 2021; 9(705410):1-19.

8. Nombela-Arrieta C, Isringhausen S. The Role of the Bone Marrow Stromal Compartment in the Hematopoietic Response to Microbial Infections. Front. Immunol. 2016; 7(689):1-12.

9. Kräter M, Jacobi A, Otto O, Tietze S, Müller K, Poitz DM*, et al.* Bone marrow niche-mimetics modulate HSPC function via integrin signaling. Sci. Rep. 2017; 7(1):2549.

10. Kidoya H, Muramatsu F, Shimamura T, Jia W, Satoh T, Hayashi Y*, et al.* Regnase-1-mediated post-transcriptional regulation is essential for hematopoietic stem and progenitor cell homeostasis. Nat. Commun. 2019; 10(1):1072.

11. Kokkaliaris KD, Scadden DT. Cell interactions in the bone marrow microenvironment affecting myeloid malignancies. Blood Adv. 2020; 4(15):3795-803.

12. Vishnu P, Aboulafia DM. Haematological manifestations of human immune deficiency virus infection. Br. J. Haematol. 2015; 171(5):695-709.

13. Liesveld JL, Sharma N, Aljitawi OS. Stem cell homing: From physiology to therapeutics. Stem Cells. 2020; 38(10):1241-53.

14. Mende N, Laurenti E. Hematopoietic stem and progenitor cells outside the bone marrow: where, when, and why. Exp. Hematol. 2021; 104:9-16.

15. Gao X, Xu C, Asada N, Frenette PS. The hematopoietic stem cell niche: from embryo to adult. Development. 2018; 145(2):dev139691.

16. Cossío I, Di Scala M, Adrover JM, Casanova-Acebes M, Weiss L, Weber C*, et al.* Replenishment of Damaged Bone Marrow Niches By Circulating Hematopoietic Stem Cells. Blood. 2017; 130(S1):3750.

17. Granick JL, Simon SI, Borjesson DL. Hematopoietic Stem and Progenitor Cells as Effectors in Innate Immunity. Bone Marrow Res. 2012; 2012(165107):1-8.

18. Quaranta P, Basso-Ricci L, Jofra Hernández R, Naldini MM, Barcella M, Pacini G*, et al.* Unveiling the Biological Role of Peripheral Blood Human Circulating Hematopoietic Stem and Progenitor Cells. Blood. 2022; 140(S1):1977-8.

19. Herd CL, Mellet J, Mashingaidze T, Durandt C, Pepper MS. Consequences of HIV infection in the bone marrow niche. Front. Immunol. 2023; 14(1163012):1-17.

20. Caiado F, Pietras EM, Manz MG. Inflammation as a regulator of hematopoietic stem cell function in disease, aging, and clonal selection. J. Exp. Med. 2021; 218(7):e20201541.

21. Cheng H, Zheng Z, Cheng T. New paradigms on hematopoietic stem cell differentiation. Protein Cell. 2020; 11(1):34-44.

22. Sanchez-Pino MD, Dean MJ, Ochoa AC. Myeloid-derived suppressor cells (MDSC): When good intentions go awry. Cell. Immunol. 2021; 362(104302):1-10.

23. Yaseen MM, Abuharfeil NM, Darmani H. Myeloid-derived suppressor cells and the pathogenesis of human immunodeficiency virus infection. Open Biol. 2021; 11(11):210216.

24. Millrud CR, Bergenfelz C, Leandersson K. On the origin of myeloid-derived suppressor cells. Oncotarget. 2017; 8(2):3649-65.

25. Rosado-Sánchez I, De Pablo-Bernal R, Rull A, Gónzalez J, Moreno S, Vinuesa D*, et al.* Increased Frequencies of Myeloid-Derived Suppressor Cells Precede Immunodiscordance in HIV-Infected Subjects. Front. Immunol. 2020; 11(581307):1-10.

26. Agrati C, Tumino N, Bordoni V, Pinnetti C, Sabatini A, Amendola A*, et al.* Myeloid Derived Suppressor Cells Expansion Persists After Early ART and May Affect CD4 T Cell Recovery. Front. Immunol. 2019; 10(1886):1-10.

27. Ma H, Xia CQ. Phenotypic and Functional Diversities of Myeloid-Derived Suppressor Cells in Autoimmune Diseases. Mediators Inflamm. 2018; 2018(4316584):1-8.

28. Wu Y, Yi M, Niu M, Mei Q, Wu K. Myeloid-derived suppressor cells: an emerging target for anticancer immunotherapy. Mol. Cancer. 2022; 21(1):184.

29. Yang Y, Li C, Liu T, Dai X, Bazhin AV. Myeloid-Derived Suppressor Cells in Tumors: From Mechanisms to Antigen Specificity and Microenvironmental Regulation. Front. Immunol. 2020; 11(1371):1-22.

30. Ademe M. Paradoxes in the phenotype, frequency and roles of myeloid-derived suppressor cells during HIV infection. HIV AIDS (Auckl.). 2020; 12:151-6.

31. Fiseha T, Ebrahim H. Prevalence and Predictors of Cytopenias in HIV-Infected Adults at Initiation of Antiretroviral Therapy in Mehal Meda Hospital, Central Ethiopia. J. Blood Med. 2022; 13:201-11.

32. Bordoni V, Bibas M, Viola D, Sacchi A, Cimini E, Tumino N*, et al.* Bone Marrow $CD34^{+}$ Progenitor Cells from HIV-Infected Patients Show an Impaired T Cell Differentiation Potential Related to Proinflammatory Cytokines. AIDS Res. Hum. Retroviruses. 2017; 33(6):590-6.

33. Bhardwaj S, Almaeen A, Ahmed Wani F, Thirunavukkarasu A. Hematologic derangements in HIV/AIDS patients and their relationship with the CD4 counts: a cross-sectional study. Int. J. Clin. Exp. Pathol. 2020; 13(4):756-63.

34. Mohammed Nuru M, Bizuayehu Wube T, Fenta DA. Magnitude and Factors Associated with Cytopenia Among Children on Highly Active Antiretroviral Therapy at Hawassa University College of Medicine and Health Science, Sidama Region, Southern Ethiopia. HIV AIDS (Auckl.). 2023; 15:145-55.

35. Gunda DW, Godfrey KG, Kilonzo SB, Mpondo BC. Cytopenias among ART-naive patients with advanced HIV disease on enrolment to care and treatment services at a tertiary hospital in Tanzania: A cross-sectional study. Malawi Med. J. 2017; 29(1):43-52.

36. Durandt C, Potgieter JC, Mellet J, Herd C, Khoosal R, Nel JG*, et al.* HIV and haematopoiesis. S. Afr. Med. J. 2019; 109(8b):40-5.

37. Lysenko V, McHugh D, Behrmann L, Rochat MA, Wilk CM, Kovtonyuk L*, et al.* Humanised mouse models for haematopoiesis and infectious diseases. Swiss Med. Wkly. 2017; 147(w14516):1-16.

38. Adesina OA, Fasola F, Adekanbi O, Ogunbosi B, Akinyemi J, Kuti MA*, et al.* Burden of Cytopaenias among Hiv Positive Pregnant Women at the University College Hospital, Ibadan. Ann Ib Postgrad Med. 2018; 16(2):99-108.

Chapter 2: Literature review

2.1. Introduction to the immune system

The immune system is defined as "an organisation of cells and molecules with specialised roles in defending against infection''[1]. The host defence systems can be divided into: (i) anatomical and physiological barriers; (ii) innate immunity; and (iii) adaptive immunity[2]. The innate and adaptive systems make up the immune system, which work in-tandem to prevent and eliminate infections. The anatomical and physiological barriers, which are the first line of defence, are only one component of innate immunity[3]. These 'anatomical barriers' include intact skin, mucosal membranes, and cilia, whilst low pH values (stomach), lysozymes (mucous secretions, tears, and saliva), complement proteins (tissues and blood), interferons and antimicrobial peptides are considered 'physiological barriers'[2, 4]. An in-depth understanding of the immune system is essential to move forward in the fight against human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS).

2.1.1. Communication within the immune system

Cytokines are important mediators of the intercellular communication within the immune system[5]. This can be observed through their continuous secretion by various immune cells during immune activity. Some cytokines have been named based on their cell of origin, such as lymphokines produced by lymphocytes and monokines produced by macrophages and/or monocytes. However, this naming convention does not apply to all cytokines. Therefore, broader terms such as interleukins (IL) and interferons (IFNs) have been introduced to encompass the diverse range of cytokines produced[6]. Cytokines can be classified into two groups, type 1, and type 2 (Table 2.1), which mediate immunoglobulin production, isotype specific responses and stimulation of B and T cells[6]. Type 1 cytokines stimulate immune activity and are often referred to as pro-inflammatory cytokines. In contrast, type 2 cytokines, which stimulate B-cell maturation and anti-parasitic activity, are known as anti-inflammatory cytokines[6, 7]. A singular cytokine can be produced by several immune cell populations and may produce different effects depending on the cell it is directed to. An example of this is IL-6 and transforming growth factor (TGF)-β which may act as pro-inflammatory or anti-inflammatory depending on the immune response generated[8, 9]. Table 2.2 highlights the major immune cell populations and the cytokines which they secrete.

Table 2.1: Examples of type 1 and 2 cytokines[8, 10-13].

Type	Activity	Cytokines
1	Pro-inflammatory	IL-1α/β, TNF-α/β, IL-2, -3, -6, -7, -8,-15, -17, -18, IFN-γ, GM-CSF, TSLP, TGF-β, leptin and SCF
2	Anti-inflammatory	IFN- α/β, IL-1RA, -4, -9, -10, -11, -13, -37, EPO and TGF-β

EPO, Erythropoietin; GM-CSF, Granulocyte–macrophage colony-stimulating factor; IFN, Interferon; IL, Interleukin; SCF, Stem cell factor; TGF, Transforming growth factor; TSLP, Thymic stromal lymphopoietin; TNF, Tumour necrosis factor.

Table 2.2: Cytokines produced by the various major immune populations[14-21].

Immune cells	Cytokines produced
Neutrophils	IL-1α, -1β, -4, -6, -10, -16, -18, IFN-γ, TNF, and MIF
Monocytes/macrophages	IL-1β, -6, -10, -12, -15, -18, -23, -27, -35, IFN-α, IFN-β, and TNF
T cells	IL-2, -3, -4, -5, -6, -8, -10, -13, -17a, -17f, -21, -22, -31, -33, -35, IFN-γ, and TNF-α
B cells	IL-1, -1β, -4, -6, -10, -12, -35, IFN-α, GM-CSF, TNF-α, -β, and TGF-β
NK cells	IL-5, -8, -10, -13, -15, -22, GM-CSF, IFN-γ, and TNF-α

IFN, Interferon; IL, Interleukin; GM-CSF, Granulocyte–macrophage colony-stimulating factor; MIF, Macrophage migration inhibitory factor; NK, Natural killer TGF; Transforming growth factor; TNF, Tumour necrosis factor.

It is important to note that immune cells that have left their region of maturation but have not encountered an activating agent such as antigens, are described as naïve. Naïve B cells are limited in their cytokine secretion post-activation whilst the opposite is true for naïve T cells[22]. B cell cytokine secretion requires additional activation signals produced by the immune microenvironment and is specific to the stage of B cell differentiation[22]. Interestingly, certain pro-inflammatory cytokines such as IL-1α/β, -6 and tumour necrosis factor (TNF)-α occur *de novo* after bacterial and/or viral infections[6]. These cytokines contribute to phagocyte, monocyte, macrophage, and neutrophil interaction with pathogens and play a role in recruiting other cells to the site of infection[6].

2.1.2. Introduction to the innate immune system

The innate immune system is the first line of defence against foreign pathogens and is fast acting to prevent any potential infection/disease from occuring[23]. If necessary, the innate immune system will stimulate an adaptive immune response to enhance protection against infection and tissue repair[24]. The innate immune system does this by utilising various receptors that are constitutively expressed to recognise non-self-antigens present on the invading pathogen[25]. The first report on the innate system by C. Janeway (1989) stated that in the presence of microbial pathogens, pattern-recognition receptors (PRRs) can identify and bind to conserved structures on pathogens. These unique structures are referred to as pathogen-associated molecular patterns (PAMPs) which induce inflammation and various immune responses[26]. In addition, PRRs also identify damage-associated molecular patterns (DAMPs) which are molecules released by damaged cells[27]. When invading pathogens enter the body, they are detected by PRRs present on cells such as macrophages and dendritic cells (DCs) which triggers an immune reponse[28]. To ensure a rapid and effective immune response against pathogens, the body employs a variety of PRRs to recognise specific features of a pathogen, rather than relying on a single PRR. This enables a higher degree of specificity in detecting and responding to pathogens[29]. By utilising multiple PRRs, the immune system can recognise a wide variety of pathogens and respond with a range of immune mechanisms, including inflammation, phagocytosis, and the activation of T and B lymphocytes.

Pattern-recognition receptors can be classified into four major sub-families: toll-like receptors (TLRs), nucleotide-binding oligomerisation domain (NODs), leucine-rich repeats (LRRs) or NOD-like receptors (NLRs)[28]. Phagocytic cells and antigen presenting cells (APCs), such as macrophages and DCs, play a significant role in the immune response to foreign antigens. This response is largely non-specific, meaning it is not targeted to a specific pathogen, but rather involves the recognition and elimination of a broad range of foreign substances[30]. In addition to PAMP and DAMP recognition, cells release various cytokines and chemokines to eliminate microbes or infected cells[3]. The cytokines released (Table 2.1) are responsible for inducing inflammation after the stimulation of PRRs. They proceed to stimulate the differentiation of helper T cells into various effector cells or regulatory T cells (Tregs), which further modulate the immune response[24, 31]. This helps to eliminate the invading pathogen and prevent tissue damage.

Initially, it was believed that the innate immune system lacks or has very limited immunological memory, resulting in reactivation of the innate system when previously encountered antigens re-emerge[32]. However, over time the innate system was observed to be capable of acquiring immunological memory, a phenomenon described as 'trained immunity'[33]. This was first reported in 1964, where a single immune response directed against one bacterial pathogen provided cross-protection for other unrelated bacterial pathogens[34]. Trained immunity refers to an enhanced innate immune response, through increased production of IFN-γ, IL-1β, IL-6 and TNF by activated macrophages, and secondary expansion of natural killer (NK) cells that precedes their degranulation resulting in long-term epigenetic reprogramming of myeloid progenitors in the bone marrow[35].

Due to the role of the innate immune system in monitoring tissue homeostasis by continuously surveying the body for signs of damage or infection, innate cytokines are currently being considered as a potential target for therapeutic intervention[24]. Damaged/dying cells often undergo different types of cell death which include apoptosis (controlled cell suicide)[36], necrosis (uncontrolled cell death that occurs as a consequence of infection/injury)[37] and autophagy (a self-degradation process that aims to eliminate damaged cells to conserve energy and nutrients)[38]. The specific response of the innate immune system to each of these pathways is determined by the type of cell death that occurred[24]. For example, macrophages are responsible for the removal of post-apoptotic cells, whereas necrotic cells produce DAMPs, that trigger inflammatory cytokines and initiate inflammation at the site of injury[24]. This further activates macrophages and myeloid derived suppressor cells (MDSCs) to help counteract inflammation and promote tissue repair[24]. Interleukin-10 and transforming growth factor (TGF) are examples of cytokines that are released by macrophages to maintain a homeostatic environment and suppress effector mechanisms in injured tissues[31]. Although the innate immune system has various mechanisms for responding to a pathogen, all of these mechanisms aim to achieve three main goals: 1) prevent infection, 2) destroy foreign pathogens, and 3) stimulate an acquired immune response[3].

2.1.3. Introduction to the adaptive immune system

The adaptive immune system is stimulated by the innate system. The term adaptive is often used interchangeably with acquired as the reaction is delayed. The delay is as a result of the highly specific antigen responses that the adaptive immune system generates, facilitated by

the tightly regulated interactions between APCs, and B and T lymphocytes, which are potent and destructive[39, 40]. The specificity ensures that antigen recognition occurs strictly with only harmful foreign pathogens and their toxic products acted upon[41]. Together with the characteristic 'slow acting/delayed' response, the adaptive immune system also retains immunological memory[23]. In essence, following pathogen elimination the adaptive immune system generates long-lived, specific memory cells that allow for a rapid and more potent response if there is a re-encounter with the antigen[32].

The adaptive immune activity begins in the primary lymphoid organs, which include the thymus and the bone marrow (BM). T lymphocytes originate in the BM but mature in the thymus, while antibody-producing B lymphocytes are produced and mature in the BM[40]. Immature B and T lymphocytes undergo significant gene rearrangements to ensure the expression of a vast variety of antigen receptors which enables these cells to recognise a wide range of potential antigens[42]. The immune cells migrate to secondary lymphoid organs such as the lymph nodes and spleen, where circulating antigens are present[40].

The highly specialised process of antigen specificity is driven by different variable (V) regions present on the immune components, such as (i) immunoglobulins, (ii) human leukocyte antigen (HLA) class I and II molecules, and (iii) T cell receptor chains (alpha, beta, gamma and delta)[5]. During T cell activation, interactions between the HLA peptide and T cell receptors induce an immune response, which include clonal expansion of T cells[43]. Additionally, cluster of differentiation (CD)4^+ T cells secrete cytokines that influence the actions of effector cells, such as $CD8^+$ T cells and B cells[43]. In the presence of a viral infection, $CD8^+$ T cells eliminate infected cells through their interaction with HLA class I via the T cell receptor[43]. In addition, activated B cells undergo maturation into plasma cells and start producing antibodies in response to stimulation by their corresponding antigens[43]. More so, these $CD4^+$ T cells are also capable of differentiating into other subsets, outside of the well-established classical T helper 1 and 2 populations[44]. These less studied subsets of immune cells include natural killer T cells (NKT), T helper 17 (Th17), follicular helper T cell (Tfh), induced Tregs (iTreg), and the regulatory type 1 cells (Tr1), as well as the potentially distinct T helper 9 (Th9) cells, all of which release distinctive cytokines[44].

However, the immune system is derived from a broader system - the haematopoietic system in which haematopoietic stem cells (HSCs) differentiate to give rise to all the blood cell

components of the body (haematopoiesis), including the immune cells[45]. Furthermore, the various cells of the immune system often function in response to a signal produced by the haematopoietic system and *vice versa*. The two systems tend to work together to maintain a cellular balance within the body and an imbalance of one system can impact the other system.

2.2. Haematopoiesis

In 1961, the experimental work of Till and McColloch demonstrated the existence of a cell in the haematopoietic system that is capable of self-renewal and differentiation into various cell types[46]. This was observed in a group of irradiated mice that underwent a BM transplantation. This resulted in visible lumps on the spleens that were discovered to be colonies that originated from a single cell[46]. This ground-breaking research formed the basis of our understanding of HSCs and subsequently haematopoiesis.

Haematopoiesis refers to the process of blood cell formation and is mediated by HSCs (Figure 2.1)[47]. Prior to birth, the term primitive haematopoiesis is used to describe haematopoiesis, whilst after-birth it is referred to as definitive haematopoiesis[48]. The process of haematopoiesis is initiated during embryonic development and occurs throughout adulthood as a constant source of cell production and replenishment of the blood system[47]. During early embryonic development, embryonic stem cells are present (inner cell mass of blastocyst), which are defined as pluripotent and are capable of differentiating into all of the body's cell lineages[49]. Primitive haematopoiesis occurs during the initial stages of HSC generation, which is limited to the yolk sac and occurs only once in life. As development progresses, early HSCs known as haematopoietic progenitor cells (HPCs) are produced in the foetal liver and eventually migrate to the BM for residence[50]. Haematopoiesis occurs exclusively in the BM after birth[51, 52]. Within the BM, the HSCs are localised to both the endosteal and vascular niches in the endosteum and sinusoidal vessels, respectively[51].

Stem cells are defined as a group of cells that are capable of self-renewal as well as differentiating into various specialised adult cells as required by the body[53]. The three main types of stem cells are embryonic stem cells, adult stem cells, and induced pluripotent stem cells[54]. Adult stem cells are multipotent, meaning they are capable of differentiating into various cell types such as osteoblasts (bone), adipocytes (adipose tissue), chondrocytes (cartilage) [55]. Haematopoietic stem cells and mesenchymal stem cells are the two main types

of adult stem cells[55] [56]. Haematopoietic stem cells have been studied extensively, making them the most well characterised stem cells. Haematopoietic stem cells differentiate into progenitor cells (HPCs) which differentiate/mature into blood cell precursors (Figure 2.1). The terms HSC and HPC are often used interchangeably due to the lack of standard criteria to distinguish between these cells. Moving forward, HSCs and HPCs will collectively be referred to as haematopoietic stem and progenitor cells (HSPCs).

As haematopoiesis is a constitutive process, it is imperative that the HSPCs reside in specialised microenvironments known as 'niches' to facilitate self-renewal and differentiation activities[57]. The niche is not only a residence for HSPCs, but also plays a role in (a) the self-renewal of HSPCs, (b) the expansion of HSPCs for embryo survival or blood cell formation, and (c) the preservation of HSPCs in the BM that ensures homeostasis and their activation during distress[58]. In the BM, two types of HSPCs have been observed: long-term re-populating HSPCs (LTRCs) and short-term repopulating HSPCs (STRCs)[59]. The LTRC-HSPCs have self-renewal capabilities and the ability to differentiate into all blood cells[60]. However, STRC-HSPCs derived from LTRC-HSPCs, have limited self-renewal characteristics and are capable of differentiating into either myeloid or lymphoid lineages during their developmental stage[60]. The LTRC-HSPCs are considered to be the most primitive stem cell. Long-term re-populating -HSPCs occupy the endosteal niche whilst STRC-HSPCs occupy the vascular niche[52]. A significant difference between the two niches is that the former consists of low oxygen levels and high calcium levels whilst the latter has high oxygen levels and low calcium levels[52]. The contrast in calcium levels is crucial for homing and retention of the cells, especially for LTRC-HSPCs that express calcium receptors to match their environment[52, 61]. Moreover, the hypoxic environment allows LTRC-HSPCs to maintain a state of quiescence, ensuring that HSPCs retain their ability to sustain haematopoiesis throughout life, reducing their cell cycle rate to avoid depletion[52, 62]. In contrast, the hyperoxia vascular niche supports the continued proliferation of the STRC-HSPCs, which actively differentiate and replenish haematopoietic cells[63].

Interestingly, HSPCs are also present in peripheral blood in negligible amounts[64, 65]. Under steady-state conditions, a small proportion of HSPCs are released from the BM into the bloodstream, with the capability to return to the BM (homing)[52, 66]. The exact role of circulating HSPCs is not fully known. There is increased evidence suggesting that HSPCs potentially play an important role during primary immune responses to various pathological

conditions[67]. Two hypotheses concerning the function of circulating HSPCs were put forward by Lapidot and Petit (2002). The first is that these circulating cells repopulate the damaged areas in the BM and sustain the thymus population[68]. It has also been reported that the number of circulating HSPCs increase in response to inflammation and tissue/organ damage[69]. The second hypothesis suggests that the circulating cells do not have a specific function, but that their presence is indicative of non-specific leakage from the BM[68].

2.2.1. Differentiation pathways

During HSPC differentiation, HSPCs give rise to multipotent progenitors (MPPs) with decreased self-renewal potential but retained ability to differentiate into multiple haematopoietic cell lineages (Figure 2.1)[70]. Differentiation of MPPs into lineage committed progeny can be divided into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), resulting in an asymmetric division process (Figure 2.1)[71, 72]. Common lymphoid progenitors use a 'direct' pathway to differentiate into B and T lymphocytes, and NK cells (Figure 2.1)[72]. Whereas the differentiation pathway of CMP is 'indirect' and produces a variety of morphologically, phenotypically and functionally distinct cell types that still require further differentiation to reach their end-point (Figure 2.1)[72]. This is observed through the differentiation of (i) granulocyte-macrophage progenitors (GMP) into neutrophils, eosinophils, basophils, and monocytes; and (ii) megakaryocyte-erythrocyte progenitors (MEP) into platelets and erythrocytes[72].

2.2.2. Characterisation of haematopoietic stem and progenitor cells phenotype

Approximately one trillion blood cells are required to replenish the blood to maintain steady-state haematopoiesis which is tightly controlled by HSPCs[73, 74]. As HSPCs are a critical cell population, it is important to be able to identify them. Currently the transmembrane phosphoglycoprotein CD34 is used to identify HSPCs, and its presence is associated with colony forming activity (long-term reconstitution and multilineage differentiation capacity after haematopoietic stem and progenitor cell transplantation)[75, 76]. However, not all HSPCs are CD34 positive ($CD34^+$) and the majority of $CD34^+$ HSPCs are progenitor cells[77]. There are numerous studies indicating the existence of sub-populations of HSPCs that do not express the CD34 marker, such as the study performed by Goodell *et al*. (1997).

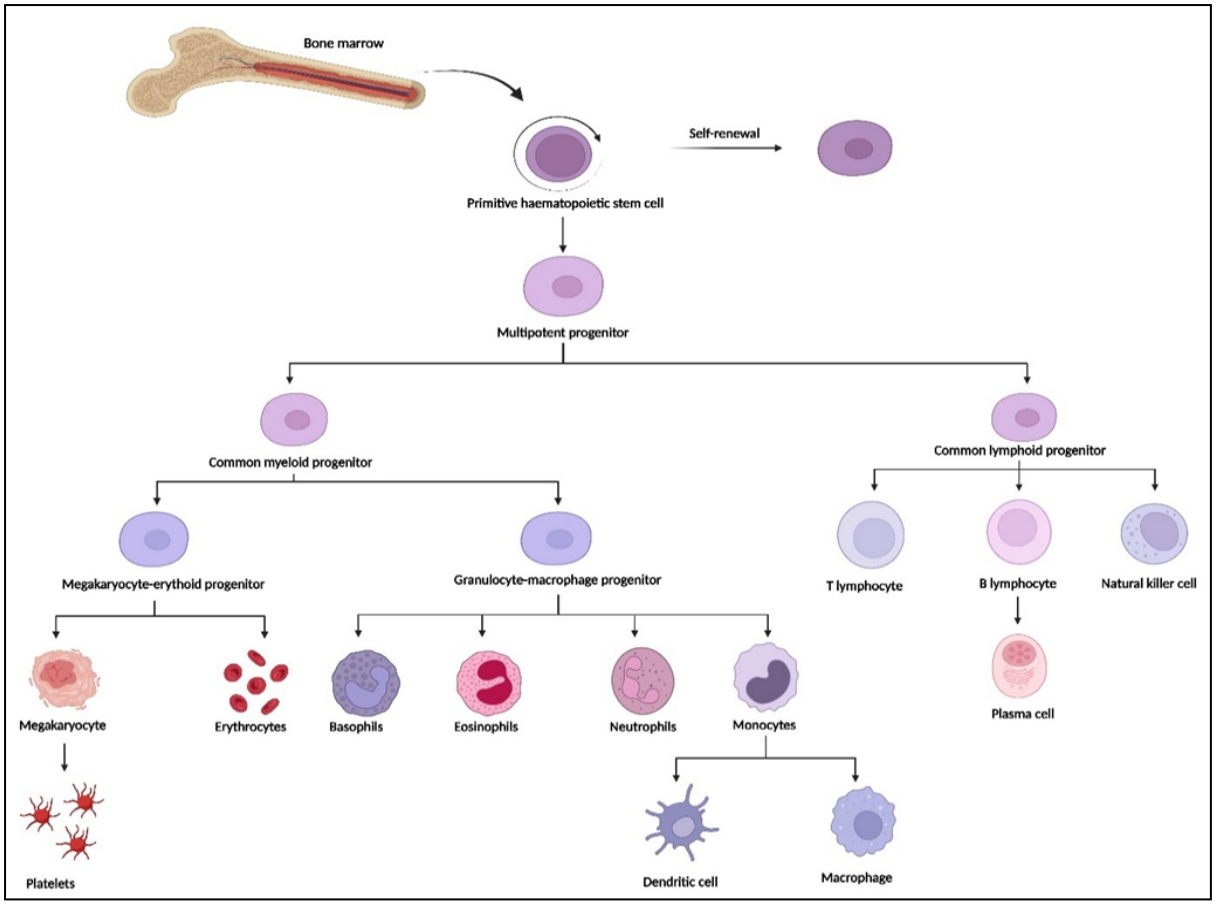


Figure 2.1: Schematic illustration of the differentiation process of haematopoietic stem and progenitor cells into mature blood cell types (haematopoiesis). Image adapted from Konieczny and Arranz (2018)[78]. Image created by Tsungai Mashingaidze using BioRender.

This study identified CD34 negative ($CD34^-$) HSPCs in mice with the ability to efflux the fluorescent deoxyribonucleic acid (DNA)-binding dye Hoechst[79]. This subpopulation, referred to as side population (SP) cells, demonstrated a higher long-term multilineage repopulating potential compared to non-SP cells[79]. The SP cells were found to be capable of differentiating into all blood lineages, indicating their primitive nature. The investigation revealed this to also be true for human cells, with more primitive cells lacking expression of the CD34 marker[79]. This lack of CD34 expression is not only observed in more primitive cells but also in mature blood cells, indicating the completion of the maturation process[80]. Prior to maturation, the majority of $CD34^+$ cells (more than 95%) are committed to a specific lineage[80]. Multipotent progenitors are $CD34^+$ and can differentiate into lymphoid and myeloid lineage cells[80]. The presence of the CD34 marker ranges from CD34 dim positive ($CD34^{+;}$ decreased number of epitopes per cell) to CD34 bright positive ($CD34^{++}$); increased levels of CD34 epitopes per cell.

Studies have shown that $CD34^{++}$ HSPCs mainly consist of immature HSPCs, whilst $CD34^{+}$ HSPCs are mostly comprised of lineage-committed progeny[81, 82].

As Goodell and colleagues (1997) continued to expand on their work, they found that upon culturing $CD34^{-}$ SP cells in BM stroma, a phenotypic conversion from $CD34^{-}$ to $CD34^{+}$ occurred[79]. This finding strongly suggests that $CD34^{-}$ HSPCs are more primitive than their $CD34^{+}$ counterparts. It became necessary to validate the $CD34^{-}$ population by other well-established phenotypic markers. Following this, the absence of lineage markers (Lin^{-}), that are present on all mature haematopoietic cell types, confirmed the $Lin^{-}CD34^{-}$ phenotype of the newly discovered sub-population[79]. The lineage cocktail is a mixture of antibodies that allows for the simultaneous detection of multiple mature cell types in a sample. It includes antibodies against T cells (CD3), B cells (CD19 and CD20), NK cells (CD16 and CD56), monocytes/macrophages (CD14 and CD16), and neutrophils (CD14, CD16 and CD56)[83, 84]. In addition, in a separate study, using a *Tie2/*GFP-reporter mouse model, it was reported that the $Tie2^{+}$ $Lin^{-}CD34^{-}$ HSPCs population resides in the BM of the mouse throughout its entire lifespan[85]. The authors previously observed that *Tie2* is highly expressed by $CD34^{-}$ HSCs and can be used as a molecular marker to trace these cells in mice[85]. The $Lin^{-}CD34^{-}$ population maintained a high level of reconstitution potential, supporting the widely accepted hypothesis that $Lin^{-}CD34^{-}$ HSPCs are at the top of the haematopoiesis hierarchy[85, 86].

The exact phenotype of primitive HSPCs remains under great debate by scientists worldwide and more research is required to make definitive conclusions. However, the $Lin^{-}CD34^{-}$ cell population exhibits a bimodal distribution of CD38, meaning that the $Lin^{-}CD34^{-}$ cell population can be further divided into $Lin^{-}CD34^{-}CD38^{-}$ or $Lin^{-}CD34^{-}CD38^{+}$ cells. In contrast, the $Lin^{-}CD34^{+}$ population is generally $CD38^{+}$ [87]. A study published by Anjos-Afonso and colleagues (2013), observed increased immaturity and quiescence of the $Lin^{-}CD34^{-}CD38^{-}$ population, placing the population at the top of the haematopoietic hierarchy. This was also confirmed by the inability of the $Lin^{-}CD34^{+}CD38^{-}$ HSPCs to produce $Lin^{-}CD34^{-}CD38^{-}$ HSPCs that maintained both differentiation and engraftment potential[88]. Therefore, the $Lin^{-}CD34^{+}CD38^{-}$ HSPCs is highlighted as the more mature, less functional population and the $Lin^{-}CD34^{-}CD38^{-}$ HSPC population as the opposite[88].

As such, the CD38 marker plays a key role in distinguishing between MPPs and committed progenitors, as MPPs are $CD38^{-}$ whilst the committed progenitors are $CD38^{+}$ [52, 89]. Multipotent

progenitors are therefore considered as the starting point in the development of lineage committed progeny (Figure 2.1)[71]. Other notable markers, such as CD90, CD49f and CD45RA are also key in distinguishing between sub-populations. However, only CD90 and CD49f are used for primitive HSPC identification (Table 2.3) in combination with CD34 and CD38[90]. Interestingly, the $Lin^-CD34^-CD38^-$ sub-population has the unique ability to bypass multiple intermediate stages in the differentiation cascade and progress directly towards the terminal phases of cell differentiation. This leads to the production of mature blood cells when cultured in the presence of specific cytokines such as flt-3 ligand (Flt3L), stem cell factor (SCF), thrombopoietin (TPO), IL-3, and hyper-IL-6[91]. This is a departure from the expected differentiation pathway where cells follow a stepwise progression towards a particular lineage.

The observation mentioned above triggered an interest in the colony forming abilities (CFA) of $CD34^-$ and $CD34^+$ HSPCs, which revealed that $CD34^-$ HSPCs have reduced CFA. However, culturing these cells under both short- and long-term culturing conditions induced $CD34^+$ expression which led to an increase in CFA[92]. A research study performed by Nakamura and colleagues confirmed the hypothesis that Lin^-CD34^- cells generate $CD34^+$ cells with CFA and severe combined immunodeficiency (SCID)-repopulating cell (SRC) ability[93]. As such, the presence of CD34 became an indicator of pluripotency in HSPCs, although as differentiation progresses, the expression of CD34 gradually decreases with the maturation of haematopoietic cell lineages[80].

Table 2.3: Haematopoietic stem and progenitor cell sub-populations and their associated cell surface markers[52, 89, 90, 94].

Cell type	Marker
HSPC	$Lin^-CD34^+CD38^-CD90^+CD45RA^-Flt3^+Rho^{-/low}$ $CD10^-CD49f^+CD7^-$
MPP	$Lin^-CD34^+CD38^-CD90^-CD45RA^-Flt3^+CD7^-CD10^-CD49f^-$
CMP	$Lin^-CD34^+CD38^+CD90^-CD45RA^-Flt3^+CD7^-CD10^-CD49f^-$
CLP	$Lin^-CD34^+CD38^+CD90^-CD45RA^-Flt3^+CD7^+CD10^-CD49f^-$

CD, Cluster of differentiation; CLP, Common lymphoid progenitor; CMP, Common myeloid progenitor; Flt3, FMS like tyrosine kinase 3; HSPC, Haematopoietic stem and progenitor cells; Lin, Lineage; MPP; Multipotent progenitor; Rho; Rhodamine 123.

2.2.3. Colony forming abilities of haematopoietic stem and progenitor cells

The ability of HSPCs to differentiate into various haematopoietic lineages, including granulocytes, erythrocytes, monocytes/macrophages, and megakaryocytes (GEMM), is an indication of multipotent colony forming unit (CFU) capacity, with these colonies referred to as CFU-GEMM[95]. The CFU-GEMM is considered to be the cell of origin of all myeloid cells[96]. Under the appropriate growth conditions (i.e. in the presence of IL-3) in semi-solid media CFU-GEMM cells will differentiate/mature into the various mature myeloid cells[97]. The continuation of CFU ability follows a hierarchal pathway that allows differentiation into various colony-forming cells (CFC), namely CFU-granulocyte/macrophage (CFU-GM), burst-forming-unit-erythroid (BFU-E), CFU-erythroid (CFU-E), and CFU-megakaryocyte (CFU-Mk)[95]. These haematopoietic progenies have increased maturity and therefore do not possess any self-renewal potential and are lineage committed[95]. As such, CFU-E divides rapidly to produce single standalone erythroblast colonies, whilst BFU-E is slow-dividing to produce larger erythroblast colonies[98]. The differentiation pattern of BFU-E is as follows: slow-dividing immature BFU-E cells differentiate into intermediate-mature BFU-E cells and then into fast dividing CFU-E cells[99].

Stimulation of the CFU-Mk unipotent precursor of platelets can be achieved in the presence of thrombopoietin (TPO), which gives rise to mature megakaryocytic cells[97, 98]. Colony-stimulating factors (CSF) such as GM-CSF, G-CSF, and macrophage (M)-CSF aid in the stimulation of CFU-GM, leading to the generation of single lineage-committed myeloid progenitors[100]. Interestingly, CFU-GM exhibit a high proliferative index due to the high turnover rate of mature granulocytes, while common lymphoid progenitors (CLPs) have a lower proliferative index. This is hypothesised to be as a result of the continuous proliferation of long-lived B and T cells during maturation[101]. All mature haematopoietic progeny (regardless of lineage) can be identified using cell surface markers, similar to those used to identify HSPCs. The table in Appendix I lists all the surface markers associated with mature haematopoietic cells.

The CFA of $CD34^+$ HSPCs can be negatively impacted by diseases and infection. The presence of HIV has been identified to adversely affect the colony forming potential of HSPCs, leading to compromised self-renewal, and impaired replenishment of blood cell lineages and various immune cells. Therefore, it is imperative to gain a deeper understanding of the impact of HIV

infection on the haematopoietic system, as well as the role of this impact on immune dysregulation.

2.3. Human immunodeficiency virus

Human immunodeficiency virus is a retrovirus that can have detrimental effects on the immune system by primarily targeting $CD4^+$ T cells. When left untreated, HIV develops into AIDS[102]. The World Health Organisation (WHO) and the Centres for Disease Control (CDC) defines AIDS as "the occurrence of one or more of a range of well-defined opportunistic diseases and infections indicative of immunodeficiency as a result of HIV infection and/or a CD4 count less than 200cells/µL"[103, 104]. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), approximately 39 million people worldwide were living with HIV in 2022, with only 29.8 million having access to antiretroviral therapy. Despite various attempts to increase HIV/AIDS awareness, approximately 1.3 million people worldwide became newly infected with HIV in 2022 and 630 000 people died of AIDS-related diseases in the same year[105]. In the combat against HIV, one of the great successes has been the implementation of combination antiretroviral treatment (cART) to prevent HIV replication, which refers to the production of infectious HIV particles and consequently the reduced infection and destruction of $CD4^+$ T cells and progression to AIDS[106]. The existing cART regimens are unable to completely eradicate the presence of HIV in the body, thus creating a need for a better understanding of the virus to allow for more targeted treatments. The current treatment options towards HIV have been observed to be effective but not curative. The reason for the insufficiency of cART to cure HIV is as a result of the HIV latent reservoir.

The term "latent HIV reservoir" refers to HIV-infected cells that contain dormant replication-competent HIV, that has the potential to become active and start replicating when the conditions are favourable[107]. A prime example of 'favourable conditions' is the abrupt discontinuation of cART. If cART is discontinued, the reservoir is activated, resulting in a significant increase in viremia that may lead to the progression of HIV infection if treatment is not resumed promptly[108]. It has been hypothesised that the persistence of the viral reservoir despite suppressive therapy is due to infection of slow-dividing cells prior to the initiation of treatment[109]. Resting memory $CD4^+$ T cells carrying integrated provirus have been identified as a viral reservoir[110]. The long-lived nature of this T cell sub-population which retains long-

term antigenic memory supports their formation as a latent HIV reservoir[111]. The half-life of HIV-infected resting $CD4^+$ T cells analysed in a longitudinal study was reported to be more than 43 months[111].

Cells of the monocyte-macrophage lineage have also been identified as latent HIV reservoirs[112]. Monocytes circulate in the blood for three days after which they migrate to tissues and differentiate into macrophages, which are more susceptible to HIV infection than their precursors[112, 113]. Macrophages act as long-term viral reservoirs that can circulate the virus, creating a stable microenvironment for viral replication and persistence[113]. Cells of the monocyte-macrophage lineage express the necessary receptors, CD4, C-C chemokine receptor type-5 (CCR5), and C-X-C motif chemokine receptor type-4 (CCR4), required for HIV binding and entry[114]. It is hypothesised that circulating monocytes frequently migrate into and out of tissues, such as the spleen and lymph nodes, where HIV infection may take place[115]. Despite only a small portion of monocytes being infected with HIV, they have the ability to sustain viral replication[116]. These macrophages then form and maintain viral reservoirs within the tissues, which contributes to the propagation of the virus [115]. In support of the above-mentioned findings, productive HIV infection has been observed in circulating monocytes of people living with HIV (PLWH) who have been receiving cART for an extensive period of time[117].

While both resting $CD4^+$ T cell and monocyte-macrophage lineage populations are well established latent reservoirs, HSPCs as a reservoir are still a subject of debate. A study by Carter and colleagues (2010) reported *in vivo* and *in vitro* infection of HSPCs, resulting in both productive and latent infection[118]. The HSPCs have an immature phenotype and a long lifespan, thus supporting the proposition of HSPCs as a reservoir[118]. The same research group further expanded their research findings and reported that HIV infection of HSPCs involves the use of CXCR4 as a co-receptor, indicating X4-tropism. Furthermore, the infected HSPCs maintained their differentiation capabilities following engraftment in transgenic mice despite the robust infection by X4-tropic viruses. This supports the idea of viral persistence and the existence of long-term, stably integrated viral reservoirs[119]. Current research on HSPCs as a reservoir supports previous findings. Renelt and colleagues (2022) conducted a comprehensive study that confirmed the robust *in vitro* infection of lineage negative (Lin^-) HSPCs by an X4-tropic strain. This study also reported significant co-expression of CD4 and

CXCR4, which increased the susceptibility of HSPCs to HIV infection[120]. In contrast, Lin^- HSPCs did not exhibit any HIV infection when exposed to R5-virus *in vitro*, possibly due to significantly lower levels of CD4 and CCR5 co-expression observed[120]. Furthermore, the detection of HIV proviral DNA in BM-derived HSPCs from individuals with detectable viremia or who were treatment-naïve at the time of enrolment supports the hypothesis of *in vivo* infection of these cells[120].

2.3.1. Human immunodeficiency virus origin and distribution

A crucial factor in HIV research is to accurately define its variations and respective subtypes. There are two main types of HIV; namely HIV type 1 (HIV-1) and HIV type 2 (HIV-2)[121]. In 1981, the insurgence of deaths from unusual opportunistic infections and rare malignancies in a group of homosexual men in the United States of America resulted in the worldwide recognition of AIDS. The causative agent of AIDS, HIV-1, originated and spread from Kinshasa, Democratic Republic of the Congo[122-124]. Five years later, HIV-2 was identified, which was distantly related to HIV-1, but closely related to simian immunodeficiency virus (SIV) observed in captive macaques[122]. Comparative studies on HIV-1 and -2 have reported that the two variants have similar transmission methods, however HIV-2 is less infectious, with a longer asymptomatic phase and slower progression to AIDS[122, 125]. Treatment strategies for HIV-2-infected persons differ significantly from those for HIV-1-infected persons. The recommendation for HIV-2 is based on $CD4^+$ T cell count and clinical status, whereas cART for HIV-1 is usually initiated upon a positive test result[126]. People living with HIV-2 often present with higher $CD4^+$ T cell counts, and longer survival times compared to people living with HIV-1, with the majority of HIV-2 infected persons being within the normal life expectancy range[126]. However, with HIV-1, the absence of treatment or poor adherence to treatment often results in an increased viral load and chronic immune depletion, which can progress to AIDS. Additionally, there is also a potential for developing multi-drug resistance, especially when not strictly adhering to the treatment regimen, which varies between individuals and ultimately can reduce life expectancy.

Geographically, HIV-1 occurs worldwide whilst HIV-2 is endemic to west Africa and to communities in Europe with socioeconomic links to west Africa, such as France and Portugal[127]. This difference in epidemiology primarily highlights the greater infectivity of HIV-1, which is more readily transmitted and predominantly responsible for the HIV pandemic[121].

Human immunodeficiency virus-1 has increased genetic diversity resulting in four groups, namely main (M), outlier (O), non-M non-O (N) and P[128, 129]. With group P being named according to HIV-1 nomenclature guidelines outlined in 2000 by Robertson and colleagues[130]. Each group originated from cross-species transmission between apes and humans[122]. The increased genetic variability is propelled by the low rate of proofreading activity of the viral reverse transcriptase, the high turnover of virions during replication and the genomic recombination occurring within infected cells[131]. The HIV-1 group M has the highest prevalence and it is the driver of the pandemic[122, 132]. Within this group, there are nine subtypes that include A – D, F, G, H, J and K[133]. Substantial variation exists (25 to 35%) between these subtypes at a genomic level, which allows researchers to distinguish one subtype from another[133, 134]. The HIV-1 subtypes B and C are the most common, with the latter accounting for the majority of infections worldwide[135].

Human immunodeficiency virus-1C is most prevalent in east and southern Africa and India whilst HIV-1B is more prevalent in Northern America, Western Europe, Latin America, and the Caribbean[133, 136]. Human immunodeficiency virus-1C is responsible for approximately 46.6% of global infections whilst subtype 1B is responsible for 12.1%[137]. Human immunodeficiency virus-1C is more transmissible than the other group M subtypes, with a lower virulence which has caused extended asymptomatic periods, thus propelling its dominance and expansion during the epidemic[138]. To date, most HIV-related research studies have used study participants based in Europe and/or North America. This has resulted in HIV-1 treatment regimens with an unintended bias for subtype B infections, which are the minority of global infections. Therefore, there is a need to increase the number of studies on subtype C, which make up most global HIV infections. A more targeted approach for subtype C may potentially curb the risk of multidrug resistance- which supports HIV virulence. A decrease in virulence would likely allow for more potent immune responses and reconstitution, against the virus, to be generated also reducing the rate of immune cell exhaustion. Additionally, dampening the virus' toxic effects on the immune system.

2.3.2. The impact of human immunodeficiency virus on the innate immune system

During infection, the recognition of HIV triggers different signalling pathways to stimulate anti-viral responses, such as induction of pro-inflammatory cytokines and chemokines. Interferons are produced in response to the presence of viruses that triggers a myriad of interferon-

stimulated genes (ISG) to create a toxic environment for the virus whilst priming an adaptive immune response[139]. In addition, the complement system is activated producing complement proteins that act against the virus by opsonisation, lysis, and inflammation, ultimately aiding in viral destruction[140]. Despite all of this, the virus still manages to evade the innate immune system through manipulating PAMPs, suppressing IFN production, antagonising the ISG products, downregulating complement receptors, impairing innate regulators and upregulating inflammatory molecules[141-143].

The innate immune response to infection is vast as DCs are activated to induce a cytokine storm, characterised by a sharp increase in IFN-α, IL-15, and inducible protein-10 (IP-10) levels ensued by a steady incline in various cytokines. Dendritic cells function as APCs by presenting antigen to B and T lymphocytes to further activate the acquired immune system. They further stimulate NK cell activity and proliferation through the release of IFN-α[144, 145]. Natural killer cells are the 'effector lymphocytes' of innate immunity recruited to fight infection[146]. These cells play a key role in the anti-viral innate immune response, as they promote the elimination of infected cells through the secretion of cytotoxic granules such as perforins, granzymes, and cytokines like IFN-γ. Perforins trigger apoptosis and IFN-γ promotes antigen presentation, leading to a more potent immune response[147-149]. Notably, NK cells do not express any antigen-specific receptors. Instead they have a variety of activation and inhibitory receptors and co-receptors that bind to class I HLA and other molecules[150].

The virus attempts to evade the immune system by downregulating HLA class I on the surfaces of infected cells to reduce the extent of NK cell activation[151]. However, NK cells remain an important part of the immune response against HIV as they can identify and kill infected cells, whilst maintaining their regulatory function. When activated, NK cells promote DC maturation and eliminate immature DCs to limit the production of suppressive Tregs, whilst further enhancing T cell polarisation and cross priming to enhance both innate and adaptive responses[145, 152, 153].

2.3.3. The impact of human immunodeficiency virus on the adaptive immune system

The $CD4^+$ T helper lymphocytes are the primary target of HIV, with the effect of HIV on this sub-population in the absence of treatment often fatal, as these helper T cells are key

mediators of cellular immunity[154, 155]. They can activate cells of the innate system, cytotoxic $CD8^+$ T cells, mediate the production of antibodies by B cells and the anti-microbial properties of macrophages, recruit neutrophils to sites of infection and inflammation, secrete appropriate cytokines, and suppress immune reactions[44, 156, 157]. In addition, this T cell subset is imperative for maintaining mucosal immunity and the formation of the long-term cellular and humoral antigen specific immunity. They orchestrate the adaptive immune response and establish life-long protection against future infections. This contributes to the overall host immune response against a vast range of pathogens[158, 159]. Therefore, the HIV-mediated rapid depletion of $CD4^+$ T cell frequency and function during acute infection has a severe impact on the immune system function causing extensive immune dysregulation[155].

Notably, the effects of HIV on the adaptive immune system are not limited to $CD4^+$ T cell populations but extend further to the cytotoxic $CD8^+$ T cells and cells of the monocyte-macrophage lineage[116, 155, 160]. The $CD8^+$ T cells usually target virus-infected cells through CD8 receptor and T-cell receptor (TCR) interactions with HLA class I molecules present on the cell surface of virus-infected cells. This results in the eradication of infected cells through either lytic (secretion of cytotoxic granules and/or induction of apoptosis) or non-lytic (secretion of soluble factors) activity[160, 161]. However, HIV has the ability to downregulate HLA class I expression essential for $CD8^+$ T cell activation, decreasing the cytotoxic activity required to clear infections[160].

Macrophages interact with B and T lymphocytes by expressing HLA class II molecules, which allows for antigen presentation[20, 162]. Consequent T cell activation stimulates a cytokine secretion cascade that in turn stimulates B cell activity. Once activated, B cells produce antibodies that bind to foreign antigens, allowing for an increased rate of phagocytosis by macrophages[20]. When infected with HIV, macrophages lose their ability to effectively present antigens and activate T cell responses against the virus. This can lead to T cell anergy or activation-induced cell death[163], ultimately resulting in viral persistence as the immune system fails to generate the potent antigen-specific T cell response necessary to suppress viral replication[164]. The continued production of viral particles results in continuous/chronic immune activation[158].

Ironically, chronic immune activation has been observed to be more detrimental to the host than beneficial. Chronic immune activation supports the rapid activation and proliferation of

naïve and central memory CD4⁺ T cells, which are key sub-populations in replenishing T cell numbers. This ultimately disrupts T cell homeostasis[155, 165-167]. As a result of this disruption, an overall and significant decrease in the number of peripheral/effector CD4⁺ T cells occurs[164, 167]. Moreover, it coincides with an increase in the number of cytotoxic CD8⁺ T cells, resulting in the reversal of the 'CD4/CD8 ratio'[168]. In healthy individuals, the CD4⁺ and CD8⁺ T cell numbers vary, but generally follow a trend of a higher CD4⁺ T cell count with a lower CD8⁺ T cell count[169]. An inversely proportional CD4/CD8 ratio and an increase in viral load collectively indicates chronic immune activation and immunosenescence[164, 168, 170]. As the CD4⁺ T cell population plays a critical role in driving antibody production, stimulating and maintaining cytolytic responses, and macrophage and NK cell activation, the decrease in CD4⁺ T cell frequency following HIV infection negatively impacts the immune system[169]. However, the use of cART minimises the impact of HIV on the immune system by decreasing the viral load and promoting recovery of the CD4⁺ T cells[171].

2.3.4. The impact of human immunodeficiency virus on haematopoiesis

The identification of primitive HSPCs has allowed for more in-depth and crucial studies to be done. One such example is the work done by Redd and colleagues (2007) that highlighted that both HIV-1B and -1C interact with HSPCs but in diverse ways[172, 173]. Furthermore, they found that the HIV-1C variant, and not -1B, has the ability to infect CD34⁺ HSPCs[172, 173]. Significant decreases in the number of CD34⁺ HSPCs have been observed in HIV-positive patients, indicating a dysregulation of haematopoiesis[174]. Currently there is controversy regarding the infection capability of true primitive HSPCs with HIV, despite reports indicating infection - these results have not been successfully replicated[59, 175]. The impairment of haematopoiesis as a result of HIV infection can also potentially be contributed to by the distinct decrease observed in the number of CD34⁺ HSPCs in patients who have tested positive for HIV[112].

During the differentiation cascade, CMPs normally would give rise to immature myeloid cells (IMCs) that differentiate into functional DCs, macrophages and granulocytes in healthy individuals[176]. However, the chronic inflammation, that occurs as a result of HIV infection prevents a sub-population of the IMCs from maturing and instead prompts them to differentiate into MDSCs, resulting in elevated levels of MDSCs in circulation[176, 177]. Myeloid derived suppressor cells are a heterogeneous population of immature and mature myeloid cells, which are capable of suppressing both innate and adaptive immune responses[177, 178].

Two major subsets of MDSCs have been described, namely monocytic-MDSCs (M-MDSCs) and polymorphonuclear-MDSCs (PMN-MDSCs)[177]. A less studied third subset is called the immature subset of MDSCs (i-MDSCs). Table 2.4 provides the cell surface markers used to identify each MDSC subset.

Table 2.4: Myeloid derived suppressor cell subsets and their associated cell surface markers[176, 179].

Cell type	Marker
M-MDSC	$Lin^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}CD15^{low/-}IL\text{-}4R\alpha^{+}$
PMN-MDSC	$Lin^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{low/-}CD14^{-}CD15^{+}CD66b^{+}$
i-MDSC	$Lin^{-}CD11b^{+}CD33^{+}HLA^{-}DR^{low/-}$

CD, Cluster of differentiation; HLA-DR, Human leukocyte antigen–DR; i, Immature; IL, Interleukin; Lin, Lineage; M, Monocytic; MDSC, Myeloid-derived suppressor cell; PMN, Polymorphonuclear.

Several studies have found no evidence of MDSCs in healthy individuals[180]. Agrati and colleagues (2019) demonstrated that PMN-MDSCs could regulate the commitment of $CD34^{+}$ HSPCs by preventing the differentiation of $CD34^{+}$ HSPCs into myeloid and erythroid lineages. Supporting the hypothesis that MDSCs have a negative effect on haematopoietic progenitors[177, 181]. The occurrence of MDSCs is an example of how HIV dysregulates the HSPC differentiation cascade and further negatively impacts the progeny.

Impaired haematopoiesis can also often lead to the manifestation of haematological abnormalities such as cytopenias. The most commonly observed cytopenias are anaemia, leukopenia, thrombocytopenia, and neutropenia[182]. They are defined as abnormally low red blood cell (RBC), white blood cell (WBC), platelet, and neutrophil counts, respectively[182]. Cytopenias may result from a defect in blood cell production in the BM or from increased peripheral blood cell destruction[183], and are frequently observed in HIV infected individuals and can be fatal if left untreated[182, 184]. The use of cART can correct cytopenia and prevent its recurrence in people living with HIV/AIDS [185].

Notably infection with HIV, during pregnancy applies an additional strain the haematopoietic system. Both HIV infection and pregnancy bring about a significant decrease in the $CD4^{+}$ T cell absolute counts and frequency[186]. However, pregnancy itself does not promote the progression HIV infection and the $CD4^{+}$ T cell counts rebound after birth[186 187]. However,

untreated HIV Infection during pregnancy may result in various undesirable outcomes such as stillbirths, preterm delivery, spontaneous miscarriages, low birth weight, and mother-to-child viral transmission[188, 189]. The haematopoietic system activity escalates during pregnancy as noted by the increase of HSPC trafficking from the BM into circulation[190, 191]. Furthermore, there is a progressive increase in plasma volumes that occurs to adapt to the meet foetal requirements[192]. However, the red blood cell mass does not match the plasma volume expansion, thereby resulting in a decrease haemoglobin concentrations, haematocrit, red blood cell counts as well as platelet counts[193]. Thereby promoting the likelihood of the occurrence of peripheral blood cytopenias such as physiological anaemia and gestational thrombocytopenia. In addition, $CD4^+$ T cell counts <350 cells/µL are associated with lower white blood cell counts thereby supporting the occurrence of neutropenia[194]

2.3.4.1. Anaemia

Anaemia significantly affects an individual's quality of life due to symptoms such as increased heart rate, fatigue, and shortness of breath[195]. It is the most frequently occurring haematological abnormality in individuals with HIV worldwide, and has been linked to HIV disease progression[196, 197]. Anaemia in HIV infection has been associated with a decline in RBC production, an increase in RBC destruction, and insufficient RBC production caused by nutrient deficiencies[198]. The causes of HIV-associated anaemia are not fully understood, however, factors such as BM infiltration, myelosuppressive effects of antiretroviral medication like azidothymidine (AZT), and a decrease in erythropoietin progenitor cells have been associated with its occurrence[199]. Azidothymidine is a common antiretroviral drug, that has been linked to the development of anaemia and the disruption of the BM niche[200]. In addition, HIV infection can directly cause anaemia by inhibiting the function of erythropoietin progenitor cells in the BM[196].

2.3.4.2. Thrombocytopenia

Thrombocytopenia is a common cytopenia in HIV-positive individuals, which occurs at any stage of infection[201]. Thrombocytopenia affects approximately 3-40% of HIV-positive individuals[202]. Human immunodeficiency virus-related thrombocytopenia is hypothesised to be multifactorial, including increased platelet destruction, impaired platelet production, and splenic platelet sequestration[203]. The impaired production of platelets may be as a result of

HIV infection of megakaryocytes that express the CD4 receptor[204]. Depending on the disease stage, the mechanisms responsible for HIV-related platelet reduction differs[201, 205]. In the early stages of HIV, when the viral load is low, an immune thrombocytopenic purpura (ITP)-like mechanism prevails, which is characterised by the presence of immune complexes, anti-platelet antibodies and increased platelet destruction[201]. However, in the advanced stages, with a high viral load and low $CD4^+$ T cell count, decreased platelet production becomes the predominant mechanism[205-207]. Human immunodeficiency virus-associated thrombocytopenia and ITP have similar clinical presentations and individuals respond to treatment in a similar manner[208]. Notably, thrombocytopenia has been associated with decreased survival rates and the development of HIV-associated conditions, such as HIV-associated dementia[209].

2.3.4.3. Neutropenia

Notably, neutrophils are a major sub-population of WBCs, comprising of 50-70% of WBCs[210, 211]. Therefore, neutropenia is a common feature of advanced HIV infection and the onset of AIDS[212]. Severe neutropenia occurs in tandem with the collapse of the adaptive immune system, which significantly increases the risk of secondary infections[213]. However, there have been instances where neutropenia has been observed in the early stages of HIV infection as well[213]. It is hypothesised that the decreased production of granulocyte colony-stimulating factor (G-CSF) and auto-immunity may contribute to the occurrence[197]. Neutropenia is commonly associated with drugs, such as AZT and the anti-viral ganciclovir, as well as certain cancer treatments[214]. As a result, individuals with neutropenia may also be susceptible to uncommon infections, such as spontaneous bacterial infections and rare fungal infections, such as aspergillosis or mucormycosis[214].

2.4. Conclusion

A broader investigation of the cellular interactions of HIV to include all blood cells and not only immune cells, may lead to a better understanding of interactions of HIV with other cells in the body. A better understanding of HIV/HSPC interactions may also assist researchers in developing a better understanding if and to what extent HSPCs play a role in latent HIV reservoirs. This may also lead to the development of new, more effective treatment options. Two goals must be achieved to cure HIV-1; (i) eradicate all viral reservoirs, (ii) develop novel

cell/gene therapy approaches[215]. To ensure that next-generation cell therapy-based treatment options for HIV are effective and safe, it is important to understand the interactions between HIV and HSPCs. The success of next-generation treatment options, such as gene therapy approaches to make blood cells resistant to HIV infection and chimeric antigen receptor (CAR) T cell immunotherapy directed against HIV-1-infected cells, depends heavily on fully functional HSPCs (gene therapy) and T cells pre- and post-modification. As an alternative to generating a cure, developing a preventative vaccine would significantly alleviate the HIV infection burden worldwide.

2.5 References

1. Delves PJ, Roitt IM. The immune system. First of two parts. N. Engl. J. Med. 2000; 343(1):37-49.

2. Turvey SE, Broide DH. Innate immunity. J. Allergy Clin. Immunol. 2010; 125(2 Suppl 2):S24-32.

3. Aristizábal B, González Á. Innate immune system [Internet]. Bogota, Colombia:El Rosario University Press; 2013 [cited: 03/02/2023]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459455/

4. Kindt TJ, Goldsby RA, Osborne BA, Kuby J. Kuby Immunology [Internet]. New York: W.H. Freeman; 2007 [cited: 2023/03/06]. Available from: https://muhammad1988adeel.files.wordpress.com/2011/04/kuby-immunology-6th-edition.pdf

5. Bellanti JA, Kadlec JV, Escobar-Gutierrez A. Cytokines and the immune response. Pediatr. Clin. North Am. 1994; 41(4):597-621.

6. Lunney JK. Cytokines orchestrating the immune response. Rev. Sci. Tech. 1998; 17(1):84-94.

7. Crews FT, Bechara R, Brown LA, Guidot DM, Mandrekar P, Oak S*, et al.* Cytokines and alcohol. Alcohol. Clin. Exp. Res. 2006; 30(4):720-30.

8. Zhang J-M, An J. Cytokines, inflammation, and pain. Int. Anesthesiol. Clin. 2007; 45(2):27-37.

9. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: From Clinical Significance to Quantification. Advanced Science. 2021; 8(15):2004433.

10. Chung KF. Cytokines. In: Barnes PJ, Drazen JM, Rennard SI, Thomson NC, editors. Asthma and COPD. 2 ed. Oxford: Academic Press; 2009. p. 327-41.

11. Levine SJ. Cytokines. In: Lennarz WJ, Lane MD, editors. Encyclopedia of Biological Chemistry. Waltham: Academic Press; 2013. p. 613-21.

12. Dembic Z. Chapter 6 - Cytokines of the Immune System: Interleukins. In: Dembic Z, editor. The Cytokines of the Immune System. Amsterdam: Academic Press; 2015. p. 143-239.

13. Silva I, Alípio C, Pinto R, Mateus V. Potential anti-inflammatory effect of erythropoietin in non-clinical studies in vivo: A systematic review. Biomed. Pharmacother. 2021; 139:111558.

14. Li Q, Wang B, Mu K, Zhang JA. The pathogenesis of thyroid autoimmune diseases: new T lymphocytes–cytokines circuits beyond the Th1− Th2 paradigm. J. Cell. Physiol. 2019; 234(3):2204-16.

15. de Gruijter NM, Jebson B, Rosser EC. Cytokine production by human B cells: role in health and autoimmune disease. Clin. Exp. Immunol. 2022; 210(3):253-62.

16. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural killer cells: development, maturation, and clinical utilization. Front. Immunol. 2018; 9(1869):1-23.

17. Gideon HP, Phuah J, Junecko BA, Mattila JT. Neutrophils express pro- and anti-inflammatory cytokines in granulomas from Mycobacterium tuberculosis-infected cynomolgus macaques. Mucosal Immunol. 2019; 12(6):1370-81.

18. Chan L, Karimi N, Morovati S, Alizadeh K, Kakish JE, Vanderkamp S*, et al.* The Roles of Neutrophils in Cytokine Storms. Viruses. 2021; 13(11):2318.

19. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood. 2011; 118(1):9-18.

20. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front. Immunol. 2014; 5(491):1-14.

21. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010; 115(11):2167-76.

22. Vazquez MI, Catalan-Dibene J, Zlotnik A. B cells responses and cytokine production are regulated by their immune microenvironment. Cytokine. 2015; 74(2):318-26.

23. McComb S, Thiriot A, Akache B, Krishnan L, Stark F. Introduction to the Immune System. In: Fulton KM, Twine SM, editors. Immunoproteomics: Methods and Protocols. New York, NY: Springer New York; 2019. p. 1-24.

24. Charles JF, Nakamura MC. Bone and the innate immune system. Curr Osteoporos Rep. 2014; 12(1):1-8.

25. Gonzalez S, Gonzalez-Rodriguez AP, Suarez-Alvarez B, Lopez-Soto A, Huergo-Zapico L, Lopez-Larrea C. Conceptual aspects of self and nonself discrimination. Self Nonself. 2011; 2(1):19-25.

26. Janeway CA. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. 1989; 54(1):1-13.

27. Amarante-Mendes GP, Adjemian S, Branco LM, Zanetti LC, Weinlich R, Bortoluci KR. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. Front. Immunol. 2018; 9(2379):2379.

28. Bortoluci KR, Medzhitov R. Control of infection by pyroptosis and autophagy: role of TLR and NLR. Cell. Mol. Life Sci. 2010; 67(10):1643-51.

29. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin. Microbiol. Rev. 2009; 22(2):240-73.

30. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 2004; 5(10):987-95.

31. Striz I, Brabcova E, Kolesar L, Sekerkova A. Cytokine networking of innate immunity cells: a potential target of therapy. Clin. Sci. (Lond.). 2014; 126(9):593-612.

32. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. Allergy Asthma Clin. Immunol. 2018; 14(Suppl 2):49.

33. Ochando J, Mulder WJM, Madsen JC, Netea MG, Duivenvoorden R. Trained immunity - basic concepts and contributions to immunopathology. Nat Rev Nephrol. 2023; 19(1):23-37.

34. Mackaness GB. The Immunological Basis of Acquired Cellular Resistance. J. Exp. Med. 1964; 120(1):105-20.

35. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. Cell Host Microbe. 2011; 9(5):355-61.

36. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer. 1972; 26(4):239-57.

37. Boulton ME, Mitter SK, Rao HV, Dunn WA. Cell Death, Apoptosis, and Autophagy in Retinal Injury. In: Ryan SJ, Sadda SR, Hinton DR, Schachat AP, Sadda SR, Wilkinson CP*, et al.*, editors. Retina. London: W.B. Saunders; 2013. p. 537-52.

38. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J. Pathol. 2010; 221(1):3-12.

39. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science. 2010; 327(5963):291-5.

40. Bonilla FA, Oettgen HC. Adaptive immunity. J. Allergy Clin. Immunol. 2010; 125(2 Suppl 2):S33-40.

41. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Innate immunity. Molecular Biology of the Cell. 4th edition: Garland Science; 2002.

42. Mirzaei HR. Adaptive Immunity [Internet]. In: Rezaei N, editor. Encyclopedia of Infection and Immunity: Elsevier; 2022. p. 39-55. [cited: 2020/05/20]. Available from: https://www.sciencedirect.com/science/article/pii/B9780128187319000288

43. Bhoj VG, Chen ZJ. Ubiquitylation in innate and adaptive immunity. Nature. 2009; 458(7237):430-7.

44. Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^+$T cells: differentiation and functions. Clin. Dev. Immunol. 2012; 2012(925135):1-12.

45. Charles A, Janeway J, Travers P, Walport M, Shlomchik M. Immunobiology: the immune system in health and disease [Internet]. New York:Garland Publishing Inc; 2001 [cited: 2023/03/10]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK10757/

46. Till JE, Mc CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 1961; 14(2):213-22.

47. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. Development. 2013; 140(12):2463-7.

48. Umeda K, Heike T, Yoshimoto M, Shiota M, Suemori H, Luo HY, *et al.* Development of primitive and definitive hematopoiesis from nonhuman primate embryonic stem cells *in vitro*. Development. 2004; 131(8):1869-79.

49. Biehl JK, Russell B. Introduction to stem cell therapy. J. Cardiovasc. Nurs. 2009; 24(2):98-103.

50. Golub R, Cumano A. Embryonic hematopoiesis. Blood Cells Mol. Dis. 2013; 51(4):226-31.

51. Tamma R, Ribatti D. Bone Niches, Hematopoietic Stem Cells, and Vessel Formation. Int. J. Mol. Sci. 2017; 18(1):151.

52. Mosaad YM. Hematopoietic stem cells: an overview. Transfus. Apher. Sci. 2014; 51(3):68-82.

53. Kolios G, Moodley Y. Introduction to stem cells and regenerative medicine. Respiration. 2013; 85(1):3-10.

54. Leuning DG, Beijer NR, Du Fossé NA, Vermeulen S, Lievers E, Van Kooten C, *et al.* The cytokine secretion profile of mesenchymal stromal cells is determined by surface structure of the microenvironment. Sci. Rep. 2018; 8(1):1-9.

55. AbuSamra DB, Aleisa FA, Al-Amoodi AS, Jalal Ahmed HM, Chin CJ, Abuelela AF*, et al.* Not just a marker: CD34 on human hematopoietic stem/progenitor cells dominates vascular selectin binding along with CD44. Blood advances. 2017; 1(27):2799-816.

56. Ng AP, Alexander WS. Haematopoietic stem cells: past, present and future. Cell Death Discov. 2017; 3(1):17002.

57. Wang LD, Wagers AJ. Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat. Rev. Mol. Cell Biol. 2011; 12(10):643-55.

58. Al-Drees MA, Yeo JH, Boumelhem BB, Antas VI, Brigden KW, Colonne CK*, et al.* Making Blood: The Haematopoietic Niche throughout Ontogeny. Stem Cells Int. 2015; 2015(571893):1-14.

59. Zhang J, Scadden DT, Crumpacker CS. Primitive hematopoietic cells resist HIV-1 infection via $p21^{Waf1/Cip1/Sdi1}$. J. Clin. Invest. 2007; 117(2):473-81.

60. Bellantuono I. Haemopoietic stem cells. Int. J. Biochem. Cell Biol. 2004; 36(4):607-20.

61. Lam BS, Cunningham C, Adams GB. Pharmacologic modulation of the calcium-sensing receptor enhances hematopoietic stem cell lodgment in the adult bone marrow. Blood. 2011; 117(4):1167-75.

62. Arai F, Suda T. Quiescent stem cells in the niche [Internet]. 2010 [cited: 2022/11/04]. Available from: https://europepmc.org/article/NBK/nbk27036

63. Yin T, Li L. The stem cell niches in bone. J. Clin. Invest. 2006; 116(5):1195-201.

64. Anversa P, Kajstura J, Leri A. Circulating progenitor cells: search for an identity. Circulation. 2004; 110(20):3158-60.

65. Bonig H, Papayannopoulou T. Mobilization of hematopoietic stem/progenitor cells: general principles and molecular mechanisms [Internet]. In: Kolonin M, Simmons P, editors. Stem Cell Mobilization. Methods in Molecular Biology. Totowa, New Jersey: Humana Press; 2012. p. 1-14. [Available from: https://www.ncbi.nlm.nih.gov/pubmed/22890918

66. Alvarez P, Carrillo E, Velez C, Hita-Contreras F, Martinez-Amat A, Rodriguez-Serrano F*, et al.* Regulatory systems in bone marrow for hematopoietic stem/progenitor cells mobilization and homing. Biomed Res Int. 2013; 2013(312656):1-12.

67. Wu WC, Sun HW, Chen HT, Liang J, Yu XJ, Wu C*, et al.* Circulating hematopoietic stem and progenitor cells are myeloid-biased in cancer patients. Proc. Natl. Acad. Sci. U. S. A. 2014; 111(11):4221-6.

68. Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp. Hematol. 2002; 30(9):973-81.

69. Ratajczak MZ, Adamiak M, Plonka M, Abdel-Latif A, Ratajczak J. Mobilization of hematopoietic stem cells as a result of innate immunity-mediated sterile inflammation in the bone marrow microenvironment—the involvement of extracellular nucleotides and purinergic signaling. Leukemia. 2018; 32(5):1116-23.

70. Chotinantakul K, Leeanansaksiri W. Hematopoietic stem cell development, niches, and signaling pathways. Bone Marrow Res. 2012; 2012(270425):1-16.

71. Lai AY, Kondo M. Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. J. Exp. Med. 2006; 203(8):1867-73.

72. Iwasaki H, Akashi K. Hematopoietic developmental pathways: on cellular basis. Oncogene. 2007; 26(47):6687-96.

73. Belyavsky A, Petinati N, Drize N. Hematopoiesis during Ontogenesis, Adult Life, and Aging. Int. J. Mol. Sci. 2021; 22(17):9231.

74. Robb L. Cytokine receptors and hematopoietic differentiation. Oncogene. 2007; 26(47):6715-23.

75. Pei X. Who is hematopoietic stem cell: $CD34^+$ or $CD34^-$? Int. J. Hematol. 1999; 70(4):213-5.

76. Rodriguez YBA, Manso BA, Forsberg EC. CFU-S assay: a historical single-cell assay that offers modern insight into clonal hematopoiesis. Exp. Hematol. 2021; 104:1-8.

77. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007; 1(6):635-45.

78. Konieczny J, Arranz L. Updates on Old and Weary Haematopoiesis. Int. J. Mol. Sci. 2018; 19(9):2567.

79. Goodell MA, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med. 1997; 3(12):1337.

80. Carvalho JM, Souza MK, Buccheri V, Rubens CV, Kerbauy J, Oliveira JS. CD34-positive cells and their subpopulations characterized by flow cytometry analyses on the bone marrow of healthy allogenic donors. Sao Paulo Med. J. 2009; 127(1):12-8.

81. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood. 1996; 87(1):1-13.

82. F Morel SS, M Travis, B Chen and A Galy. . Primitive hematopoietic cells in murine bone marrow express the CD34 antigen. Blood. 1996; 88(10):3774-84.

83. Feldman S, Kasjanski R, Poposki J, Hernandez D, Chen JN, Norton JE*, et al.* Chronic airway inflammation provides a unique environment for B cell activation and antibody production. Clin. Exp. Allergy. 2017; 47(4):457-66.

84. BioLegend [Internet]. FITC anti-human Lineage Cocktail BioLegend; [updated 2021/07/09; cited 2022/05/06]. Available from: https://www.biolegend.com/de-de/products/fitc-anti-human-lineage-cocktail-cd3-cd14-cd16-cd19-cd20-cd56-6689?GroupID=GROUP28

85. Ito K, Turcotte R, Cui J, Zimmerman SE, Pinho S, Mizoguchi T*, et al.* Self-renewal of a purified $Tie2^+$ hematopoietic stem cell population relies on mitochondrial clearance. Science. 2016; 354(6316):1156-60.

86. Hofer T, Rodewald HR. Differentiation-based model of hematopoietic stem cell functions and lineage pathways. Blood. 2018; 132(11):1106-13.

87. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat. Med. 1998; 4(9):1038-45.

88. Anjos-Afonso F, Currie E, Palmer HG, Foster KE, Taussig DC, Bonnet D. $CD34^-$ cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell Stem Cell. 2013; 13(2):161-74.

89. Cimato TR, Furlage RL, Conway A, Wallace PK. Simultaneous measurement of human hematopoietic stem and progenitor cells in blood using multicolor flow cytometry. Cytometry B Clin. Cytom. 2016; 90(5):415-23.

90. Lysenko V, McHugh D, Behrmann L, Rochat MA, Wilk CM, Kovtonyuk L*, et al.* Humanised mouse models for haematopoiesis and infectious diseases. Swiss Med. Wkly. 2017; 147(w14516):1-16.

91. Fujisaki T, Berger MG, Rose-John S, Eaves CJ. Rapid differentiation of a rare subset of adult human $lin^-CD34^-CD38^-$ cells stimulated by multiple growth factors *in vitro*. Blood. 1999; 94(6):1926-32.

92. Sonoda Y. Human CD34-negative hematopoietic stem cells: The current understanding of their biological nature. Exp. Hematol. 2021; 96:13-26.

93. Nakamura Y, Ando K, Chargui J, Kawada H, Sato T, Tsuji T*, et al. Ex vivo* generation of $CD34^+$ cells from $CD34^-$ hematopoietic cells. Blood. 1999; 94(12):4053-9.

94. Schippel N, Sharma S. Dynamics of human hematopoietic stem and progenitor cell differentiation to the erythroid lineage. Exp. Hematol. 2023; 123:1-17.

95. Wognum B, Yuan N, Lai B, Miller CL. Colony forming cell assays for human hematopoietic progenitor cells [Internet]. In: Helgason C, Miller C, editors. Basic Cell Culture Protocols: Methods in Molecular Biology. Totowa, New Jersey: Humana press; 2013. p. 267-83. [cited: 2020/06/12]. Available from: https://doi.org/10.1007/978-1-62703-128-8_17

96. Cora MC, Latimer K, Travlos GS. Bone Marrow. In: Suttie AW, editor. Boorman's Pathology of the Rat. Boston: Academic Press; 2018. p. 495-519.

97. Wasnik S, Tiwari A, Kirkland MA, Pande G. Chapter Three - Osteohematopoietic Stem Cell Niches in Bone Marrow. In: Jeon KW, editor. Int. Rev. Cell Mol. Biol.: Academic Press; 2012. p. 95-133.

98. Kimura N, Mak TW. Isolation and characterization of an erythroid cell line highly inducible to form erythroid burst-like colonies. J. Cell. Physiol. 1986; 128(1):41-6.

99. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995; 83(1):59-67.

100. Metcalf D, Burgess AW. Clonal analysis of progenitor cell commitment of granulocyte or macrophage production. J. Cell. Physiol. 1982; 111(3):275-83.

101. Bryder D, Rossi DJ, Weissman IL. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am. J. Pathol. 2006; 169(2):338-46.

102. Fauci AS. The Human Immunodeficiency Virus: Infectivity and Mechanisms of Pathogenesis. Science. 1988; 239(4840):617-22.

103. World Health Organization. Weekly Epidemiological Record: Acquired immunodeficiency syndrome (AIDS). WHO/CDC case definition for AIDS.Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire; 61(10):69-74. Available from: https://iris.who.int/handle/10665/225725

104. Centers for Disease Control and Prevention. Monitoring selected national HIV prevention and care objectives by using HIV surveillance data—United States and 6 dependent areas, 2019. HIV Surveillance Supplemental Report. Atlanta, Georgia, USA. 2021; Document number: 2. [updated: May 2021. cited: 2023/11/30.] Available from: https://www.cdc.gov/hiv/library/reports/hiv-surveillance/vol-26-no-2/content/technical-notes.html#print

105. UNAIDS. Global HIV & AIDS statistics - Fact Sheet. World Health Organisation; 2023. Available from: https://www.unaids.org/en/resources/fact-sheet

106. Kartikeyan S, Bharmal RN, Tiwari RP, Bisen PS. HIV and AIDS: Basic elements and priorities. The Netherlands: Springer; 2007.

107. Kamori D, Ueno T. HIV-1 Tat and viral latency: what we can learn from naturally occurring sequence variations. Front. Microbiol. 2017; 8(80):1-7.

108. Chun TW, Davey RT, Jr., Engel D, Lane HC, Fauci AS. Re-emergence of HIV after stopping therapy. Nature. 1999; 401(6756):874-5.

109. Falcinelli SD, Ceriani C, Margolis DM, Archin NM. New Frontiers in Measuring and Characterizing the HIV Reservoir. Front. Microbiol. 2019; 10(2878):1-17.

110. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. *In vivo* fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat. Med. 1995; 1(12):1284-90.

111. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T*, et al.* Latent infection of $CD4^+$ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. 1999; 5(5):512-7.

112. Alexaki A, Liu Y, Wigdahl B. Cellular reservoirs of HIV-1 and their role in viral persistence. Curr HIV Res. 2008; 6(5):388-400.

113. Kedzierska K, Crowe SM. The role of monocytes and macrophages in the pathogenesis of HIV-1 infection. Curr. Med. Chem. 2002; 9(21):1893-903.

114. Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proc. Natl. Acad. Sci. U. S. A. 1999; 96(9):5215-20.

115. Wong ME, Jaworowski A, Hearps AC. The HIV Reservoir in Monocytes and Macrophages. Front. Immunol. 2019; 10(1435):1-16.

116. Bergamaschi A, Pancino G. Host hindrance to HIV-1 replication in monocytes and macrophages. Retrovirology. 2010; 7(31):1-17.

117. Lambotte O, Taoufik Y, de Goer MG, Wallon C, Goujard C, Delfraissy JF. Detection of infectious HIV in circulating monocytes from patients on prolonged highly active antiretroviral therapy. J. Acquir. Immune Defic. Syndr. 2000; 23(2):114-9.

118. Carter CC, Onafuwa-Nuga A, McNamara LA, Riddell Jt, Bixby D, Savona MR*, et al.* HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. Nat. Med. 2010; 16(4):446-51.

119. Carter CC, McNamara LA, Onafuwa-Nuga A, Shackleton M, Riddell Jt, Bixby D*, et al.* HIV-1 utilizes the CXCR4 chemokine receptor to infect multipotent hematopoietic stem and progenitor cells. Cell Host Microbe. 2011; 9(3):223-34.

120. Renelt S, Schult-Dietrich P, Baldauf HM, Stein S, Kann G, Bickel M*, et al.* HIV-1 infection of long-lived hematopoietic precursors *in vitro* and *in vivo*. Cells. 2022; 11(19):2968.

121. Nyamweya S, Hegedus A, Jaye A, Rowland-Jones S, Flanagan KL, Macallan DC. Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis. Rev. Med. Virol. 2013; 23(4):221-40.

122. Sharp PM, Hahn BH. Origins of HIV and the AIDS pandemic. Cold Spring Harb. Perspect. Med. 2011; 1(1):a006841.

123. Gallagher J. Aids: Origin of pandemic'was 1920s Kinshasa' [Internet]. BBC News. 2014/10/03. Available from:https://www.bbc.com/news/health-29442642

124. Worobey M, Watts TD, McKay RA, Suchard MA, Granade T, Teuwen DE*, et al.* 1970s and 'Patient 0' HIV-1 genomes illuminate early HIV/AIDS history in North America. Nature. 2016; 539(7627):98-101.

125. Campbell-Yesufu OT, Gandhi RT. Update on Human Immunodeficiency Virus (HIV)-2 infection. Clin. Infect. Dis. 2011; 52(6):780-7.

126. Esbjornsson J, Mansson F, Kvist A, da Silva ZJ, Andersson S, Fenyo EM*, et al.* Long-term follow-up of HIV-2-related AIDS and mortality in Guinea-Bissau: a prospective open cohort study. Lancet HIV. 2018; 6(1):e25-e31.

127. Berzow D, Descamps D, Obermeier M, Charpentier C, Kaiser R, Guertler L*, et al.* Human Immunodeficiency Virus-2 (HIV-2): A Summary of the Present Standard of Care and Treatment Options for Individuals Living with HIV-2 in Western Europe. Clin. Infect. Dis. 2021; 72(3):503-9.

128. Williams ME, Zulu SS, Stein DJ, Joska JA, Naudé PJ. Signatures of HIV-1 subtype B and C Tat proteins and their effects in the neuropathogenesis of HIV-associated neurocognitive impairments. Neurobiol. Dis. 2020; 136(104701):1-12.

129. Geretti AM. HIV-1 subtypes: epidemiology and significance for HIV management. Curr. Opin. Infect. Dis. 2006; 19(1):1-7.

130. Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK*, et al.* HIV-1 nomenclature proposal. Science. 2000; 288(5463):55-6.

131. Hué S, Clewley JP, Cane PA, Pillay D. HIV-1 pol gene variation is sufficient for reconstruction of transmissions in the era of antiretroviral therapy. AIDS. 2004; 18(5):719-28.

132. Leoz M, Feyertag F, Kfutwah A, Mauclere P, Lachenal G, Damond F*, et al.* The two-phase emergence of non pandemic HIV-1 group O in Cameroon. PLoS Pathog. 2015; 11(8):e1005029.

133. Taylor BS, Sobieszczyk ME, McCutchan FE, Hammer SM. The challenge of HIV-1 subtype diversity. N. Engl. J. Med. 2008; 358(15):1590-602.

134. Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V. Evolutionary and immunological implications of contemporary HIV-1 variation. Br. Med. Bull. 2001; 58(1):19-42.

135. Shevchenko AK, Zhernakova DV, Malov SV, Komissarov A, Kolchanova SM, Tamazian G*, et al.* Genome-wide association study reveals genetic variants associated with HIV-1C infection in a Botswana study population. Proc. Natl. Acad. Sci. U. S. A. 2021; 118(47):e2107830118.

136. Sarabia I, Bosque A. HIV-1 Latency and Latency Reversal: Does Subtype Matter? Viruses. 2019; 11(12):1104.

137. Hemelaar J, Elangovan R, Yun J, Dickson-Tetteh L, Fleminger I, Kirtley S*, et al.* Global and regional molecular epidemiology of HIV-1, 1990-2015: a systematic review, global survey, and trend analysis. Lancet Infect. Dis. 2019; 19(2):143-55.

138. Matume ND, Tebit DM, Bessong PO. HIV-1 subtype C predicted co-receptor tropism in Africa: An individual sequence level meta-analysis. AIDS Res. Ther. 2020; 17(1):5.

139. Rustagi A, Gale M, Jr. Innate antiviral immune signaling, viral evasion and modulation by HIV-1. J. Mol. Biol. 2014; 426(6):1161-77.

140. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. Cell Res. 2010; 20(1):34-50.

141. Yin X, Langer S, Zhang Z, Herbert KM, Yoh S, Konig R*, et al.* Sensor Sensibility-HIV-1 and the Innate Immune Response. Cells. 2020; 9(1):254.

142. Murugaiah V, Yasmin H, Pandit H, Ganguly K, Subedi R, Al-Mozaini M*, et al.* Innate Immune Response Against HIV-1. In: Kishore U, editor. Microbial Pathogenesis: Infection and Immunity. Cham: Springer International Publishing; 2021. p. 23-58.

143. Altfeld M, Gale M, Jr. Innate immunity against HIV-1 infection. Nat. Immunol. 2015; 16(6):554-62.

144. Carrington M, Alter G. Innate immune control of HIV. Cold Spring Harb. Perspect. Med. 2012; 2(7):a007070.

145. Ferlazzo G, Morandi B. Cross-Talks between Natural Killer Cells and Distinct Subsets of Dendritic Cells. Front. Immunol. 2014; 5:159.

146. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat. Immunol. 2008; 9(5):503-10.

147. Belizario JE, Neyra JM, Setubal Destro Rodrigues MF. When and how NK cell-induced programmed cell death benefits immunological protection against intracellular pathogen infection. Innate Immun. 2018; 24(8):452-65.

148. Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat. Rev. Immunol. 2007; 7(4):279-91.

149. Ivashkiv LB. IFNgamma: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. Nat. Rev. Immunol. 2018; 18(9):545-58.

150. Iannello A, Debbeche O, Samarani S, Ahmad A. Antiviral NK cell responses in HIV infection: I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS. J. Leukoc. Biol. 2008; 84(1):1-26.

151. Natarajan K, Dimasi N, Wang J, Mariuzza RA, Margulies DH. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. Annu. Rev. Immunol. 2002; 20(1):853-85.

152. Biasin M, Clerici M, Piacentini L. Innate immunity in resistance to HIV infection. J. Infect. Dis. 2010; 202 (3 Suppl 3):S361-5.

153. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J. Exp. Med. 2002; 195(3):343-51.

154. Aavani P, Allen LJS. The role of CD4 T cells in immune system activation and viral reproduction in a simple model for HIV infection. Appl Math Model. 2019; 75:210-22.

155. Okoye AA, Picker LJ. CD 4+ T-cell depletion in HIV infection: mechanisms of immunological failure. Immunol. Rev. 2013; 254(1):54-64.

156. Swain SL, McKinstry KK, Strutt TM. Expanding roles for $CD4^+$ T cells in immunity to viruses. Nat. Rev. Immunol. 2012; 12(2):136-48.

157. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008; 112(5):1557-69.

158. d'Ettorre G, Paiardini M, Ceccarelli G, Silvestri G, Vullo V. HIV-associated immune activation: from bench to bedside. AIDS Res. Hum. Retroviruses. 2011; 27(4):355-64.

159. Peng X, Ouyang J, Isnard S, Lin J, Fombuena B, Zhu B*, et al.* Sharing CD4+ T Cell Loss: When COVID-19 and HIV Collide on Immune System. Front. Immunol. 2020; 11:596631.

160. Gulzar N, Copeland KF. CD8+ T-cells: function and response to HIV infection. Curr HIV Res. 2004; 2(1):23-37.

161. Sewell AK, Price DA, Oxenius A, Kelleher AD, Phillips RE. Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. Stem Cells. 2000; 18(4):230-44.

162. Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: coordinators of immune and inflammatory responses. Annu. Rev. Biochem. 1990; 59(1):783-836.

163. Hewson T, Lone N, Moore M, Howie S. Interactions of HIV-1 with antigen-presenting cells. Immunol. Cell Biol. 1999; 77(4):289-303.

164. Deeks SG, Walker BD. The immune response to AIDS virus infection: good, bad, or both? J. Clin. Invest. 2004; 113(6):808-10.

165. Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RM. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. The Journal of Immunology. 2002; 169(6):3400-6.

166. Hellerstein MK, Hoh RA, Hanley MB, Cesar D, Lee D, Neese RA*, et al.* Subpopulations of long-lived and short-lived T cells in advanced HIV-1 infection. J. Clin. Invest. 2003; 112(6):956-66.

167. Paiardini M, Muller-Trutwin M. HIV-associated chronic immune activation. Immunol. Rev. 2013; 254(1):78-101.

168. Serrano-Villar S, Wu K, Hunt PW, Lok JJ, Ron R, Sainz T*, et al.* Predictive value of CD8+ T cell and CD4/CD8 ratio at two years of successful ART in the risk of AIDS and non-AIDS events. EBioMedicine. 2022; 80(104072):1-11.

169. Cao H, Walker BD. Immunopathogenesis of HIV-1 infection. Clin. Dermatol. 2000; 18(4):401-10.

170. Konadu KA, Anderson JS, Huang MB, Ali SA, Powell MD, Villinger F*, et al.* Hallmarks of HIV-1 pathogenesis are modulated by Nef's Secretion Modification Region. J. AIDS Clin. Res. 2015; 6(7):476.

171. Lu W, Mehraj V, Vyboh K, Cao W, Li T, Routy JP. CD4:CD8 ratio as a frontier marker for clinical outcome, immune dysfunction and viral reservoir size in virologically suppressed HIV-positive patients. J. Int. AIDS Soc. 2015; 18(1):20052.

172. Redd AD, Avalos A, Essex M. Infection of hematopoietic progenitor cells by HIV-1 subtype C, and its association with anemia in southern Africa. Blood. 2007; 110(9):3143-9.

173. Redd AD, Avalos A, Phiri K, Essex M. Effects of HIV type 1 infection on hematopoiesis in Botswana. AIDS Res. Hum. Retroviruses. 2007; 23(8):996-1003.

174. Alexaki A, Wigdahl B. HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination. PLoS Pathog. 2008; 4(12):e1000215.

175. Griffin DO, Goff SP. Restriction of HIV-1-based lentiviral vectors in adult primary marrow-derived and peripheral mobilized human CD34+ hematopoietic stem and progenitor cells occurs prior to viral DNA integration. Retrovirology. 2016; 13(1):14.

176. Ma H, Xia CQ. Phenotypic and Functional Diversities of Myeloid-Derived Suppressor Cells in Autoimmune Diseases. Mediators Inflamm. 2018; 2018(4316584):1-8.

177. Agrati C, Tumino N, Bordoni V, Pinnetti C, Sabatini A, Amendola A*, et al.* Myeloid Derived Suppressor Cells Expansion Persists After Early ART and May Affect CD4 T Cell Recovery. Front. Immunol. 2019; 10(1886):1-10.

178. Lv M, Wang K, Huang XJ. Myeloid-derived suppressor cells in hematological malignancies: friends or foes. J. Hematol. Oncol. 2019; 12(1):105.

179. Ostrand-Rosenberg S, Fenselau C. Myeloid-derived suppressor cells: immune-suppressive cells that impair antitumor immunity and are sculpted by their environment. J. Immunol. 2018; 200(2):422-31.

180. He Y-M, Li X, Perego M, Nefedova Y, Kossenkov AV, Jensen EA*, et al.* Transitory presence of myeloid-derived suppressor cells in neonates is critical for control of inflammation. Nat. Med. 2018; 24(2):224-31.

181. Chen X, Eksioglu EA, Zhou J, Zhang L, Djeu J, Fortenbery N*, et al.* Induction of myelodysplasia by myeloid-derived suppressor cells. J. Clin. Invest. 2013; 123(11):4595-611.

182. Kyeyune R, Saathoff E, Ezeamama AE, Loscher T, Fawzi W, Guwatudde D. Prevalence and correlates of cytopenias in HIV-infected adults initiating highly active antiretroviral therapy in Uganda. BMC Infect. Dis. 2014; 14(1):496.

183. Opie J. Haematological complications of HIV infection: forum-review. S. Afr. Med. J. 2012; 102(6):465-8.

184. Fan L, Li C, Zhao H. Prevalence and Risk Factors of Cytopenia in HIV-Infected Patients before and after the Initiation of HAART. Biomed Res Int. 2020; 2020(3132589):1-10.

185. Talargia F, Getacher L. Thrombocytopenia and Associated Factors Among HIV Infected Patients in Pre-and Post-Anti-Retroviral Therapy, North East Ethiopia. J. Blood Med. 2021; 12:741-8.

186. Watanabe M, Iwatani Y, Hidaka Y, Mitsuda N, Amino N. Changes in soluble CD4 and CD8 proteins in healthy pregnant and postpartum women. Am. J. Reprod. Immunol. 1996; 36(4):220-7.

187. Wall KM, Rida W, Haddad LB, Kamali A, Karita E, Lakhi S*, et al.* Pregnancy and HIV Disease Progression in an Early Infection Cohort from Five African Countries. Epidemiology. 2017; 28(2):224-32.

188. Tukei VJ, Hoffman HJ, Greenberg L, Thabelo R, Nchephe M, Mots'oane T*, et al.* Adverse Pregnancy Outcomes Among HIV-positive Women in the Era of Universal Antiretroviral Therapy Remain Elevated Compared With HIV-negative Women. The Pediatric Infectious Disease Journal. 2021; 40(9):821-6.

189. Anoje C, Aiyenigba B, Suzuki C, Badru T, Akpoigbe K, Odo M*, et al.* Reducing mother-to-child transmission of HIV: findings from an early infant diagnosis program in south-south region of Nigeria. BMC Public Health. 2012; 12(1):184.

190. Inra CN, Zhou BO, Acar M, Murphy MM, Richardson J, Zhao Z*, et al.* A perisinusoidal niche for extramedullary haematopoiesis in the spleen. Nature. 2015; 527(7579):466-71.

191. Gonzalez S, Amat L, Azqueta C, Madrigal JA, Laïlla JM, Garcia J*, et al.* Factors modulating circulation of hematopoietic progenitor cells in cord blood and neonates. Cytotherapy. 2009; 11(1):35-42.

192. Soma-Pillay P, Nelson-Piercy C, Tolppanen H, Mebazaa A. Physiological changes in pregnancy. Cardiovasc. J. Afr. 2016; 27(2):89-94.

193. Gangakhedkar GR, Kulkarni AP. Physiological Changes in Pregnancy. Indian J. Crit. Care Med. 2021; 25(Suppl 3):S189-s92.

194. Abdulqadir I, Ahmed SG, Kuliya AG, Tukur J, Yusuf AA, Musa AU. Hematological parameters of human immunodeficiency virus positive pregnant women on antiretroviral therapy in Aminu Kano Teaching Hospital Kano, North Western Nigeria. J Lab Physicians. 2018; 10(1):60-3.

195. Castro L, Goldani LZ. Iron, folate and vitamin B12 parameters in HIV-1 infected patients with anaemia in southern Brazil. Trop. Doct. 2009; 39(2):83-5.

196. Huibers MHW, Bates I, McKew S, Allain TJ, Coupland SE, Phiri C*, et al.* Severe anaemia complicating HIV in Malawi; Multiple co-existing aetiologies are associated with high mortality. PLoS One. 2020; 15(2):e0218695.

197. Firnhaber C, Smeaton L, Saukila N, Flanigan T, Gangakhedkar R, Kumwenda J*, et al.* Comparisons of anemia, thrombocytopenia, and neutropenia at initiation of HIV antiretroviral therapy in Africa, Asia, and the Americas. Int. J. Infect. Dis. 2010; 14(12):e1088-e92.

198. Durandt C, Potgieter JC, Mellet J, Herd C, Khoosal R, Nel JG*, et al.* HIV and haematopoiesis. S. Afr. Med. J. 2019; 109(8b):40-5.

199. Bayleyegn B, Woldu B, Yalew A, Asrie F. Magnitude and associated factors of peripheral cytopenia among HIV-infected children attending at University of Gondar Specialized Referral Hospital, Northwest Ethiopia. PLoS One. 2021; 16(3):e0247878.

200. Takuva S, Maskew M, Brennan AT, Sanne I, MacPhail AP, Fox MP. Anemia among HIV-Infected Patients Initiating Antiretroviral Therapy in South Africa: Improvement in Hemoglobin regardless of Degree of Immunosuppression and the Initiating ART Regimen. J. Trop. Med. 2013; 2013(162950):1-6.

201. Getawa S, Aynalem M, Bayleyegn B, Adane T. The global prevalence of thrombocytopenia among HIV-infected adults: A systematic review and meta-analysis. Int. J. Infect. Dis. 2021; 105:495-504.

202. Enawgaw B, Alem M, Addis Z, Melku M. Determination of hematological and immunological parameters among HIV positive patients taking highly active antiretroviral treatment and treatment naïve in the antiretroviral therapy clinic of Gondar University Hospital, Gondar, Northwest Ethiopia: a comparative cross-sectional study. BMC Hematol. 2014; 14(1):8.

203. Zetterberg E, Neuhaus J, Baker JV, Somboonwit C, Llibre JM, Palfreeman A*, et al.* Platelet count kinetics following interruption of antiretroviral treatment. AIDS. 2013; 27(1):59-68.

204. Pretorius E. Platelets in HIV: A Guardian of Host Defence or Transient Reservoir of the Virus? Front. Immunol. 2021; 12(649465):1-12.

205. Najean Y, Rain JD. The mechanism of thrombocytopenia in patients with HIV infection. J. Lab. Clin. Med. 1994; 123(3):415-20.

206. Gordon O. Challenges in Platelet Functions in HIV/AIDS Management [Internet]. In: Samuel O, editor. Future Opportunities and Tools for Emerging Challenges for HIV/AIDS Control. Rijeka: IntechOpen; 2022. p. 1-22. [cited: 2023-07-05]. Available from: https://doi.org/10.5772/intechopen.105731

207. Nka AD, Sosso SM, Fokam J, Bouba Y, Teto G, Simo Rachel R*, et al.* Thrombocytopenia according to antiretroviral drug combinations, viremia and CD4 lymphocytes among HIV-infected patients in Cameroon: a snapshot from the City of Yaoundé. BMC Res. Notes. 2019; 12(632):1-5.

208. Sebitloane H. Thrombocytopenia during pregnancy in women with HIV infection receiving no treatment. S. Afr. Med. J. 2016; 106(2):210-3.

209. Vannappagari V, Nkhoma ET, Atashili J, Laurent SS, Zhao H. Prevalence, severity, and duration of thrombocytopenia among HIV patients in the era of highly active antiretroviral therapy. Platelets. 2011; 22(8):611-8.

210. Tigner A, Ibrahim SA, Murray I. Histology, [Internet]. Treasure Island, Florida: StatPearls Publishing [Internet]; 2020 [cited: 2023/02/22]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK563148/

211. Lederer AK, Bartsch F, Moehler M, Gassmann P, Lang H. Morbidity and Mortality of Neutropenic Patients in Visceral Surgery: A Narrative Review. Cells. 2022; 11(20):3314.

212. Munyazesa E, Emile I, Mutimura E, Hoover DR, Shi Q, McGinn AP*, et al.* Assessment of haematological parameters in HIV-infected and uninfected Rwandan women: a cross-sectional study. BMJ Open. 2012; 2(6):e001600.

213. Shi X, Sims MD, Hanna MM, Xie M, Gulick PG, Zheng YH*, et al.* Neutropenia during HIV infection: adverse consequences and remedies. Int. Rev. Immunol. 2014; 33(6):511-36.

214. Fekene TE, Juhar LH, Mengesha CH, Worku DK. Prevalence of cytopenias in both HAART and HAART naive HIV infected adult patients in Ethiopia: a cross sectional study. BMC Hematol. 2018; 18(8):1-11.

215. Gillespie SL, Chinen J, Paul ME. Human Immunodeficiency Virus Infection and Acquired Immunodeficiency Syndrome. In: Rich RR, Fleisher TA, Schroeder HW, Weyand CM, Corry DB, Puck JM, editors. Clinical Immunology. 6th ed: Elsevier; 2023. p. 525-41.

Chapter 3: Functionality of circulating CD34⁺ haematopoietic stem and progenitor cells

3.1. Introduction

Haematopoietic stem and progenitor cells (HSPCs) are multipotent cells and are responsible for producing all haematological cell lineages. According to literature, approximately 100 billion new haematopoietic and immune cells are generated in adults every day, highlighting the robust nature of HSPCs[1]. Haematopoietic stem and progenitor cells are located within the bone marrow (BM), where they are tightly regulated to protect against mutations and to promote self-renewal and differentiation[2]. Haematopoietic stem and progenitor cells are heterogeneous with varying self-renewal capacities and levels of lineage commitment. This diversity exists even among cells that share common phenotypic markers, such as cluster of differentiation (CD)34[3, 4].

The downstream differentiation activity of BM-derived HSPCs leads to the production of common myeloid progenitors (CMP) and/or common lymphoid progenitors (CLP)[5]. The former differentiates into granulocyte-monocyte progenitors (GMP) and/or megakaryocyte-erythroid progenitors (MEPs), whilst the latter gives rise to T, B, and natural killer (NK) cells[5]. There is some literature that suggests HSPCs have the ability to differentiate directly into the megakaryocyte lineage, bypassing sequential differentiation steps (lineage bias)[6]. As the lineage commitment process continues, their multipotent capacity gradually decreases until they mature into distinct blood cell types[7,8].

At steady-state, a small amount of HSPCs egress to enter into circulation[9]. These HSPCs are referred to as circulating HSPCs and are the cell population of interest in this chapter. Two hypotheses concerning the function of circulating HSPCs were put forward by Lapidot and Petit (2002). The first is that these circulating cells repopulate the damaged areas in the BM and sustain the thymus population[10]. The second hypothesis suggests that these circulating cells lack a specific function but that their presence is indicative of non-specific leakage from the BM[10]. Interestingly, the number of circulating HSPCs rapidly increases in the presence of stressors, such as inflammation, infection, and/or injury (emergency haematopoiesis), as they are forced to transition from quiescence to differentiation[11-13]. In a study by Xu and colleagues (2022), it was reported that circulating HSPCs grown in a three-dimensional cell culture were

rapidly differentiating at a rate similar to the HSPCs in the BM whilst maintaining their long-term reconstitution capacity[14]. Treatment with cytokines, such as granulocyte colony-stimulating factor (G-CSF) and/or C-X-C chemokine receptor type-4 (CXCR4) antagonists (Plerixafor) is frequently used in HSPC transplantation to mobilise large numbers of HSPCs from the BM into circulation for successful reconstitution[15]. Literature suggests that circulating HSPCs maintain their plasticity, although the extent of this remains to be fully elucidated.

The exact mechanisms by which human immunodeficiency virus (HIV) affects the haematopoietic system are not fully known, but various mechanisms have been suggested. Some of the suggested mechanisms include: (1) HIV-mediated elimination of HSPCs in the BM niche through direct infection of HSPCs or HIV-induced apoptosis of HSPCs; (2) HIV-mediated disruption of the stromal cell network in the BM by direct infection of these cells by HIV, leading to HSPC death; or (3) HIV-mediated disruption of haematopoiesis-associated cell signalling events in the BM which negatively impacts on HSPC proliferation and differentiation[16]. Independent of the mechanisms involved, the end result remains the same - the reduction of HSPCs in the BM or altered proliferation and differentiation potential of their progeny[16].

The purpose of this part of the study was to quantify and describe the phenotypic characteristics of circulating HSPCs in peripheral blood of treatment-naïve, HIV-positive individuals and compare it to HIV-negative controls. This chapter will discuss the differences between the circulating HSPCs in the HIV-positive and HIV-negative (control) groups, focussing on (1) frequency, assessed using flow cytometry, and (2) functionality, evaluated through colony forming unit (CFU) assays. The colonies observed were also phenotypically characterised to assess the differentiation potential of circulating HSPCs more accurately across various cell lineages, including erythroid, granulocyte, monocyte, megakaryocyte, and lymphoid cell lineage.

3.2. Methodology

3.2.1 Study design

A comparative cross-sectional study was performed. Individuals were enrolled between November 2020 and November 2022 from the Antenatal Clinic (ANC) at Dr George Mukhari

Academic Hospital (DGMAH) in Ga-Rankuwa, Pretoria, South Africa. All participants enrolled provided written informed consent and donated approximately 15 millilitres (mL) of peripheral blood. Furthermore, all participants enrolled were pregnant. Using the donated peripheral blood, a full blood count with white cell differential (FBCD) was performed to assess for the presence of haematological abnormalities. The FBCD was performed as outlined in (*section 5.2.3)* in Chapter 5. *Associations of circulating haematopoietic stem and progenitor cells with haematological and immunological parameters in treatment-naïve HIV-positive pregnant women.*

Throughout this chapter, "treatment-naïve, HIV-positive," (study group 1) refers to individuals who were diagnosed with HIV at time of enrolment (n=13) and/or have not received combined antiretroviral-treatment (cART) for at least six months prior to enrolment in the study (defaulted; n = 0). Exclusion criteria for this cohort includes individuals currently on cART and those who have defaulted from cART less than six months ago. Study group 2 (control) consisted of women from the same clinic who tested negative for HIV (inclusion criteria) (n=25). Exclusion criteria for this cohort includes individuals who present with haematological abnormalities observed on the FBC. Individuals younger than 18 years of age, regardless of HIV-status, were excluded from the study.

This chapter investigates the quantity of circulating HSPCs present in peripheral blood and the phenotypic characteristics of the circulating HSPCs compared between the treatment-naïve, HIV positive and HIV-negative (control) groups. Furthermore, this chapter assesses the colony forming abilities (CFA) of the circulating HSPCs and the phenotypic characteristics of the resultant colonies compared between the treatment-naïve, HIV-positive and HIV-negative (control) groups.

3.2.2. Ethics approval

The research study received ethics approval from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (protocol number: 738/2020; Appendix II), and Sefako Makgatho Health Sciences University Research Ethics Committee (reference number: SMUREC/M/86/2020:IR). Approval was also received from the Dr George Mukhari Academic Laboratory of the National Health Laboratory Services (NHLS) as well as from the Dr George Mukhari Academic Hospital (DGMAH).

3.2.3. Study participant enrolment

3.2.3.1. Consent

Informed consent was obtained from all study participants by the clinical staff at the Ante-natal Clinic (ANC), DGMAH prior to peripheral blood collection. The patient information leaflet and informed consent documents are included as Appendix III. All participants, treatment-naïve, HIV-positive and HIV-negative (control), were recruited from the ANC, DGMAH.

3.2.3.2. HIV counselling and confirmation

A rapid HIV test was used to confirm the HIV status of all individuals enrolled in the study. HIV testing forms part of the routine screening of patients visiting the clinic. All participants underwent mandatory pre-HIV test counselling from HIV counsellors employed by the clinic, and those who tested positive for HIV received post-HIV test counselling from the same counsellors.

3.2.3.3. Study participant demographics

The mean [standard deviation (SD)] age of the treatment-naïve, HIV-positive cohort was 35.82 ± 8.59 (ranging from 20 to 39). Furthermore, in the treatment-naïve, HIV-positive cohort (n=13), 7.69% (n=1) of the women were in their first trimester, 7.69% (n=1) in the second trimester, 61.54% (n=8) in the third trimester, and data for the remaining 23.08% (n=3) was unavailable. One individual in this group presented with high blood pressure. The remaining 92.31% (n=12) had no comorbidities (self-reported).

The mean [standard deviation (SD) age of the HIV-negative (control) group cohort was 33.00 ± 5.47 (ranging from 19 to 47). Furthermore, in the HIV-negative (control) group cohort (n=25), none of the women were in their first trimester, 56% (n=14) in the second trimester, 40% (n=10) in the third trimester, and data for the remaining 4% (n=1) was unavailable. Amongst the HIV-negative (control) group, 68% (n=17) had no comorbidities, whilst 12% (n=3) presented with high blood pressure, another 12% (n=3) suffered from asthma and 4% (n=1) had gestational diabetes.

3.2.4. Experiments

All samples obtained four ethylenediamine tetra-acetic acid (EDTA)-vacutainer tubes [two 3 millilitre (mL) tubes and two 6 mL tubes] of peripheral blood were strictly processed as described in a standard operating procedure (SOP) attached as Appendix IV. Ethylenediamine tetra-acetic acid containing blood collection tubes were used to prevent the blood samples from clotting as EDTA functions as a blood anti-coagulant[17]. Each sample was processed on the day of collection. Experiments that involved peripheral blood and peripheral blood mononuclear cells (PBMNCs) were performed on the day of collection.

3.2.4.1. Distribution of peripheral blood samples for research purposes

The contents of the two 6 mL EDTA tubes were pooled into a single sterile 15 mL centrifuge tube (Falcon®, Corning, New York, USA) and the total volume of blood was recorded. From this, a 100 microlitre (µL) aliquot of peripheral blood was used for the Basic Immune Profile assessment described in *Chapter 4: Enumeration of immune cell and myeloid derived suppressor cell populations*. In addition, a 4 mL peripheral blood aliquot was transferred into sterile 1.5 mL microcentrifuge tubes (1 mL per tube) (Lasec®, Cape Town, South Africa) for plasma isolation and storage. The remaining peripheral blood was used for peripheral blood mononuclear cell (PBMNC) isolation, as described in *Section 3.2.4.2. Isolation of peripheral blood mononuclear cells.* The two 3 mL EDTA-tubes were used to obtain a FBCD and viral load (*section 5.2.3 in* Chapter 5. *Associations of circulating haematopoietic stem and progenitor cells with haematological and immunological parameters in treatment-naïve HIV-positive pregnant women).*

3.2.4.2. Isolation of peripheral blood mononuclear cells

Circulating HSPCs are contained within the PBMNC fraction. Circulating HSPCs are rare cell populations with an expected frequency in PBMNCs of 0.0170±0.0020% in healthy controls[12]. Therefore, to enrich for HSPCs, the PBMNC population was isolated, and used for the enumeration of the circulating $CD34^+$ HSPC populations.

Peripheral blood mononuclear cells were isolated using density-gradient centrifugation. The peripheral blood was layered carefully onto Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) in 15 mL centrifuge tubes at a Histopaque®-1077 to blood ratio of 1:1. The layered

samples were centrifuged (SL16R centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 300 times gravitational force (x g) for 30 minutes, without applying a brake. Four distinct layers formed, as illustrated in Figure 3.1, one of which was the desired PBMNC population.

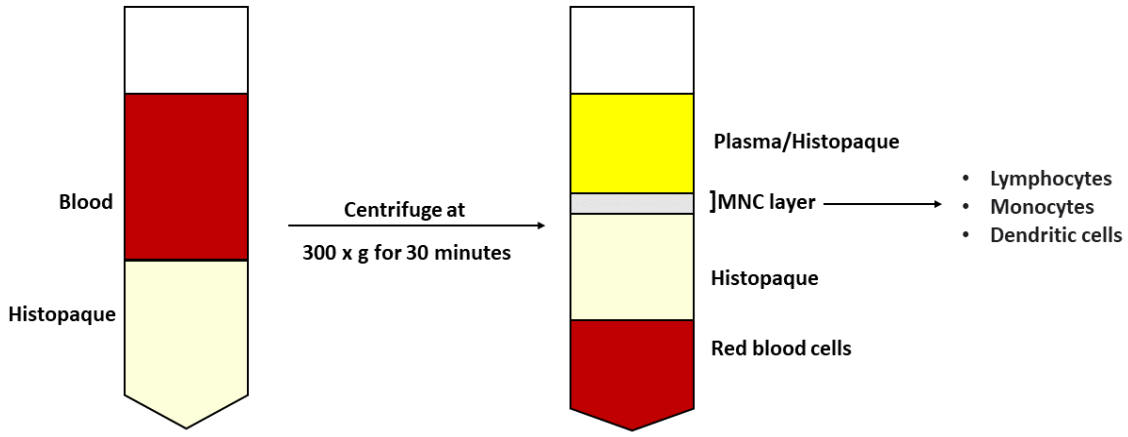


Figure 3.1: A schematic illustration of the various layers that form during density-gradient centrifugation of peripheral blood. Image created by Tsungai Mashingaidze using Microsoft PowerPoint. MNC, Mononuclear cells; x g, Times gravitational force.

The PBMNC layer was transferred to a new 15 mL centrifuge tube and washed with sterile phosphate-buffered saline (PBS) [PBS, pH 7.4; 137 millimolar (mM) NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4; Sigma-Aldrich, St. Louis, MO, USA] by filling the tube with PBS followed by centrifugation at 300 x g for 10 minutes. The supernatant was carefully aspirated to not disturb the cell pellet. Next, the cell pellet was resuspended in the residual supernatant. VersaLyse™ lysing solution (Beckman Coulter, Miami, FL, USA) (2 mL) was added to the cell suspension, vortexed to mix and incubated for 15 minutes at room temperature (RT). After incubation, the tube was filled with sterile PBS, centrifuged at 300 x g for 10 minutes, and the supernatant was aspirated. The PBMNC pellet was resuspended in 1 mL of sterile PBS.

3.2.4.3. Enumeration of peripheral blood mononuclear population

From the PBMNC suspension, a 50 µL aliquot was transferred into a flow cytometry tube to which 10 µL of 7-aminoactinomycin D (7-AAD) and 10 µL of CD45-fluorescein isothiocyanate (FITC) were added followed by incubation for 15 minutes at RT. Thereafter, 3 mL of sterile PBS was added to the tube followed by centrifugation at 300 x g for 10 minutes. The supernatant was aspirated, and the cell pellet was resuspended in the residual PBS. The cell suspension volume was adjusted by adding 200 µL of sterile PBS to the tube. Flow-count™ fluorospheres

(50 µL; Beckman Coulter, Miami, FL, USA) were added to the tube followed by analysis using the CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA) to obtain the $CD45^+$ PBMNC count.

3.2.4.4. Circulating $CD34^+$ haematopoietic stem and progenitor cell frequency and absolute counts

3.2.4.4.1. Colour compensation for the haematopoietic stem and progenitor cell panel

When more than one fluorochrome is used simultaneously, 'spill over' of emission spectrums into detector channels other than the primary detector channel may occur. These interfering (spill-over) signals must be removed, during a process known as colour compensation[18]. To adequately calculate the compensation, single-colour-stained control cells, i.e., cells individually stained with the respective fluorochromes present in the multi-colour panel used for the specific assay, are used. This allows for the generation of a colour compensation matrix that is applied to samples during analysis to correct for any interference (spill-over) that occurred. For the HSPC panel, a colour compensation matrix was generated using VersaComp™ Antibody Capture beads (Beckman Coulter, Miami, FL, USA) (as outlined below) stained with individual monoclonal antibodies (mABs), listed in Table 3.1.

The VersaComp™ Antibody Capture kit consists of two vials of 3.0-3.4 micrometre (µm) beads in suspension at a concentration of approximately 1 x 10^7 beads/mL. The first vial contained VersaComp™ Antibody Capture Negative Beads which acts as a negative staining control, as these beads do not have the ability to bind fluorochrome-conjugated antibodies[19]. The second vial contained VersaComp™ Antibody Capture Positive Beads, which are coated with an immunoglobulin (IgG)-binding agent that allows binding of all mouse and rat isotypes, hamster IgG and rabbit polyclonal antibodies[19].

Nine flow cytometry tubes, each tube containing an individual monoclonal antibody, were prepared for monoclonal antibodies listed in Table 3.1. One drop of negative and one drop of positive VersaComp™ beads were added to each tube. The tubes were vortexed for 5-10 seconds, followed by a 15-minute incubation at RT. After incubation, 3 mL of PBS was added to the tubes followed by centrifugation at 300 x g for 10 minutes. The supernatant was aspirated, and the bead pellet was resuspended in the remaining supernatant. An additional

500 µL of PBS was added to each tube. The colour compensation setup feature on the CytoFLEX software or the BD FACSAria™ Fusion cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) software (Diva version 8.0) was launched and the tubes were acquired to generate a colour compensation matrix. The colour compensation matrix was saved and applied during acquisition of patient samples ($CD34^+$ circulating HSPC template).

3.2.4.4.2. Standardisation setup for the haematopoietic stem and progenitor cell panel

Standardisation of the circulating $CD34^+$ HSPC template allowed for correcting day-to-day technical variation of instrument settings by automatically adjusting signal gains to ensure that the baseline intensities (5% variance allowed) in each detector channel were consistent from day to day. This was achieved by applying the adjusted settings (usually minor adjustments) to the template used to analyse samples before sample acquisition was started.

To generate a standardisation template, a forward scatter (FSC) versus (v) side scatter (SSC) plot, and one-parameter histograms were created according to the mABs listed in Table 3.1 (a histogram for each detector/fluorochrome). The optimised template of interest, such as the circulating $CD34^+$ HSPC template, was used as base template. Flow-Set™ Pro fluorospheres (Beckman Coulter, Miami, FL, USA) were acquired using the CytoFLEX flow cytometer. A region was drawn around the intact fluorospheres on the FSC v SSC plot and assigned as a gate to the aforementioned one-parameter histograms. For the one-parameter histograms, linear regions were created across each of the fluorescent peaks. The Flow-Set™ Pro fluorospheres lot number was recorded and the respective median fluorescent intensities (MFIs) were added to the standardisation target library (library is template specific), creating an application-specific (circulating $CD34^+$ HSPC) standardisation template which was saved.

Before sample acquisition the standardisation software feature was launched, the circulating $CD34^+$ HSPC standardisation settings and lot number of the Flow-Set™ Pro in use were selected, followed by acquisition of Flow-Set™ Pro fluorospheres. If standardisation passed, the adjusted settings were applied to the circulating $CD34^+$ HSPC template.

3.2.4.4.3. Sample preparation and acquisition for the haematopoietic stem and progenitor cell panel

To prepare the sample for the HSPC panel, the staining approach, as described below, was used, using the mAB panels listed in Tables 3.1 and 3.2 (isotypic control tube). An isotype control was selected to match the immunoglobulin class and fluorochrome of the mAB for which it needed to serve as a non-specific staining control[20].

The frequency and absolute counts of the HSPC populations were determined by adding PBMNC suspension to a new flow cytometry tube, containing the mABs listed in Table 3.1. The volume of suspension added to the tube was calculated to ensure that a minimum of 1 x 10^6 PBMNCs were added to the tube. Analysis of a minimum of 1 x 10^6 PBMNCs was done in an attempt to ensure adequate enumeration of the HSPC sub-populations. The tube was vortexed for 10 seconds and incubated for 15 minutes at RT. After incubation, 3 mL of sterile PBS were added to the tube followed by centrifugation at 300 x g for 10 minutes. The supernatant was aspirated without disturbing the cell pellet. The cell pellet was then resuspended in the residual PBS and 250 µL of sterile PBS were added. Analysis was performed on the BD FACSAria™ Fusion cell sorter. Acquiring the sample using the BD FACSAria™ Fusion cell sorter enabled simultaneous phenotypic assessment and cell sorting of lineage-negative (Lin^-)/$CD34^{++}$ HSPCs for the CFU assay (section 3.2.3.6. *Colony-forming unit assay)*.

When technical difficulties arose with the BD FACSAria™ Fusion cell sorter, the CytoFLEX flow cytometer was used for the phenotypic assessment using a standardised protocol. In such cases, the entire sample was acquired, and isolation of Lin^-CD34^{++} HSPCs for the CFU assay was performed using magnetic bead isolation techniques (*section 3.2.3.5*).

Table 3.1: The panel of fluorochrome-conjugated monoclonal antibodies and the volume used for the circulating $CD34^+$ haematopoietic stem and progenitor cell panel.

Marker	Fluorochrome	Volume of mAB (µL)
Lin	FITC	7,5
CD45RA	APC	7,5
CD38	ECD	5
CD34	APC-AF700	5
CD90	BV510	5

Table 3.1 continues.

CD49f	SB780	5
CD10	BV660	5
CD133/1	PE-violet 770	2
CD117	PE	1

APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; BV, Brilliant violet; CD, Cluster of differentiation; ECD, R- Phycoerythrin-Texas red®-X; FITC, Fluorescein isothiocyanate; Lin, Lineage; mAB, Monoclonal antibody; PE, Phycoerythrin; SB, Super bright. Volumes were determined through monoclonal antibody titration experiments, performed by Dr Durandt, my supervisor. This was done before the start of the project.

Table 3.2: Panel of fluorochrome-conjugated monoclonal antibodies and the volume used for the isotypic control tube for the circulating CD34⁺ haematopoietic stem and progenitor cell panel.

Marker	**Fluorochrome**	**Volume of mAB (µL)**
Mouse IgG1	APC	7,5
Lin	FITC	7,5
Mouse IgG1	BV510	5
Rat IgG2a	SB780	5
Mouse IgG1	BV660	5
CD34	APC-AF700	5
Mouse IgG1	PE	1

APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; BV, Brilliant violet; CD, Cluster of differentiation; FITC, Fluorescein isothiocyanate; Ig, Immunoglobulin; Lin, Lineage; mAB, Monoclonal antibody; PE, Phycoerythrin; SB, Super bright. Volumes were determined through monoclonal antibody titration experiments, performed by Dr Durandt, my supervisor. This was done before the start of the project.

3.2.4.4.4. Gating strategy to enumerate circulating CD34⁺ HSPCs

The gating strategy used for the enumeration of the circulating CD34⁺ HSPCs is illustrated in Figure 3.2 and for the simultaneous phenotypic assessment is illustrated in Figure 3.3.

An ungated FSC vs SSC plot was generated to obtain the lymphocyte population (region of interest “Lymphocytes'') (Figure 3.2A). The “Lymphocyte'' region was used as the input gate in Figure 3.2B [SSC vs Zombie violet (ZV)] to detect the viable cells (region of interest “Viable”). Next, a Lin-FITC vs CD34-Alexa fluor (AF)-700 plot, gated on the viable cells was created as

HSPCs are identifiable by a Lin^- and a $CD34^+$ phenotype[21, 22]. Circulating HSPCs were identified as Lin^-CD34^{++} (Figure 3.2C).

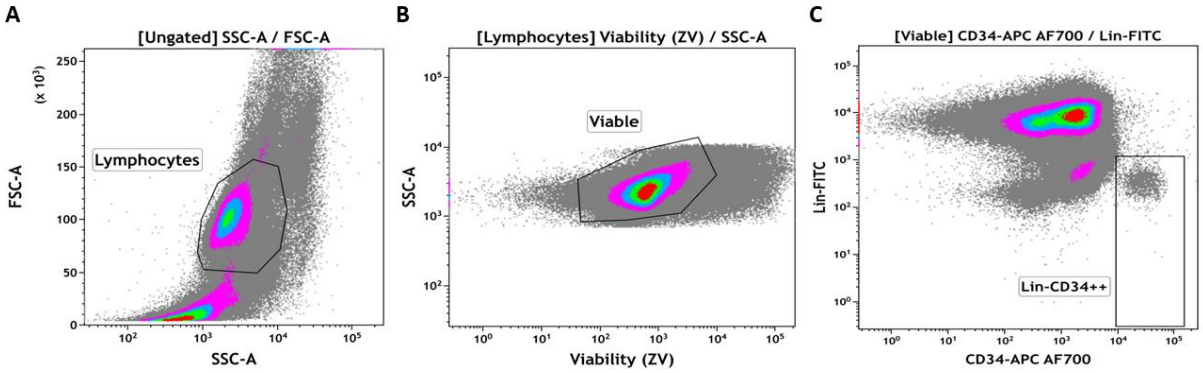


Figure 3.2: Representative flow cytometry plots to illustrate the gating strategy used to identify and enumerate circulating $CD34^+$ haematopoietic stem and progenitor cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; FITC, Fluorescein isothiocyanate; FSC, Forward scatter; Lin, Lineage; SSC, Side scatter; ZV, Zombie violet.

3.2.4.4.5. Gating strategy to phenotypically characterise circulating $CD34^+$ haematopoietic stem and progenitor cells

An ungated FSC vs SSC plot was generated to obtain the lymphocyte population (“Lymphocytes''; Figure 3.3A). The Lin^-CD34^{++} population was identified using a Lin-FITC vs CD34-AF-700 plot (Figure 3.3B). The ‘’Lymphocyte’’ region was used as the input gate to the latter plot. Next a Lin-FITC vs Zombie violet (ZV) plot was generated (Figure 3.3C). The Lin-FITC vs ZV plot (Figure 3.3C) was gated on the Lin^-CD34^{++} region which allowed for the inclusion of only viable Lin^-CD34^{++} cells in downstream analysis.

Single-parameter histograms were generated for all cell surface markers as listed in Table 3.1. This allowed for the identification of populations that stained negative and positive for the respective markers and were used to generate the tree plot (Figure 3.3D). Initially phenotypic permutations using all seven additional markers (CD10, CD38, CD45RA, CD49f, CD90, CD117 and CD133) were attempted. However, it resulted in many phenotypically distinct sub-populations with less than 25 events per cluster. Cell populations with less than 25 events per cluster required additional verification of the validity of the cluster that fell outside the scope of this project. For this reason, it was decided to limit the phenotypic characterisation to CD38, CD133 and CD117. Tree plots were used to display the different phenotypic permutations, i.e.,

co-expression profiles of CD38, CD133 and CD117 (Figure 3.3D). The viable Lin^{-}/CD34^{++} cells were used as input gate to the tree plot.

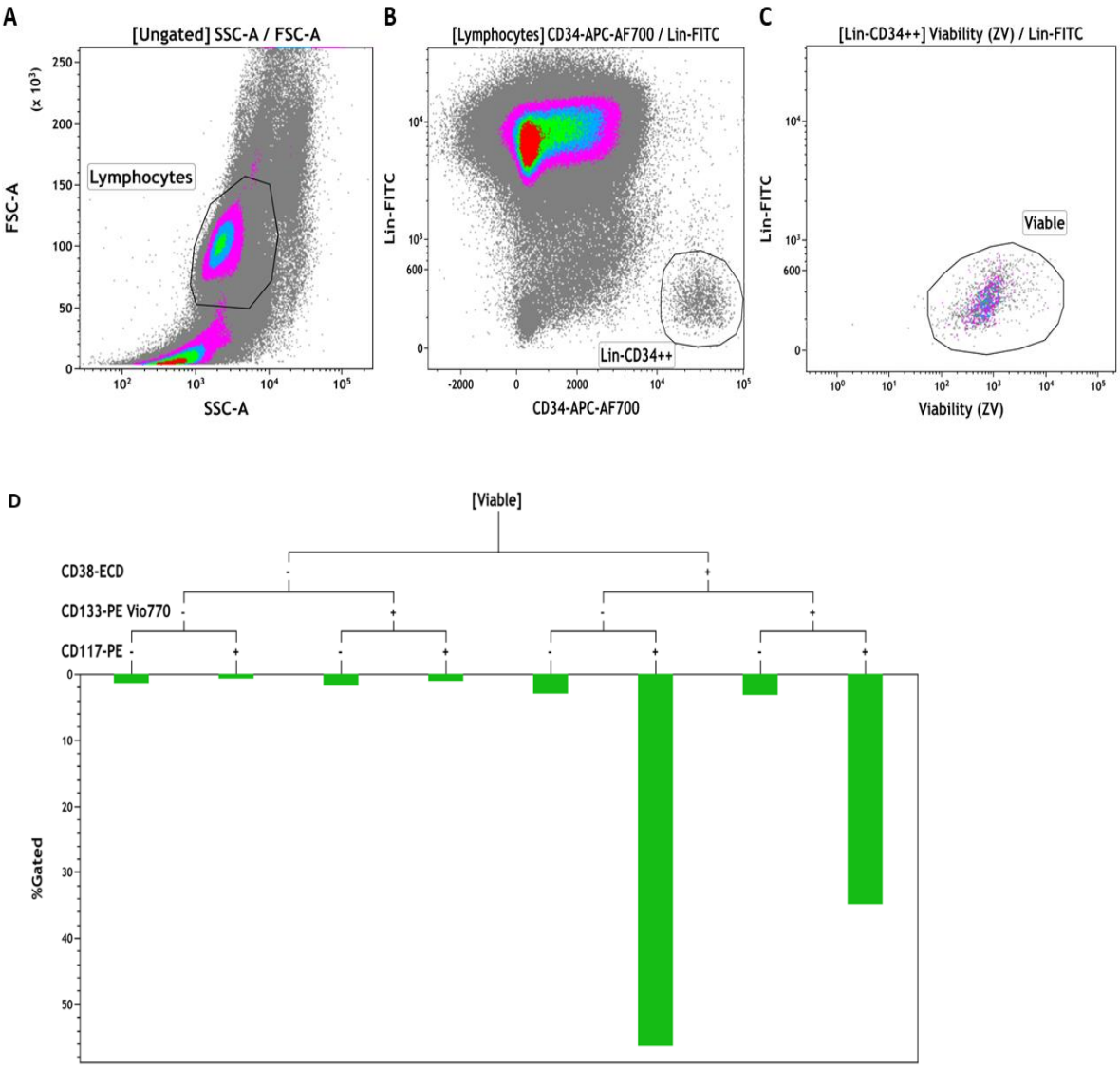


Figure 3.3: Representative flow cytometry plots [(A-C) density plots and (D) tree plot] to illustrate the gating strategy used to outline and phenotypically characterise circulating Lin^{-}CD34^{++} haematopoietic stem and progenitor cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FITC, Fluorescein isothiocyanate; FSC, Forward scatter; Lin, Lineage; PE, Phycoerythrin; SSC, Side scatter; Vio, Violet, ZV, Zombie violet.

3.2.4.5. Magnetic bead isolation

During this project, we encountered some technical difficulties with the BD FACSAria™ Fusion cell sorter, which impeded its proper functioning and impacted on the project. As we were not able to use fluorescent-activated cell sorting (FACS) for some samples, magnetic bead

isolation was attempted as an alternative method for selecting all Lin^- cells. This was done using the Lineage Cell Depletion Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

The Lineage Cell Depletion Kit employs an indirect magnetic labelling system, where the $CD34^+$ HSPCs are not labelled with microbead-conjugated mABs. Instead, they are eluted out of the column, while the Lin^+ (lineage committed) cells, such as T cells, B cells, natural killer (NK) cells, dendritic cells, monocytes, and red blood cells (RBCs) are retained[23]. It is important to note that the $CD34^+$ HSPCs are typically lineage negative.

3.2.4.5.1. Sample preparation for magnetic bead isolation

3.2.4.5.1.1. Sample enumeration

Firstly, the total number of $CD45^+$ cells in the sample preparation was determined by staining an aliquot of the PBMNC population with CD45-FITC/CD34-Phycoerythrin (PE) (Stem-Kit Enumeration kit, Beckman, Coulter, Miami, USA) (as outlined in *section 3.2.3.3 Enumeration of PBMNC population*). Flow-count™ fluorospheres were added at a 1:1 ratio of PBMNCs to Flow-Count™ fluorospheres. The total number of cells determined the appropriate buffer volumes that were used, that is, 3 mL of buffer was required for every 10^8 labelled cells in the ensuing magnetic labelling and separation steps.

3.2.4.5.1.2. Magnetic labelling

As per the manufacturer's instructions, the PBMNC suspension was centrifuged (SL16R centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 300 x g for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in 40 µL of cold complete Roswell Park Memorial Institute-1640 medium [(RPMI-1640; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS) (RPMI-1640/10% medium)] per 10^8 total cells. It is important to note that the RPMI buffer was kept cold to reduce non-specific staining[24].

The resuspended cell pellet was stained with 10 µL of the biotin-conjugated antibody cocktail [CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (glycophorin A)] vortexed and incubated for 10 minutes at 4°C. After incubation, a wash was performed by adding 750 µL of cold RPMI-1640/10% medium to the cell suspension, followed by centrifugation at 300 x g for 10 minutes. The supernatant was discarded, and the cell pellet

was resuspended in 80 µL cold RPMI-1640/10% medium, then stained with 20 µL of anti-biotin microbeads for 15 minutes at 4°C. Next, the sample was washed using 750 µL cold RPMI-1640/10% medium and centrifuged at 300 x g for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in 500 µL of cold RPMI-1640/10% medium.

3.2.4.5.1.3. Magnetic separation

For magnetic separation, the MACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and LS MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) were used.

The LS MACS columns were used for the isolation of $CD34^+$ HSPCs from peripheral blood samples. The column was inserted into the separator and rinsed with 3 mL of cold complete RPMI-1640 medium, which was followed by carefully pipetting the cell suspension onto the column. The complete RPMI-1640 medium was allowed to run through the column and the resulting effluent was collected in a 15 mL Falcon® tube. The flow-through was enriched for Lin^- cells. While still on the magnetic stand, the column was washed three times with 3 mL of cold complete RPMI-1640 medium, and the flow-through was collected in the same tube as the initial effluent mentioned above. Care was taken to ensure that all the medium passed through the column before removing it from the stand. The cells remaining in the LS column represented the Lin^+ population. The Lin^- cell suspension was centrifuged at 300 x g for 10 minutes and the supernatant was carefully discarded without disturbing the cell pellet. The remaining cell pellet was resuspended in 500 µL of cold complete RPMI-1640 medium for later use in the CFU assay *(*section 3.2.4.6 *Colony-forming unit assay*).

3.2.4.6. Colony-forming unit assay

3.2.4.6.1. Assessing colony formation of Lin^-CD34^{++} haematopoietic stem and progenitor cells

The Lin^-CD34^{++} HSPCs were sorted for the CFU assay using the BD FACSAria™ Fusion cell sorter. In a 48-well plate, 500 µL of pre-warmed MethoCult™ SF4436 (STEMCELL™ Technologies, Vancouver, Canada) was pipetted into three wells (Figure 3.4). MethoCult™ is a semi-solid, serum-free methylcellulose-based Iscove's Modified Dulbecco's medium (IMDM) that promotes an optimal environment for HSPC differentiation into the various progeny. It contains a range of preselected growth factors [bovine serum albumin, 2-mercaptoethanol, recombinant human insulin and human transferrin (iron-saturated)] and cytokines [Stem cell

factor (SCF), interleukin (IL)-3, IL-6, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF)][25, 26]. A total of 50 $Lin^{-}CD34^{++}$ HSPCs were sorted into each of the wells containing MethoCult™. Figure 3.2 depicts the gating strategy used to identify and enumerate the $Lin^{-}CD34^{++}$ HSPC population.

If the sample was prepared using magnetic bead isolation (outlined in *section 3.2.4.5*), and the proportion of $Lin^{-}CD34^{++}$ HSPCs in the Lin^{-} fraction was <60%, the sample was excluded from further processing for the CFU assay. However, if the proportion of $Lin^{-}CD34^{++}$ HSPCs in the Lin^{-} fraction was >60%, the calculated volume (see Equation 3.1) was pipetted into MethoCult™ medium in triplicate in a 48-well plate for the CFU assay.

Equation 3.1: Volume of cell suspension to be aliquoted into MethoCult™ media.

$$Volume\ required\ (\mu L) = \frac{Total\ number\ of\ cells\ in\ suspension\ (cells/\mu L)}{Number\ of\ cells\ required\ for\ plating (cells)}$$

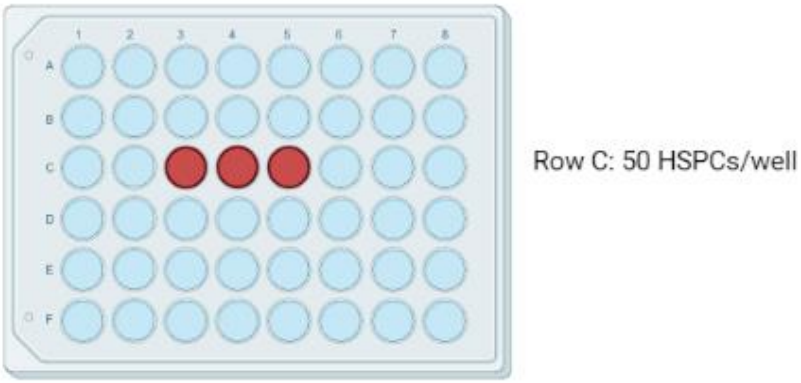


Figure 3.4: A schematic illustration of the experimental layout used for the colony forming units assay. Wells C3-C5 (red) indicate the wells containing the MethoCult™ medium and $Lin^{-}CD34^{++}$ haematopoietic stem and progenitor cell. The remaining wells contained PBS. Image created by T Mashingaidze using Microsoft PowerPoint. HSPCs, Haematopoietic stem and progenitor cells.

The viscous consistency of the semi-solid MethoCult™ medium resulted in the sorted cells being located in the same position within the wells. To ensure even distribution of cells throughout the wells, the plate was vortexed for 15 seconds, taking care not to over-vortex as it may cause the cells to be distributed too close to the well walls, making colony identification difficult. Furthermore, sterile PBS was added to the surrounding empty wells to prevent the MethoCult™ medium from drying out during the 14-day incubation period.

The plate was incubated for 14 days in a 37°C/5% CO_2 water-jacketed incubator (Thermo Fisher Scientific) to allow for differentiation. The resulting colonies were counted using light microscopy (Axio vert A1, ZEISS, Oberkochen, Germany). All the colonies observed in each well were counted and grouped according to the appropriate colony types [i.e., CFU-Granulocyte and Macrophage (GM), CFU-Granulocyte, Erythrocyte, Macrophage and Megakaryocyte (GEMM), CFU- Erythroid (E) or burst forming unit (BFU)-E]. The colonies were identified using commercial colony forming guides that outlined the morphological characteristics associated with the various types of colonies[25, 27]. Table 3.3 is a modified CFU atlas that provides a summary of the morphological descriptions of the various colonies. For each patient sample, the total number of CFU-GM, CFU-GEMM, CFU-E or BFU-E colonies present in each well was counted and recorded in a Microsoft Excel document. The total number of the different colony types (CFU-GM, CFU-GEMM, CFU-E/BFU-E; sum of the three wells) was collated and used to obtain the average for the HIV-positive and control group. After microscopic assessment, the wells were pooled together to phenotypically characterise the resulting colonies.

3.2.4.6.2. Phenotypic characterisation of differentiated colonies

3.2.4.6.2.1. Colour compensation and standardisation setup for phenotypic characterisation of colonies

The colour compensation matrix was determined using VersaComp™ Antibody Capture beads as outlined in *section 3.2.4.4.1 Colour compensation for the HSPC panel* and the mABs listed in *Table 3.4: Panel of fluorochrome-conjugated mABs used to assess viability and phenotypically characterise the differentiated progeny of Lin^{-}CD34^{++} HSPCs*.

An application-specific (CFU phenotypic analysis) standardisation template was generated using Flow-Set™ fluorospheres (as outlined in *section 3.2.4.4.2 Standardisation setup for the HSPC template*) with one-parameter histograms generated in alignment with the mABs listed in Table 3.4. The standardised instrument settings (gains) were applied to the CFU template prior to sample acquisition using the CytoFLEX flow cytometer.

3.2.4.6.2.2. Staining procedure and sample acquisition

An immunophenotypic assay was performed to confirm the phenotype of the colonies observed in the CFU assay. All colonies in the wells were pooled for the phenotypic assessment. Two tubes were prepared: (i) a viability tube stained with 7-AAD and (ii) a phenotypic tube stained with mABs listed in Table 3.4. The markers and the cell types identified by the respective markers are listed in Table 3.5.

Phosphate-buffered saline (500 µL) was added to the MethoCult™-containing wells that contained visible colonies using the microscope (Axio vert A1, Zeiss, Oberkochen, Germany). The MethoCult™ and PBS were thoroughly mixed by pipetting the well content up and down several times to break down and dilute the semi-solid MethoCult™ layer. After which all three wells (Figure 3.4) were rinsed with 1 mL PBS and the contents of the three wells were pooled into a single 15 mL Falcon® tube. This was done to ensure that all cells were transferred from the wells into the 15 mL tube. The tube was centrifuged at 300 x g for 10 minutes, the supernatant aspirated and the cell pellet resuspended in 300 µL of PBS. This suspension was used for further processing of both viability and phenotypic analysis.

Aliquots (100 µL) of the aforementioned suspension were added into two separate flow cytometry tubes; tube 1, stained with 7-AAD only (viability tube) and tube 2, a phenotypic analysis flow cytometry tube stained with mABs listed in Table 3.4. Thereafter, both tubes were incubated for 15 minutes at RT, centrifuged at 300 x g for 10 minutes, supernatant aspirated and the cell pellet resuspended in 200 µL of PBS. Flow-count™ fluorospheres (100 µL) were added to the viability tube. A separate viability tube was stained as both PC5.5 (which is present in the phenotypic tube) and 7-AAD share the same detector channel. Therefore, to appropriately determine the sample viability, a separate tube was required. Dead cells stain positive for 7-AAD, whilst viable cells stain negative for 7-AAD, due to the inability of the dye to cross intact cell membranes. For the viability tube up to 3000 events were acquired in the "Cal" (intact beads) region and up to 100 000 viable cells were acquired for the phenotypic tube.

The mature lymphocytes (see Table 3.5) were identified as a whole and not as different cell types. To do this effectively, all mature lymphocytes were identified using monoclonal antibodies conjugated with the same fluorochrome, PC5.5, which allowed for the

identification of the lymphocyte cell populations that consisted of T cells (CD3), B cells (CD19 and CD20) and NK cells (CD56). The hypothesis put forward at the beginning of the project was that only a minor population of the differentiated cells would express these mature lymphoid markers as the commercial MethoCult™ medium favours myeloid differentiation[26, 28]. Hence the decision to identify total lymphocytes, rather than identifying the different lymphocyte populations separately.

3.2.4.6.2.3. Gating strategy for the phenotypic characterisation of progenitor cells

A density plot of FSC vs SSC plot was generated. Two distinct cell populations were observed which were labelled “Viable” and “Dead” based on their 7-AAD staining profiles (Figure 3.5A). Next, two SSC vs 7-AAD-PC5.5 plots were generated. The first SSC vs 7-AAD-PC5.5 plot was gated on the population named “Viable”, while the second SSC vs 7-AAD-PC5.5 plot was gated on the population named “Dead” (Figure 3.5B and C, respectively).

Three distinctive sub-populations, based on their 7-AAD staining intensity were observed: negative, intermediate, and positive. Cells are classified as viable when they prevent 7-AAD from crossing their extracellular membrane but are classified as dead when 7-AAD is able to cross compromised extracellular membranes to intercalate into double-stranded DNA[29]. All populations outlined were quantified and the average mean ± SD values as a percentage of the viable and dead cells respectively, were calculated and compared against each other.

The flow cytometry templates used to distinguish between CFU sub-populations (erythroid, myeloid, and lymphoid) are indicated in Figures 3.6 (erythroid), 3.7 (myeloid), and 3.8 (lymphoid), respectively. The respective populations were characterised from only the “Viable” (predominantly 7-AAD negative) population.

Table 3.3: General descriptions and representative micrographs, adapted from STEMCELL™ Technologies[25, 27] used for colony identification in colony forming unit-assays. Representative micrographs [4X objective (left) and 10X objective (right)] were extracted from STEMCELL™ Technologies- Wallchart: Identification of Colonies Derived from Human Haematopoietic Progenitor Cells[27].

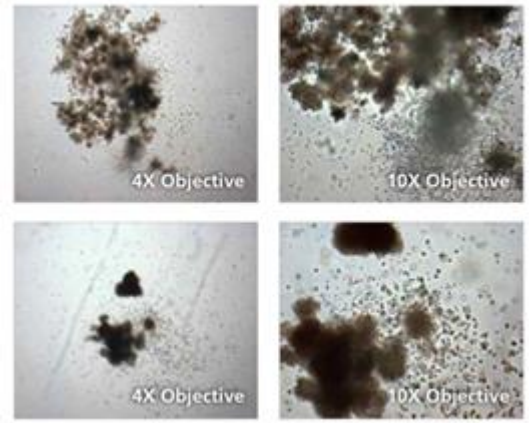
Colony name	Representative image	Identifying characteristics
CFU-GEMM CFU-granulocyte, erythrocyte, macrophage, megakaryocyte.	CFU-GEMM  	**Lineage:** Myeloid. **Progeny:** Multi-lineage (granulocyte, erythrocyte, macrophage & megakaryocyte). **Density:** Large colonies consisting of > 500 cells. **Appearance:** Tight clusters towards the centre with sparser surrounding cells. Colonies may appear to have a brown tint due to erythroid cells in the centre.

Table 3.3 continues

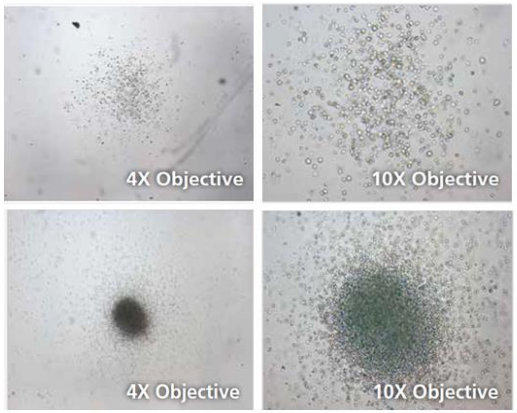
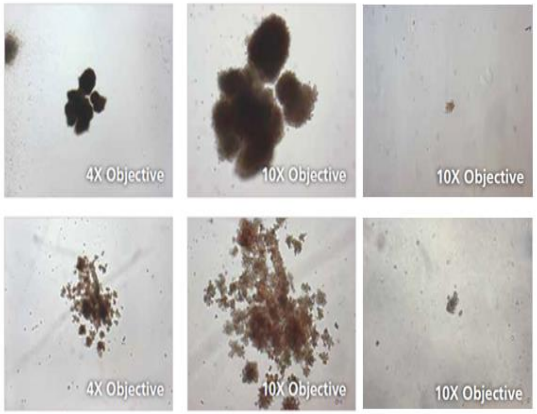
CFU-GM CFU-granulocyte, macrophage.	CFU-GM  	**Lineage:** Myeloid. **Progeny:** Granulocyte and macrophage. **Density:** A minimum 40 granulocytes and/or macrophages in a single colony. **Appearance:** Moderate clusters towards the centre of the colony with more sparse colonies surrounding the moderate clusters. No distinct colour, appear translucent.
B/CFU-E Burst/CFU-erythroid.	B/CFU-E  	**Lineage:** Erythroid. **Progeny:** Single lineage (erythrocytes). **Density:** A single cluster may contain approximately 200 erythroblasts (BFU-E > 200 cells; CFU-E < 200 cells). **Appearance:** Red to brown colour.

Table 3.4: Panel of fluorochrome-conjugated monoclonal antibodies used to assess viability and phenotypically characterise the differentiated progeny of $Lin^{-}CD34^{++}$ haematopoietic stem and progenitor cells.

	Marker	Fluorochrome	Volume of mAB (µL)
Tube 1: Viability	7-AAD		5
Tube 2: Phenotype	CD15	KO	10
	CD235a	PE	5
	CD71	APC-AF750	5
	CD41	ECD	5
	CD14	APC-AF700	5
	CD33	APC	5
	CD19	PC5.5	5
	CD20		5
	CD3		2.5
	CD56		2.5

7-AAD, 7-Aminoactinomycin-D; APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; KO, Krome orange; mAB, Monoclonal antibody; PC, Phycoerythrin-cyanine; PE, Phycoerythrin.

Table 3.5: The cell lineages identified by the respective fluorochrome-conjugated monoclonal antibodies used in the colony forming units immunophenotypic panel.

Marker	Fluorochrome	Cell Lineage
CD15	KO	Myeloid (Granulocytes)
CD235a (Glycophorin A)	PE	Erythroid
CD71	APC-AF750	Erythroid (immature; nucleated). It is also expressed by a subset of proliferating cells[17].
CD41	ECD	Megakaryocytes
CD14	APC-AF700	Myeloid (Monocytes)
CD33	APC	Myeloid (monocytes and granulocytes)
CD3	PC5.5	Lymphoid (T cells)
CD19		Lymphoid (B cells)
CD20		Lymphoid (B cells)
CD56		Lymphoid (NK and NKT cells)

APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; KO, Krome orange; PE, Phycoerythrin; PC, Phycoerythrin-cyanine.

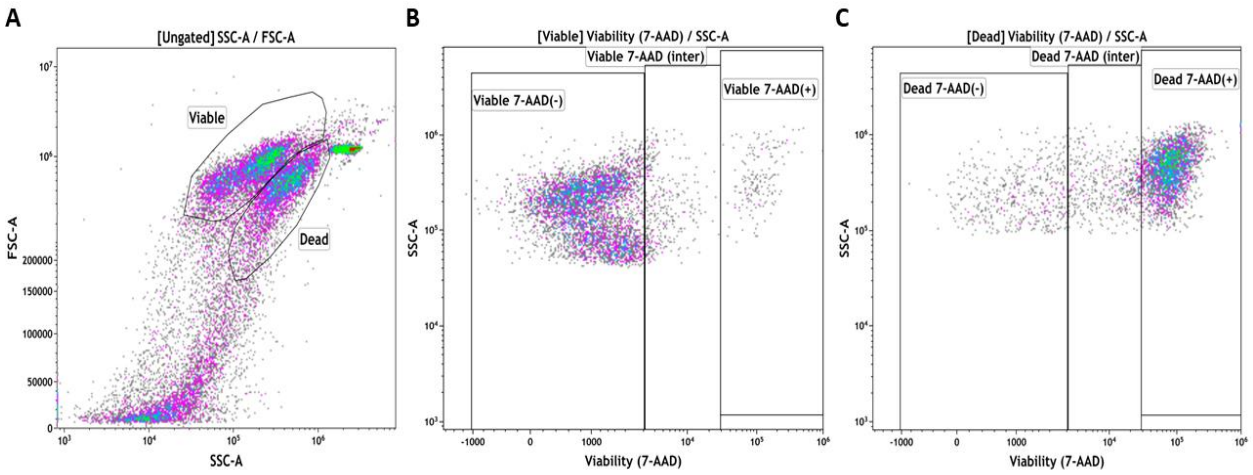


Figure 3.5: Representative flow cytometry plots to illustrate the gating strategy used to determine cell viability of the Lin^{-}CD34^{++} HSPCs isolated from PBMNCs using the CFU assay. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. 7-AAD, 7-Aminoactinomycin-D; A, Area; FSC, Forward scatter; Inter, Intermediate; SSC, Side scatter.

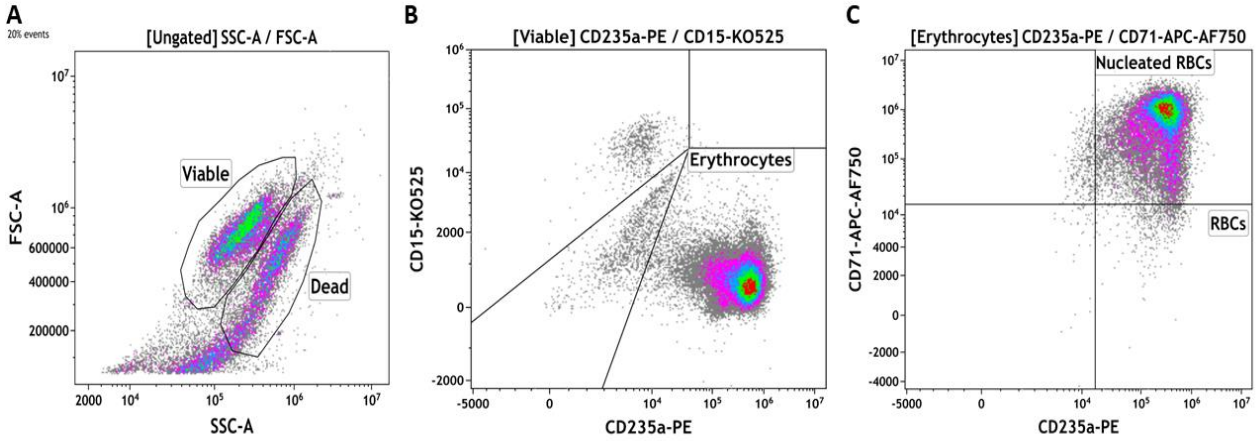


Figure 3.6: Representative flow cytometry plots to illustrate the sequential gating strategy used to identify and enumerate erythroid lineage cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; FSC, Forward scatter; KO, Krome orange; RBC; Red blood cells; PE, Phycoerythrin; SSC, Side scatter.

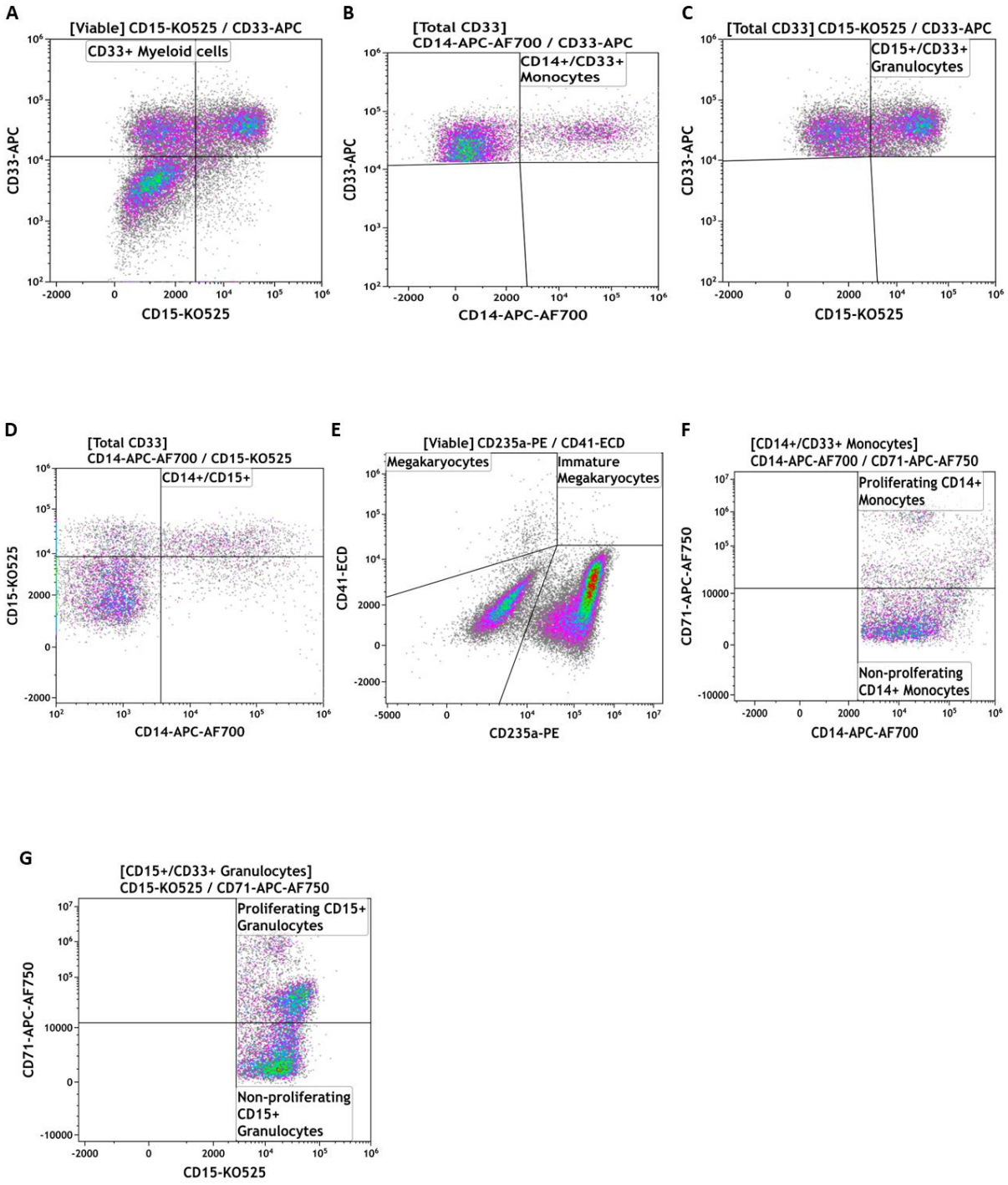


Figure 3.7: Representative flow cytometry plots to illustrate the sequential gating strategy used to identify and enumerate myeloid lineage cells. A Boolean gate "Total CD33" was created using the Boolean string of '(Viable AND CD15^{+}CD33^{+}) OR (Viable AND CD15^{-}CD33^{+})'. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FSC, Forward scatter; KO, Krome orange; PE, Phycoerythrin; SSC, Side scatter.

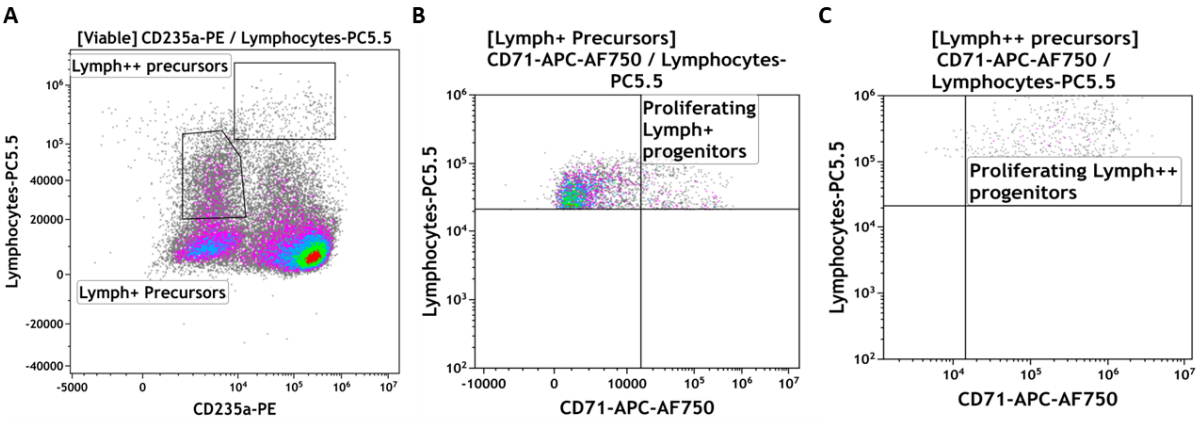


Figure 3.8: Representative flow cytometry plots to illustrate the sequential gating strategy used to identify and enumerate lymphoid lineage cells. Image created by T Mashingaidze using Kaluza Analysis software, version 2.1 and compiled using Microsoft PowerPoint. A, Area; APC-AF, Allophycocyanin-Alexa Fluor; CD, Cluster of differentiation; FSC, Forward scatter; Lymph, Lymphocytes; PC, Phycoerythrin-cyanine; SSC, Side Scatter.

3.2.5. Statistical considerations

The data was analysed using the Kaluza analysis software version 2.1 (Beckman Coulter, USA). GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform non-parametric Mann-Whitney U-tests to compare the variable means between Treatment-naïve, HIV-positive, and HIV-negative (control) groups. Values were considered significantly different when the p-value was less than 0.05 (indicating two tailed p-value) test. All p-values were calculated precisely to account for ties in values, rather than being approximated. Significance was denoted as *, **, ***, and ****, representing p-values of <0.05, <0.01, <0.001, and <0.0001, respectively.

3.4. Results

3.4.1. Quantification and phenotypic assessment of peripheral blood CD34⁺ haematopoietic stem and progenitor cell sub-populations

Flow cytometric analysis was used to determine the phenotype of the BM-derived circulating CD34⁺ HSPCs and their frequency. The phenotypic characterisation was performed to assess whether HIV exposure altered the sub-population distribution of circulating HSPCs. Whilst the quantification was performed to assess the impact of HIV-1 on the frequency of circulating CD34⁺ HSPCs, i.e., whether there was an increase or decrease or negligible change in

circulating Lin$^-$/CD34^{++} HSPC numbers present in peripheral blood between the two study groups.

The percentage (mean ± SD) and absolute counts (mean ± SD) of Lin$^-$CD34^{++} HSPCs were compared between the treatment-naïve, HIV-positive, and HIV-negative (control) groups. The percentage Lin$^-$CD34^{++} HSPCs was significantly lower in the treatment-naïve, HIV-positive group (0.09 ± 0.06%) compared to the HIV-negative (control) group (0.21 ± 0.21%) (p=0.0096) (Figure 3.9A). The average Lin$^-$CD34^{++} absolute cell count of the HIV-positive group was also significantly lower (314.5 ± 224.2 cells/µL) compared to what was observed for the HIV-negative (control) group (815.5 ± 676.2 cells/µL) (p=0.023) (Figure 3.9B).

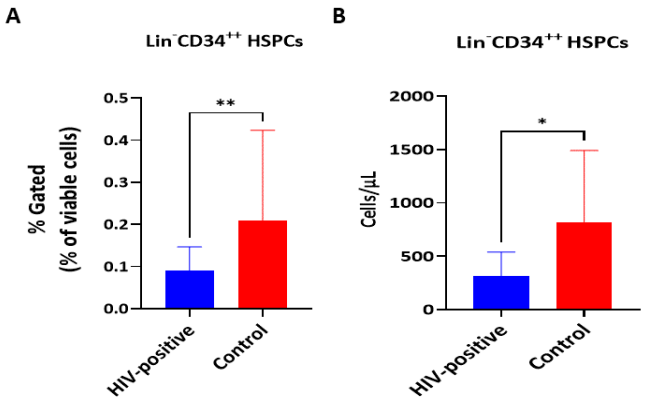


*Figure 3.9: Enumeration of circulating Lin$^-$/CD34^{++} haematopoietic stem and progenitor cells present in peripheral blood of treatment-naïve, HIV-positive (n=13), and HIV-negative (control) groups (n=25). Control refers to the HIV-negative (control) group. Bar graphs were used to demonstrate the (A) mean percentage of viable peripheral blood mononuclear cells, and (B) mean absolute cell count (cells/µL), with the error bars indicating the standard deviation (SD). Significance was reported as * and **, indicating p-values of <0.05 and <0.01, respectively.*

As mentioned before, the phenotypic characterisation of Lin$^-$CD34^{++} HSPCs was limited to three additional cell surface markers: CD38, CD117, CD133. The tree plot identified eight different co-expression permutations (Figure 3.3D) and the frequencies of the various sub-populations are listed in Table 3.6. The CD38$^+$CD133$^-$CD117$^+$ and the CD38$^+$CD133$^+$CD117$^+$ phenotypes were the most abundant phenotypes (Table 3.6). Differences in the distribution (mean percentage mean ± SD) of these different phenotypic profiles were compared between the treatment-naïve, HIV-positive, and HIV-negative (control) groups (Table 3.6). No statistically significant differences were observed between the two groups (Table 3.6).

Table 3.6: CD38, CD117, and CD133 co-expression profiles observed in the treatment-naïve, HIV-positive and HIV-negative (control) groups. Results are expressed as the mean percentage with standard deviation of viable Lin$^-$CD34^{++} haematopoietic stem and progenitor cells.

	Mean ± SD		
	HIV-positive (%) n=13	**Control (%) n=25**	**p-value**
CD38$^-$CD133$^-$CD117$^-$	5.67±8.66	1.84±2.15	0.23
CD38$^-$CD133$^-$CD117$^+$	0.57±1.33	0.39±0.65	0.49
CD38$^-$CD133$^+$CD117$^-$	0.70±0.98	0.19±0.36	0.12
CD38$^-$CD133$^+$CD117$^+$	0.40±0.54	0.52±1.70	0.56
CD38$^+$CD133$^-$CD117$^-$	9.02±6.71	7.61±9.11	0.43
CD38$^+$CD133$^-$CD117$^+$	44.35±29.01	44.56±30.11	0.98
CD38$^+$CD133$^+$CD117$^-$	1.69±2.21	1.45±1.91	0.74
CD38$^+$CD133$^+$CD117$^+$	37.59±39.96	43.42 ±36.70	0.67

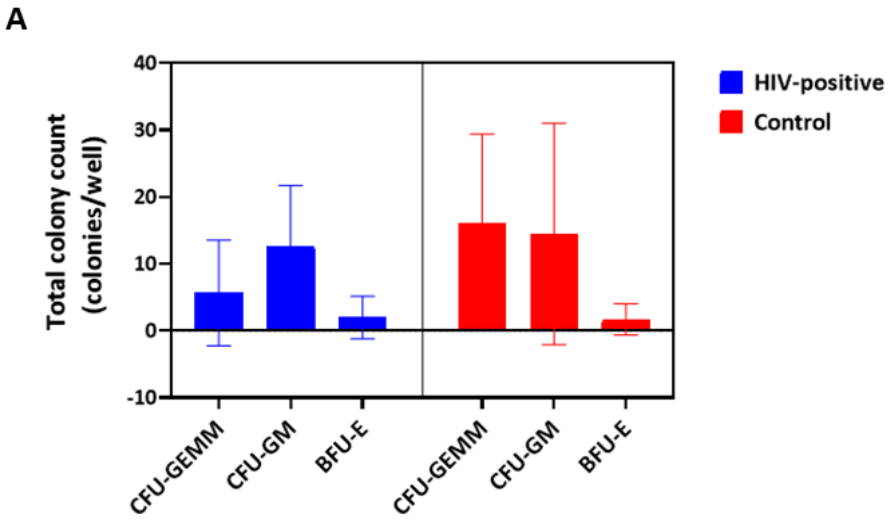
CD, Cluster of differentiation; HIV, Human immunodeficiency virus; SD, Standard deviation.

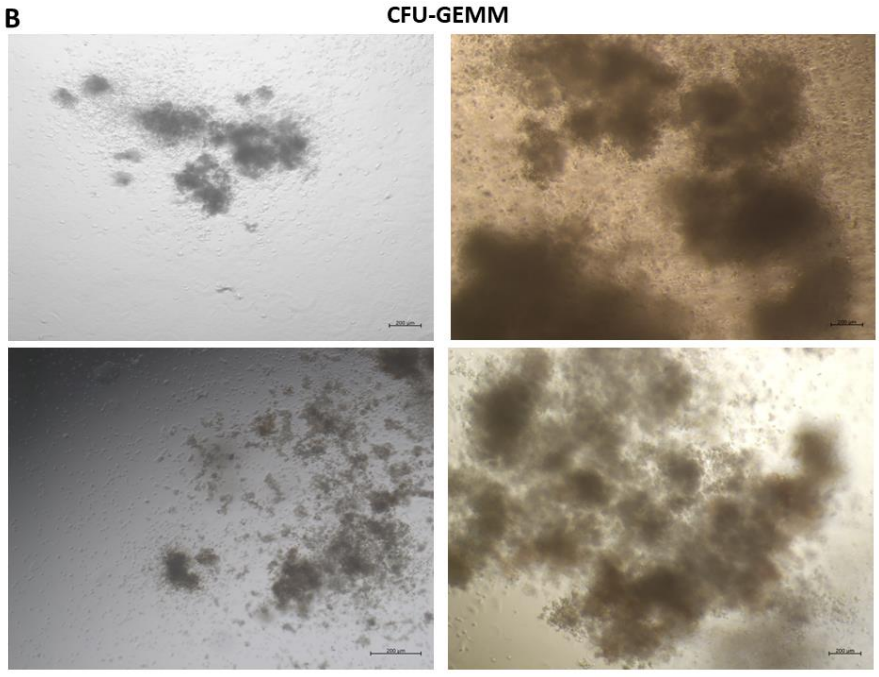
3.4.2. Functionality of circulating Lin$^-$CD34^{++} haematopoietic stem and progenitor cells

The impact of HIV-1 exposure on the differentiation potential of circulating Lin$^-$CD34^{++} HSPCs was investigated using the CFU assay. The ability of CD34$^+$ HSPCs to regenerate and replenish blood and immune cell populations outlines their functional capacity.

The resulting colonies were identified according to the modified atlas outlined in Table 3.3 and counted manually using a microscope (Axio vert A1, Zeiss, Oberkochen, Germany). Figure 3.10A displays the total colony count/well for the HIV-positive and HIV-negative (control) groups. Within the HIV-positive group, an average of 12.67±9.07 total CFU-GM colonies were observed, followed by 5.67±7.89 total CFU-GEMM colonies and 2.00±3.16 total BFU-E colonies. For the HIV-negative (control) group, 16.13±13.24 total CFU-GEMM, 14.46±16.56 total CFU-GM, and 1.708±2.331 total BFU-E were observed. No CFU-E colonies were observed in either the HIV-positive group or the control group.

An image of each identified colony was captured and used to verify the initial identification and count of colonies. Figures 3.10B-D display randomly selected images that serve as representative examples of the different colonies observed after the 14-day incubation period.





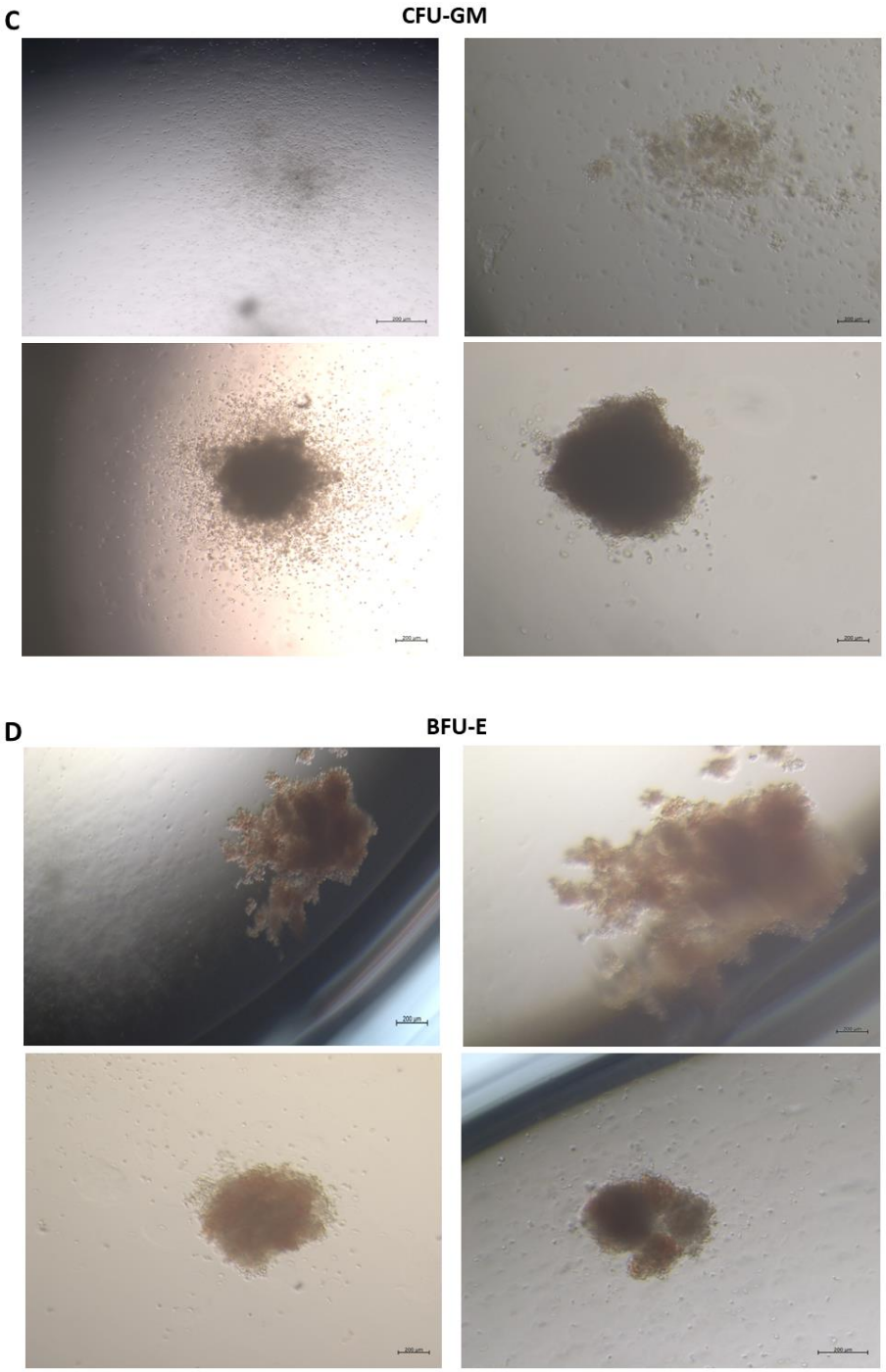


Figure 3.10: Assessment of the functionality of the isolated Lin$^-$CD34^{++} haematopoietic stem and progenitors, plated on semi-solid MethoCult™ medium after 14-day incubation. (A) Identification and enumeration of colonies observed. Bar graphs were used to denote the average (mean) number of the different colony types observed, with the error bars indicating the standard deviation between samples. (B-D) Representative images of the different types of colonies observed. Images were captured using a 5X (left) and 10X objective (right). Control refers to the HIV-negative (control) group. B, burst; CFU, colony forming unit; E, erythroid; GEMM, granulocyte erythroid macrophage megakaryocyte; GM, granulocyte macrophage.

Two distinct populations labelled “viable” and “dead” in the SSC vs FSC plot (Figure 3.4A) were consistently observed when the colonies were phenotypically characterised. Table 3.7A displays the percentage of the total “viable” cells and ”dead” cells obtained from the FSC vs SSC plots (Figure 3.4A). Percentages are the proportions of all events (ungated). The percentage ‘’viable’’ cells was 16.15 ± 10.67% (HIV-positive) and 14.46 ± 5.82% (HIV-negative control), whilst the percentage “dead” cells was 15.88 ± 8.85% (HIV-positive) and 19.12 ± 5.79% (HIV-negative control). No statistically significant differences were observed between the HIV-positive and HIV-negative (control) groups.

Table 3.7B highlights the percentage of viable and dead cells present in the “viable” and ”dead” regions of both the HIV-positive and HIV-negative (control) groups, supporting the assignment of the region labels to their respective populations. In the “viable” regions, only 2.68 ± 2.44% (HIV-positive) and 3.71 ± 4.10% (HIV-negative control) stained strongly positive for 7-AAD, i.e., was dead, while in the “dead” region 61.83 ± 20.28% (HIV-positive) and 54.25 ± 16.29% (HIV-negative control) stained strongly positive for 7-AAD.

The cells that expressed intermediate levels of 7-AAD (7-AAD^{intermediate}) started to display compromised cell membranes, which allowed low levels of 7-AAD to cross the cell membrane. It is also possible that these are actively proliferating cells with less rigid (more fluidic) cell membranes.

Table 3.7A: Mean percentages of total viable and dead cells obtained from the colony forming unit viability analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group.

Mean ± SD			
HIV-positive		**Control**	
Viable region (%)	**Dead region (%)**	**Viable region (%)**	**Dead region (%)**
16.15±10.67	15.88±8.85	14.46±5.82	19.12±5.79

Table 3.7B: Mean percentages of viable and dead cells obtained from colony forming unit viability analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group.

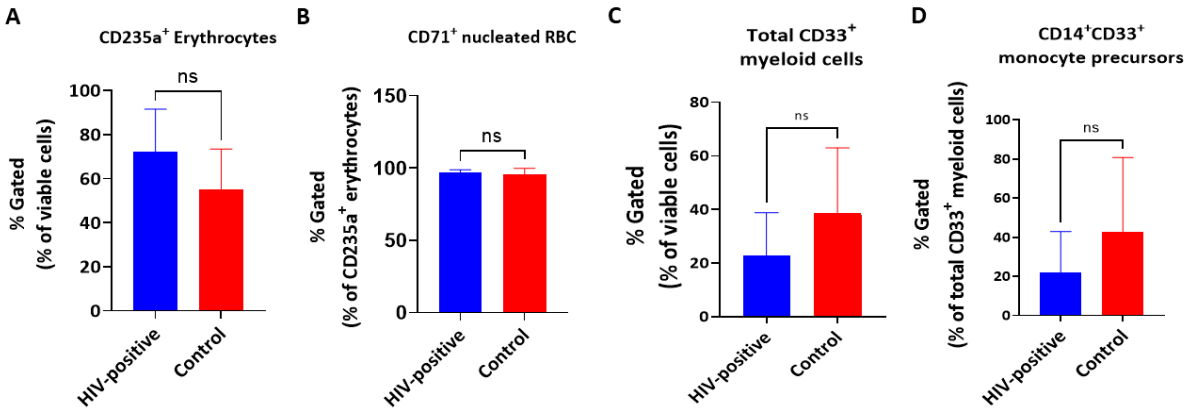
7-AAD stain	Mean ± SD			
	HIV-positive		Control	
	Viable region (%)	Dead region (%)	Viable region (%)	Dead region (%)
7-AAD^{-}	70.11±36.81	25.15±13.96	78.60±16.58	24.57±17.41
7-AAD^{intermediate}	26.87±37.59	12.73±6.38	17.26±15.99	18.57±5.45
7-AAD^{+}	2.68±2.44	61.83±20.28	3.71±4.10	54.25±16.29

Of the "viable" cell population, 72.17 ± 19.24% (HIV-positive group) and 55.06 ± 18.30% (HIV-negative, control group) expressed CD235a (glycophorin A), i.e., were CD235a^{+} erythrocytes (Figure 3.11A). Of these CD235a^{+} erythrocytes, 96.55 ± 2.03% (HIV-positive group) and 95.63 ± 4.09 (HIV-negative, control group) expressed CD71 (Figure 3.11B). Notably, CD71 is expressed by nucleated red blood cells (nRBCs; an immature erythroid cell also referred to as a normoblast)[30, 31]. Furthermore, CD71 is also expressed on all proliferating cells[32]. Next, the proportion of myeloid cells present in the "viable" region was investigated. The presence of the CD33 marker, coupled with the progressive reduction of the CD34 marker is indicative of myeloid commitment by haematopoietic progenitor cells[33]. As such, 22.72 ± 16.10% (HIV-positive group) and 38.53 ± 24.46% (HIV-negative, control group) of the "viable cells" expressed the CD33 myeloid marker (Figure 3.11C). Of the "total CD33^{+} myeloid cells", 22.15 ± 20.83% (HIV-positive group) and 42.93 ± 37.82% (HIV-negative, control group) expressed the CD14 marker (Figure 3.11D), which is predominantly used to identify monocytic cells[32]. Furthermore, 35.23 ± 17.51% (HIV-positive group) and 52.88 ± 26.25% (HIV-negative, control group) of the aforementioned cells were proliferating based on CD71 expression (Figure 3.11E). Next, 36.24 ± 22.40% (HIV-positive group) and 48.37 ± 15.83% (HIV-negative, control group) of the "total CD33^{+} myeloid cells" expressed the CD15 marker (Figure 3.11F), which is used to identify granulocytes[32], with 57.28 ± 8.77% (HIV-positive group) and 66.06 ± 17.50% (HIV-negative, control group) of the same cells ("total CD33^{+} myeloid cells") proliferating based on CD71 expression (Figure 3.11G). Notably, 6.67 ± 10.57% (HIV-positive group) and 21.34 ± 16.78% (HIV-negative, control group) of the "total CD33^{+} myeloid cells" co-expressed CD14 and CD15 markers (Figure 3.11H). The function of the dual positive CD14/CD15 cells

(Figure 3.11H) is not yet well understood, however, Maneta and colleagues (2022) suggest that they work in an immunosuppressive capacity[34].

Next, 8.72 ± 7.37% (HIV-positive group) and 8.91 ± 8.25% (HIV-negative, control group) of the "viable" cells, expressed high levels of the CD41 marker (Figure 3.11I), which is used to identify megakaryocytes[32].

As mentioned before, the percentage of lymphoid cells present in the "viable" region was assessed by identifying cells that express CD3, CD19, CD20, or CD56. Two distinct lymphoid cell populations were identified based on the intensity of expression. $Lymph^{+}$ cells displayed low/intermediate expression levels of the lymphoid markers, while the $Lymph^{++}$ cells displayed high levels of expression, i.e., showed increased MFI levels. The $Lymph^{+}$ precursor encompasses 8.97 ± 4.92% (HIV-positive group) and 8.36 ± 5.98% (HIV-negative, control group) of the "viable" cells (Figure 3.11J). Of the $Lymph^{+}$ cells, 35.62 ± 8.56% (HIV-positive group) and 44.37 ± 27.57% (HIV-negative, control group) were proliferating, based on CD71 expression (Figure 3.11K). Next, 2.90 ± 2.24% (HIV-positive group) and 3.74 ± 5.29% (HIV-negative, control group) of the "viable" cells were $Lymph^{++}$ (Figure 3.11L). While 93.06 ± 9.74% (HIV-positive group) and 95.56 ± 5.24% (HIV-negative, control group) of the $Lymph^{++}$ cells were proliferating based on CD71 expression (Figure 3.11M). No statistical significance was observed between the study groups for any of the populations investigated.



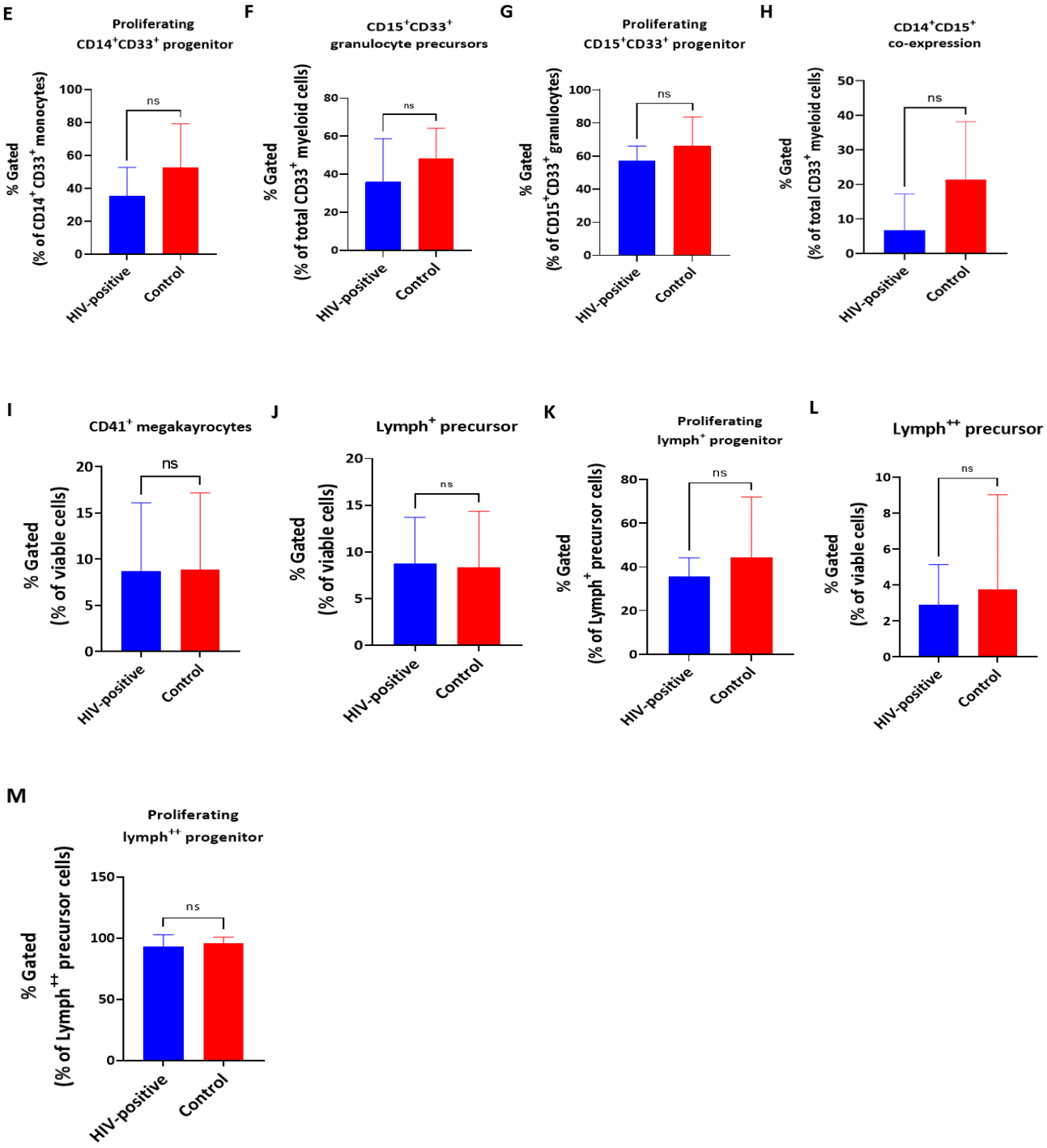


Figure 3.11: Quantification of the erythroid, myeloid, and lymphoid precursors and progeny compared between treatment-naïve, HIV-positive, and HIV-negative (control) groups. Bar graphs were used to demonstrate the mean percentage of either viable cells or as indicated by the y-axis title. Control refers to HIV-negative (control) group. The error bars indicate the standard deviation for both groups. (A-B) Erythroid progeny. (C-I) Myeloid progeny and their respective proliferating cells. (J-M) Lymphoid precursors and their proliferating lymphoid progenitors.CD, cluster of differentiation; HIV, human immunodeficiency virus; Lymph, lymphocyte; ns, non-significant.

3.5. Discussion

Haematological abnormalities, such as anaemia, thrombocytopenia, and neutropenia are frequently associated with HIV infection and disease progression[35]. The occurrence of HIV-associated haematological abnormalities, such as cytopenia, has been suggested as an indicator of ongoing dysregulated haematopoiesis in the BM[36, 37].

All blood cell lineages originate from HSPCs that reside in the BM. Under steady-state conditions, a small proportion of HSPCs is released from the BM into the peripheral blood, retaining the ability to return to the BM[4, 38]. These peripheral blood HSPCs are referred to as circulating HSPCs.

The purpose of this chapter was to determine the frequency of circulating HSPCs present in the peripheral blood of treatment-naïve HIV-positive and HIV-negative (control) patients and to further delineate on their functionality i.e., are they able to differentiate into the various blood cell types. The data obtained in this chapter demonstrate a significant decrease in the frequency circulating $Lin^{-}CD34^{++}$ HSPCs in the presence of HIV infection. Furthermore, there were no distinct differences in the phenotype of the circulating $Lin^{-}CD34^{++}$ HSPCs between both groups. Moreover, the circulating $Lin^{-}CD34^{++}$ HSPCs maintained their functionality as demonstrated by the production of viable CFU-GM, CFU-GEMM and BFU-E colonies in both treatment-naïve HIV-positive and HIV-negative (control) groups. With no differences being observed in the phenotype of the resultant colonies in either group.

Several studies have shown that circulating HSPCs are increased during host defence responses, and stress[39,40]. Redd and colleagues (2007) reported a significant increase in the circulating $CD34^{+}$ count in the presence of HIV infection [41]. Similarly, to the present study, the cohort investigated by Redd and colleagues (2007) were treatment-naïve, HIV-positive, however the findings Redd and colleagues (2007) is in contrast to what was observed in this study. Our data demonstrated a significantly lower frequency in percentage and absolute cell count of the circulating $Lin^{-}CD34^{++}$ HSPCs in the HIV-positive cohort compared to the control group (Figure 9A and B, respectively).

Our findings align with the observations of Sauce and colleagues (2011), who noted a decrease in the number of circulating $CD34^{+}$ HSPCs in an HIV-positive 'elite controller (EC) progressors'

group. They defined this group as individuals with long-term, undetectable infection and a gradual decrease in $CD4^+$ T cell count compared to healthy controls[42]. Bordoni and colleagues (2015) observed that circulating Lin^-CD34^+ HSPCs are decreased at baseline [prior to the initiation of combination antiretroviral therapy (cART)] which increase over time with cART usage[43]. Adams and colleagues (1999) observed a lower percentage of circulating $CD34^+$ cells in a treatment-naïve HIV-positive cohort, which significantly increased upon the initiation of cART when monitored over a six-month period[44].

Notably, Menkova-Garnier and colleagues (2016) reported non-significant differences between the frequency of peripheral $CD34^+$ HSPCs between HIV-negative (control) participants, and immunological responders (high $CD4^+$ T cell recovery) and non-responders (poor $CD4^+$ T cell recovery)[45]. Alternatively, Costantini and colleagues (2009) reported a non-significant difference in the frequency of peripheral blood $CD34^+$ cells at baseline and after treatment in the HIV-positive group when compared with HIV-negative controls[46].

It is important to note that the HIV-positive cohorts investigated in the aforementioned studies at enrolment were treatment-naïve and consisted of a heterogenous population of both male and females who were not pregnant. Furthermore, upon initiation of treatment, the HIV-positive cohorts were followed throughout the duration of the respective studies. Whereas, in the present study, individuals were not followed-up once treatment was initiated. The focus of this study was to report on findings of a treatment-naïve cohort. The impact of ARVs on the parameters reported fell outside the scope of this study.

The transmembrane phosphoglycoprotein, CD34, was used to identify the circulating HSPCs in the peripheral blood. Cluster of differentiation-34 expression decreases as the differentiation cascade continues, with higher CD34 expression indicating a more immature state of the cells[47]. In addition to expressing CD34, HSPCs do not express markers associated with lineage commitment, such as CD3, CD14, CD16, CD19, CD20, and CD56, resulting in the Lin-negative phenotype. Of these Lin^-CD34^+ cells, the $CD38^+CD133^-CD117^+$ and $CD38^+CD133^+CD117^+$ were the dominating phenotypes (Table 3.6). The CD38 marker is associated with the initiation of HSPC differentiation, and is strongly expressed by lineage committed haematopoietic progenitors, as well as mature lymphocytes[48]. During HIV infection, increased expression of CD38 on lymphocytes is indicative of ongoing abnormal T

cell activation and disease progression, as CD38 expression (on $CD8^+$ T cells) directly correlates with plasma viral levels[48, 49]. Uncommitted, immature haematopoietic stem cells (HSCs) lack CD38 expression[50, 51]. The dominant phenotypes ($CD38^+CD133^-CD117^+$ and $CD38^+CD133^+CD117^+$) observed in this study, suggest that circulating HSPCs initiated differentiation, but had not yet undergone lineage commitment. The CD133 expression is predominantly associated with immature HSPCs[52], however, Cimato and colleagues (2019) reported expression to be split between $CD133^+$ and $CD133^-$ within the immature HSPC population (classified as $CD34^+CD38^{dim}$) and further found downstream multi-potent progenitors (MPPs) to be predominantly $CD133^-$ in healthy individuals[53]. The CD117 marker (c-kit) is expressed brightly by up to 50% of BM-derived $CD34^+$ HSPCs, ranging from immature to lineage committed cells[54,55]. Cluster of differentiation-117 is crucial in modulating HSPC function, as its interactions with stem cell factor (SCF) mediate HSPC proliferation and differentiation[56]. Circulating $CD34^+$ HSPCs are classified as short-term (ST)-HSPCs due to their finite self-renewal potential of approximately one month, as opposed to their BM-residing counterparts, long-term (LT)-HSPCs, which have an approximate self-renewal potential of three to four months[57, 58]. Maillard and colleagues (2020) demonstrated this using the long-term culture-initiating cell (LTC-IC) assay. They reported a striking depletion in differentiation capacity of $CD34^+CD38^-CD45^+CD117^{low/neg}$ cells within one month whilst their counterparts, $CD34^+CD38^-CD45^+CD117^{hi}$ cells produced cobblestone-area forming cells for up to five months. Taking all of this in consideration, circulating HSPCs are likely to be a heterogeneous population of cells at different stages of differentiation.

In this study, we found no difference in the viability of the Lin^-CD34^{++} HSPCs between the HIV-positive and control groups (Tables 3.7A and B). Next, the functionality of the circulating HSPCs was determined. The Lin^-CD34^{++} circulating HSPCs, irrespective of the HIV status of their source, were able to form CFU-GM, CFU-GEMM and BFU-E colonies, with no statistically significant differences observed between the two study groups. Phenotypic characterisation of the colonies formed further revealed no statistically significant differences between the HIV-negative and HIV-positive cohorts. Bordoni and colleagues (2020) reported a decrease in the colony-forming abilities (CFA) of $CD34^+$ HSPCs from the BM of HIV-positive individuals on steady ART regimes[59]. The HIV-positive cohort investigated in the Bordoni and colleagues (2020) study consisted of both males and females (not pregnant) which achieved virological

suppression. The authors used similar experimental techniques in the assessment of the CFA of $CD34^+$ HSPCs that were used in this study. Furthermore, negative correlations were observed by Redd and colleagues (2007) between (i) the circulating viral p24 antigen levels and the frequency of the $CD34^+$ cells and (ii) between the circulating viral p24 antigen levels and the CFA of the $CD34^+$ cells[41]. Sauce and colleagues (2011) observed that circulating HSPCs isolated from HIV-positive EC progressors formed significantly less 'white' colonies CFU-GM and CFU-GEMM compared to the circulating HSPCs of non-progressors[42]. The lack of statistically significant differences observed in this study can be attributed to the limited number of patients in each group, as well as the high variability observed between patients in each group.

Overall, the data demonstrate that in the presence of HIV, circulating Lin^-CD34^{++} HSPCs are decreased at baseline but maintain their functionality. The lower frequencies observed may be due to circulating HSPCs returning to the BM at a faster rate to assist in repairing HIV-induced damage of the BM. The occurrence of HIV-associated haematological abnormalities is indicative of the pivotal role the BM plays in HIV replication and persistence[60]. As such, to ensure that steady-state haematopoiesis in the BM is restored, efficient homing of circulating HSPC is required[61]. Alternatively, the lower frequencies observed may be due to increased rates of migration to the thymus in an attempt to compensate for the HIV-associated loss of $CD4^+$ T cells. Guo and colleagues (2016) demonstrated that the levels of $CD4^+$ T cells in treatment-naïve HIV-positive individuals directly correlated to the CFA of HSPCs[62]. Furthermore, Redd and colleagues (2007) observed a positive correlation between the frequency of $CD34^+$ cells and $CD4^+$ T cell count in the HIV-positive cohort, but this positive correlation was absent in the HIV-negative control group[41].

In conclusion, the observed decrease in circulating Lin^-CD34^{++} HSPCs was likely due to the presence of HIV infection, dysregulating steady-state haematopoiesis and in so doing, impacting circulating Lin^-CD34^{++} HSPC numbers. A limitation of this study is that the treatment-naïve, HIV-positive participants were not followed up after the initiation of treatment to observe the response of the Lin^-CD34^{++} cell frequency during initiation of ART. Furthermore, the participants in the study were pregnant, and pregnancy itself induces its own physiological changes onto the haematopoietic system.

3.6. References

1. Comazzetto S, Shen B, Morrison SJ. Niches that regulate stem cells and hematopoiesis in adult bone marrow. Dev. Cell. 2021; 56(13):1848-60.

2. Kouroukli O, Symeonidis A, Foukas P, Maragkou MK, Kourea EP. Bone Marrow Immune Microenvironment in Myelodysplastic Syndromes. Cancers (Basel). 2022; 14(22):5656.

3. Hérault L, Poplineau M, Mazuel A, Platet N, Remy É, Duprez E. Single-cell RNA-seq reveals a concomitant delay in differentiation and cell cycle of aged hematopoietic stem cells. BMC Biol. 2021; 19(19):1-20.

4. Mende N, Laurenti E. Hematopoietic stem and progenitor cells outside the bone marrow: where, when, and why. Exp. Hematol. 2021; 104:9-16.

5. Sioud M. Microbial sensing by haematopoietic stem and progenitor cells: Vigilance against infections and immune education of myeloid cells. Scand. J. Immunol. 2020; 92(5):e12957.

6. Carrelha J, Meng Y, Kettyle LM, Luis TC, Norfo R, Alcolea V*, et al.* Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. Nature. 2018; 554(7690):106-11.

7. Loughran SJ, Haas S, Wilkinson AC, Klein AM, Brand M. Lineage commitment of hematopoietic stem cells and progenitors: insights from recent single cell and lineage tracing technologies. Exp. Hematol. 2020; 88:1-6.

8. Kato. H, Igarashi. K. To be red or white: lineage commitment and maintenance of the hematopoietic system by the ''inner myeloid''. Haematologica. 2019; 104(10):1919-27.

9. Bae J, Choi S-P, Isono K, Lee JY, Park S-W, Choi C-Y*, et al.* Phc2 controls hematopoietic stem and progenitor cell mobilization from bone marrow by repressing *Vcam1* expression. Nat Commun. 2019; 10(1):3496.

10. Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp. Hematol. 2002; 30(9):973-81.

11. Ratajczak MZ. Circulating Stem Cells in Physiology and Pathology - Recent Studies Published in Stem Cell Reviews and Reports. Stem Cell Rev Rep. 2018; 14(5):627-8.

12. Wang P, Wang J, Li YH, Wang L, Shang HC, Wang JX. Phenotypical Changes of Hematopoietic Stem and Progenitor Cells in Sepsis Patients: Correlation With Immune Status? Front. Pharmacol. 2020; 11(640203):1-9.

13. Chavakis T, Wielockx B, Hajishengallis G. Inflammatory Modulation of Hematopoiesis: Linking Trained Immunity and Clonal Hematopoiesis with Chronic Disorders. Annu. Rev. Physiol. 2022; 84:183-207.

14. Xu Y, Zeng X, Zhang M, Wang B, Guo X, Shan W*, et al.* Efficient expansion of rare human circulating hematopoietic stem/progenitor cells in steady-state blood using a polypeptide-forming 3D culture. Protein Cell. 2022; 13(11):808-24.

15. de Kruijf EJF, Fibbe WE, van Pel M. Cytokine-induced hematopoietic stem and progenitor cell mobilization: unraveling interactions between stem cells and their niche. Ann. N. Y. Acad. Sci. 2020; 1466(1):24-38.

16. Durandt C, Potgieter JC, Mellet J, Herd C, Khoosal R, Nel JG*, et al.* HIV and haematopoiesis. S. Afr. Med. J. 2019; 109(8b):40-5.

17. Banfi G, Salvagno GL, Lippi G. The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes. Clin. Chem. Lab. Med. 2007; 45(5):565-76.

18. Oldaker TA. Quality Control in Clinical Flow Cytometry. Clin. Lab. Med. 2007; 27(3):671-85.

19. Beckman Coulter Inc [Internet]. VersaComp™ Antibody Capture Bead Kit. Beckman Coulter Inc; [updated 2013/08/01; cited 2023/01/06]. Available from: https://www.beckman.com/search#q=B22804&t=coveo-tab-techdocs

20. Cunliffe J, Derbyshire N, Keeler S, Coldwell R. An approach to the validation of flow cytometry methods. Pharm. Res. 2009; 26(12):2551-7.

21. Velten L, Haas SF, Raffel S, Blaszkiewicz S, Islam S, Hennig BP*, et al.* Human haematopoietic stem cell lineage commitment is a continuous process. Nat. Cell Biol. 2017; 19(4):271-81.

22. Hughes MR, Canals Hernaez D, Cait J, Refaeli I, Lo BC, Roskelley CD*, et al.* A sticky wicket: Defining molecular functions for CD34 in hematopoietic cells. Exp. Hematol. 2020; 86:1-14.

23. Mizokami T, Hisha H, Okazaki S, Takaki T, Wang XL, Song CY*, et al.* Preferential expansion of human umbilical cord blood-derived CD34-positive cells on major histocompatibility complex-matched amnion-derived mesenchymal stem cells. Haematologica. 2009; 94(5):618-28.

24. Cato MH, Yau IW, Rickert RC. Magnetic-based purification of untouched mouse germinal center B cells for *ex vivo* manipulation and biochemical analysis. Nat. Protoc. 2011; 6(7):953-60.

25. STEMCELL™ Technologies. Human Colony-Forming Unit (CFU) Assays Using MethoCult™ Technical manual. Vancouver, Canada. 2023; Document number: 10000005589. [updated:

2023/02/01. cited: 2023/10/01.] Available from: https://cdn.stemcell.com/media/files/manual/10000005589-MAN_02.pdf

26. STEMCELL™ Technologies. MethoCult™ SH H4436: Serum-free methylcellulose-based medium with recombinant cytokines for human cells. Product information sheet. Vancouver, British Columbia, Canada. 2023; Document number: 29540. [updated: 2018/01/01. cited: 2023/10/01.] Available from: https://cdn.stemcell.com/media/files/pis/29540-PIS_2_3_2.pdf

27. STEMCELL™ Technologies. Wallchart: Human Hematopoietic Progenitors. Identification of Colonies Derived from Human Hematopoietic Progenitor Cells. Vancouver, British Columbia 2018; Document number: 29877. [updated: 2023/01/01. cited: 2023/10/01.] Available from: https://www.stemcell.com/media/files/wallchart/WA10002-Colonies_Derived_Human_Hematopoietic_Progenitors.pdf

28. Wognum AW, Szilvassy SJ. Hematopoietic Stem and Progenitor Cells (HSPCs): Isolation, Culture, and Assays. Educational Materials [Internet]. 2015; version 6.0.0. Available from: https://cdn.stemcell.com/media/files/minireview/MR29068-Hematopoietic_Stem_and_Progenitor_Cells.pdf.

29. López-Chicón P, Riba-Tietz T, Fariñas O, Gelber P-E, Casaroli-Marano R-P, Vilarrodona A. A Comparative Study Using Fluorescent Confocal Microscopy and Flow Cytometry to Evaluate Chondrocyte Viability in Human Osteochondral Allografts. Bioengineering. 2022; 9(10):515.

30. Luchini C, Parcesepe P, Nottegar A, Parolini C, Mafficini A, Remo A*, et al.* CD71 in Gestational Pathology: A Versatile Immunohistochemical Marker With New Possible Applications. Appl. Immunohistochem. Mol. Morphol. 2016; 24(3):215-20.

31. Marsee DK, Pinkus GS, Yu H. CD71 (Transferrin Receptor): An Effective Marker for Erythroid Precursors in Bone Marrow Biopsy Specimens. Am. J. Clin. Pathol. 2010; 134(3):429-35.

32. Naeim F, Rao NP, Song SX, Phan RT. Principles of Immunophenotyping [Internet]. Atlas of Hematopathology (Second Edition): Academic Press; 2018. p. 29-56. [cited: 2023/10/26]. Available from: https://www.sciencedirect.com/science/article/pii/B9780128098431000024

33. Garnache-Ottou F, Chaperot L, Biichle S, Ferrand C, Remy-Martin J-P, Deconinck E*, et al.* Expression of the myeloid-associated marker CD33 is not an exclusive factor for leukemic plasmacytoid dendritic cells. Blood. 2005; 105(3):1256-64.

34. Maneta E, Fultang L, Taylor J, Pugh M, Jenkinson W, Anderson G*, et al.* G-CSF induces $CD15^{+}CD14^{+}$ cells from granulocytes early in the physiological environment of pregnancy and the cancer immunosuppressive microenvironment. Clin Transl Immunology. 2022; 11(5):e1395.

35. Fan L, Li C, Zhao H. Prevalence and Risk Factors of Cytopenia in HIV-Infected Patients before and after the Initiation of HAART. Biomed Res Int. 2020; 2020(3132589):1-10.

36. Vishnu P, Aboulafia DM. Haematological manifestations of human immune deficiency virus infection. Br. J. Haematol. 2015; 171(5):695-709.

37. Abdullah I, Subramony N, Musekwa E, Nell EM, Alzanad F, Chetty C*, et al.* Indications and diagnostic value of bone marrow examination in HIV-positive individuals: A 3-year review at Tygerberg Hospital. S Afr J Infect Dis. 2021; 36(1):273.

38. Alvarez P, Carrillo E, Velez C, Hita-Contreras F, Martinez-Amat A, Rodriguez-Serrano F*, et al.* Regulatory systems in bone marrow for hematopoietic stem/progenitor cells mobilization and homing. Biomed Res Int. 2013; 2013(312656):1-12.

39. Kumari A, Golan K, Khatib-Massalha E, Kollet O, Lapidot T. Mechanisms of Hematopoietic Stem and Progenitor Cells Bone Marrow Homing and Mobilization [Internet]. In: Bonnet D, editor. Advances in Stem Cells and their Niches: Elsevier; 2017. p. 85-121. [cited: 2023/11/4]. Available from: https://www.sciencedirect.com/science/article/pii/S2468509716300033

40. Heidt T, Sager HB, Courties G, Dutta P, Iwamoto Y, Zaltsman A*, et al.* Chronic variable stress activates hematopoietic stem cells. Nat. Med. 2014; 20(7):754-8.

41. Redd AD, Avalos A, Phiri K, Essex M. Effects of HIV type 1 infection on hematopoiesis in Botswana. AIDS Res. Hum. Retroviruses. 2007; 23(8):996-1003.

42. Sauce D, Larsen M, Fastenackels S, Pauchard M, Ait-Mohand H, Schneider L*, et al.* HIV disease progression despite suppression of viral replication is associated with exhaustion of lymphopoiesis. Blood. 2011; 117(19):5142-51.

43. Bordoni V, Casetti R, Viola D, Abbate I, Rozera G, Sacchi A*, et al.* Early ART in primary HIV infection may also preserve lymphopoiesis capability in circulating haematopoietic progenitor cells: a case report. J. Antimicrob. Chemother. 2015; 70(5):1598-600.

44. Adams GB, Pym AS, Poznansky MC, McClure MO, Weber JN. The *in vivo* effects of combination antiretroviral drug therapy on peripheral blood $CD34^+$ cell colony-forming units from HIV type 1-infected patients. AIDS Res. Hum. Retroviruses. 1999; 15(6):551-9.

45. Menkova-Garnier I, Hocini H, Foucat E, Tisserand P, Bourdery L, Delaugerre C*, et al.* P2X7 Receptor Inhibition Improves CD34 T-Cell Differentiation in HIV-Infected Immunological Nonresponders on c-ART. PLoS Pathog. 2016; 12(4):e1005571.

46. Costantini A, Giuliodoro S, Butini L, Silvestri G, Leoni P, Montroni M. Abnormalities of erythropoiesis during HIV-1 disease: a longitudinal analysis. J. Acquir. Immune Defic. Syndr. 2009; 52(1):70-4.

47. AbuSamra DB, Aleisa FA, Al-Amoodi AS, Jalal Ahmed HM, Chin CJ, Abuelela AF*, et al.* Not just a marker: CD34 on human hematopoietic stem/progenitor cells dominates vascular selectin binding along with CD44. Blood Adv. 2017; 1(27):2799-816.

48. Lu L, Wang J, Yang Q, Xie X, Huang Y. The role of CD38 in HIV infection. AIDS Res. Ther. 2021; 18(11):1-14.

49. José Miguel Benito ML, Sara Lozano, Pilar Martinez, Juan González-Lahoz, Vincent Soriano. CD38 expression on CD8 T lymphocytes as a marker of residual virus replication in chronically HIV-infected patients receiving antiretroviral therapy. AIDS Res. Hum. Retroviruses. 2004; 20(2):227-33.

50. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G*, et al.* Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science. 2016; 351(6269):aab2116.

51. Anjos-Afonso F, Currie E, Palmer HG, Foster KE, Taussig DC, Bonnet D. $CD34^-$ cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell Stem Cell. 2013; 13(2):161-74.

52. Sharma S, Cabana R, Shariatmadar S, Krishan A. Cellular volume and marker expression in human peripheral blood apheresis stem cells. Cytometry Part A. 2008; 73A(2):160-7.

53. Cimato TR, Conway A, Nichols J, Wallace PK. CD133 expression in circulating hematopoietic progenitor cells. Cytometry B Clin. Cytom. 2019; 96(1):39-45.

54. Maillard L, Sanfilippo S, Domenech C, Kasmi N, Petit L, Jacques S*, et al.* $CD117^{hi}$ expression identifies a human fetal hematopoietic stem cell population with high proliferation and self-renewal potential. Haematologica. 2020; 105(2):e43-e7.

55. Carvalho JM, Souza MK, Buccheri V, Rubens CV, Kerbauy J, Oliveira JS. CD34-positive cells and their subpopulations characterized by flow cytometry analyses on the bone marrow of healthy allogenic donors. Sao Paulo Med. J. 2009; 127(1):12-8.

56. Shin JY, Hu W, Naramura M, Park CY. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. J. Exp. Med. 2014; 211(2):217-31.

57. Anjos-Afonso F, Bonnet D. Human $CD34^+$ hematopoietic stem cell hierarchy: how far are we with its delineation at the most primitive level? Blood. 2023; 142(6):509-18.

58. Cheng H, Zheng Z, Cheng T. New paradigms on hematopoietic stem cell differentiation. Protein Cell. 2020; 11(1):34-44.

59. Bordoni V, Tartaglia E, Refolo G, Sacchi A, Grassi G, Antinori A*, et al.* Per2 Upregulation in Circulating Hematopoietic Progenitor Cells During Chronic HIV Infection. Front Cell Infect Microbiol. 2020; 10(362):1-8.

60. Alexaki A, Wigdahl B. HIV-1 Infection of Bone Marrow Hematopoietic Progenitor Cells and Their Role in Trafficking and Viral Dissemination. PLoS Pathog. 2008; 4(12):e1000215.

61. Zhang W, Ruan L. Recent advances in poor HIV immune reconstitution: what will the future look like? Front. Microbiol. 2023; 14(1236460):1-14.

62. Guo X, He S, Lv X, Ding H, Li S, Kang J*, et al.* The role of HIV-1 in affecting the proliferation ability of HPCs derived from BM. J. Acquir. Immune Defic. Syndr. 2016; 71(5):467-73.

Chapter 4: Enumeration of immune cell and myeloid derived suppressor cell populations

4.1. Introduction

The immune cell populations that constitute the immune system are generated by haematopoietic stem and progenitor cells (HSPCs), the drivers of haematopoiesis. During the haematopoietic differentiation cascade, immune cells are produced by common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) (Figure 2.1; Chapter 2). The former produce granulocytes (basophils, eosinophil, and neutrophils), monocyte/macrophages, dendritic cells (DCs), and mast cells, whilst the latter produces B and T lymphocytes as well as natural killer cells (NK)[1].

Immune cells secrete a wide variety of pro- and anti-inflammatory cytokines and chemokines, which impact on haematopoietic cell function. In the presence of human immunodeficiency virus (HIV), the cell signalling becomes dysregulated, skewing HSPC differentiation[2]. One example of this is the HIV-mediated decrease in granulocyte-colony stimulating factor (G-CSF), which impairs the differentiation capacity of granulocyte-macrophage progenitors (GMP) that produce neutrophils, consequently causing neutropenia[2].

The presence of HIV infection causes a significant depletion of the cluster of differentiation (CD)4^+ T helper cells, an expansion of $CD8^+$ cytotoxic T cells, and chronic immune activation and inflammation, resulting in immune dysfunction[3-5]. This HIV-mediated destruction of the $CD4^+$ T cells is detrimental to the immune system as this population is key in modulating adaptive immune responses through the recruitment of various immune cells, secretion of chemokine and cytokines, as well as exerting its own cytotoxic activity[6-8]. Furthermore, the persistent, elevated levels of $CD8^+$ T cells observed in HIV infection results in T cell exhaustion, where there is a loss of anti-viral activity, an increase in expression of inhibitory receptors, and an impediment of the differentiation of effector cells into functional memory cells[9]. Cluster of differentiation-8^+ T cell exhaustion is damaging to the immune system as $CD8^+$ T cells are essential in the execution of an anti-viral immune response[10]. As such, the HIV-mediated dysfunction of T cells is linked with disease progression and incomplete immune reconstitution[11].

The progression of HIV is monitored using the CD4$^+$ T helper cell count (CD4 count), and viral load (VL)[12]. According to the Centers of Disease Control and Prevention (CDC), a normal CD4 count is ≥500 cells per microlitre (cells/µL) (HIV infection stage 1). In chronic HIV infection, CD4 counts range between 200-499 cells/µL (HIV infection stage 2). In the absence of treatment, CD4 counts may decrease below 200 cells/µL (HIV infection stage 3), which is classified as acquired immunodeficiency syndrome (AIDS)[13]. Viral replication is inhibited upon initiation of combination antiretroviral therapy (cART), resulting in an increase in the CD4 count and a decrease in the viral load (VL)[14-16]. Furthermore, cART supports immune reconstitution, as both memory and naïve CD4$^+$ T cell frequencies increase whilst T cell activation marker levels decrease, allowing the immune system to return to homeostasis[16].

However, there remains residual levels of inflammation and immune activation (immune dysfunction) as a consequence of low-level viral replication that does not allow for complete normalisation[17, 18]. This immune dysregulation is monitored by the CD4$^+$ T helper/CD8$^+$ cytotoxic T cell ratio (CD4/CD8 ratio)[17]. In immunocompetent individuals, the CD4/CD8 ratio is typically used to assess immunosenescence and is generally ≥1.5. However, in treatment-naïve HIV-positive individuals the ratio becomes inverted (<1)[19, 20]. The CD4/CD8 ratio has been suggested as a biomarker for disease progression, as it is associated with T cell activation, immunosenescence, chronic inflammation, and occurrence of non-AIDS-defining events, such as cardiovascular disease, liver disease, malignancies among others[19, 20].

In the bone marrow (BM), CMPs give rise to 'immature myeloid cells' (IMCs). In healthy individuals, these IMCs enter the periphery and differentiate into functional DCs, macrophages, and granulocytes[21, 22]. However, in the presence of pathological conditions, such as cancer, chronic inflammation, and HIV infection, IMCs follow a different differentiation pathway and differentiate into myeloid-derived suppressor cells (MDSCs)[21, 22]. This results in elevated levels of MDSCs in circulation[21, 22].

Myeloid derived suppressor cells are defined as a heterogeneous population of immature and mature myeloid cells, which exert immunosuppressive activity on T cells and NK cells[22, 23]. Two major subsets of MDSCs have been described, namely monocytic-MDSCs (M-MDSCs) and polymorphonuclear-MDSCs (PMN-MDSCs)[24]. There is also a third subset, known as the immature subset of MDSCs (i-MDSCs)[24]. The phenotype of the three subsets is outlined in

Table 2.4: Chapter 2. Generally, MDSCs express the CMP phenotype ($CD11b^{+}CD33^{+}HLA\text{-}DR^{-/lo}$) and are further distinguished by the expression of the CD14 and CD15 markers, where M-MDSCs express CD14, PMN-MDSCs express CD15 and the i-MDSCs express neither CD14 nor CD15[22, 25].

The expansion of PMN-MDSCs is suggested to occur during the first six months of infection [primary HIV infection (PHI)] whilst M-MDSC expansion is suggested to occur in the chronic stages of HIV infection (CHI)[11]. However, MDSCs have been extensively researched in cancer settings, and there is a need for more research pertaining to MDSCs in HIV. Earlier studies such as Vollbrecht and colleagues (2012) reported an increase in the frequency of PMN-MDSCs in a treatment-naïve, HIV-positive cohort, when compared to HIV-negative controls[26]. The PMN-MDSC levels decreased after six weeks' treatment[26].

To obtain a better understanding on the expansion of HIV, Garg and colleagues (2013) generated two separate cocultures: (1) PBMNCs with heat-inactivated HIV and (2) PBMNCs and infectious HIV[27]. Their results demonstrated that both heat-inactivated and infectious HIV were capable of stimulating an expansion in MDSCs and an increase in the pro-inflammatory cytokine, interleukin (IL)-6 *in vitro*[27]. The investigators found a positive correlation between M-MDSC frequency and levels of IL-6, suggesting that IL-6 plays an essential role in the expansion of M-MDSCs in an HIV-positive individual[25]. Singh and colleagues (2021) reported higher frequencies of M-MDSCs in a cohort of HIV-positive, virally suppressed individuals, mediated by IL-6 when compared to HIV-negative controls[25]. Notably, Lei and colleagues (2016) observed increased frequencies of M-MDSCs in individuals co-infected with Hepatitis C virus (HCV) and HIV when compared to non-infected controls[28]. Furthermore, the individuals mono-infected with HIV presented with MDSC levels greater than HCV mono-infected individuals but comparable to HCV/HIV co-infected individuals[28]. This result is indicative of the role HIV plays in MDSC expansion.

Myeloid derived suppressor cells exert their immunosuppressive properties on T cells and other immune cells through a combination of various mechanisms[29]. These include but are not limited to the production/expression of inducible nitric oxide synthase (iNOS), arginase 1 (ARG1), reactive oxygen species (ROS), phosphorylated signal transducer and activator of

transcription 3 (pSTAT-3), programmed death-ligand 1 (PD-L1), IL-4 receptor-α (IL-4Rα), IL-10 and, transforming growth factor-β (TGF-β)[29, 30].

The objective of this chapter was to quantify the major immune cell populations (T cells, B cells, NK cells, monocytes, and neutrophils) and the MDSC sub-populations in a treatment-naïve HIV-positive cohort and to compare it to HIV-negative (control) groups. This chapter will discuss the differences in frequency of the abovementioned cell populations and the impact of HIV infection on the immune system.

4.2. Methodology

4.2.1 Study design

The study design (*section 3.2.1*) for this chapter is described in Chapter 3: *Functionality of circulating CD34⁺ haematopoietic stem and progenitor cells.* All study participants enrolled were pregnant.

This chapter uses flow cytometry to quantify major immune cell populations (T cells, B cells, NK cells, monocytes, and neutrophils) the peripheral blood in a treatment-naïve, HIV-positive group (n=13) and compares it to HIV-negative (control; n=25) group. This chapter also investigates the quantity of MDSC sub-populations present in peripheral blood using flow cytometry in a treatment-naïve, HIV-positive group (n=8) and compared to HIV-negative (control) (n=18) group.

The MDSC panel was not performed on all samples donated as it became clear that more than 1 x 10^6 cells need to be analysed to have confidence in the MDSC results. It was not possible to increase the number of cells to be analysed without compromising the other assays that were performed. An additional ethylenediamine tetra-acetic acid (EDTA)-vacutainer tube of blood was required for processing to address this limitation. Above-mention is the reason for the decrease in the number of study participants in the MDSC sub-population investigation.

4.2.2. Study participant enrolment

The ethics approval (*section 3.2.2*), informed consent (*section 3.2.3.1*), HIV counselling and confirmation (*section 3.2.3.2*), and study participant demographics (*section 3.2.3.3*) for this study are described in Chapter 3: *Functionality of circulating CD34⁺ haematopoietic stem and progenitor cells.*

4.2.2. Experiments

The experimentation and distribution of peripheral blood samples for research purposes is also described in Chapter 3: *Functionality of circulating CD34⁺ haematopoietic stem and progenitor cell, sections 3.2.4* and *3.2.4.1,* respectively.

4.2.3. Basic immune profile

The DuraClone Basic Immunophenotyping kit (Beckman Coulter, Miami, FL, USA) was used to determine the frequency (%) and absolute counts (cells/µL) of the major immune cell populations as outlined in Table 4.1. One DuraClone Basic Immunophenotyping kit consists of 25 ready-to-use phenotyping tubes and three colour compensation sets. The resulting frequencies and absolute counts were compared between treatment-naïve, HIV-positive and HIV-negative (control) patients. The assay was performed on the CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA).

4.2.3.1. Colour compensation setup for basic immune profile

Each colour compensation set consists of eight ready-to-use tubes, each containing a single monoclonal antibody (mAB) conjugated with a fluorochrome present in the basic immunophenotyping test (ready-to-use combination) tube. Each tube contains an antibody conjugated to a different fluorochrome. The fluorochromes are listed in Table 4.1.

Peripheral blood (100 µL) was added to each tube. The tubes were vortexed for 5-10 seconds, followed by incubation for 15 minutes at room temperature (RT), protected from direct light exposure to prevent light dependent degradation of the fluorochromes[31]. After incubation, 2 millilitres (mL) of VersaLyse™ lysing solution (Beckman Coulter, Miami, FL, USA) was added and the tubes were vortexed for 10 seconds, followed by another 15 minutes incubation at RT. Thereafter, the tubes were centrifuged at 300 times gravitational force (x g) for 10 minutes,

using a SL16R centrifuge (Thermo Fisher Scientific, Waltham, MA USA). The supernatant was carefully discarded, and the cell pellet was resuspended in the remaining VersaLyse™ lysing solution. A wash step was performed by adding 3 mL of sterile phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4; Sigma-Aldrich, St Louis, MO, USA) to each tube, followed by centrifugation at 300 x g for 10 minutes. The supernatant was carefully discarded without disturbing the cell pellet, which was then resuspended in 300 µL of sterile PBS.

The *colour compensation setup* function on the CytoFLEX software was launched and the tubes were acquired to generate a colour compensation matrix, which was saved and applied to all patient samples.

Table 4.1: The panel of fluorochrome-conjugated monoclonal antibodies and the cell populations these markers identify.

.

Marker	Fluorochrome	Cell type
CD16	FITC	Neutrophils
CD56	PE	NK cells
CD19	ECD	B cells
7AAD	PC5.5	Viable cells (negative staining)
CD14	PC7	Monocytes
CD4	APC	T helper cells
CD8	APC-AF700	Cytotoxic T cells
CD3	APC-AF750	Total T cells
CD45	KO	Leukocytes (mature)
CD10	BV711	Neutrophils

APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; BV, Brilliant violet; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FITC, Fluorescein isothiocyanate; KO, Krome orange; NK, Natural killer; PE, Phycoerythrin; PC, Phycoerythrin-cyanine.

4.2.3.3. Sample preparation to obtain a basic immune profile

The sample was prepared by adding 100 µL of peripheral blood to the ready-to-use DuraClone Basic Immune Profile tube containing dried antibodies (proprietary formulation, Beckman Coulter). The antibodies in the DuraClone tube are listed in Table 4.1. 7-Aminoactinomycin D

(7-AAD) is not present in the ready-to-use DuraClone Basic Immune Profile tube and was added separately as described below.

After adding the blood to the tube, the tube was vortexed for 5-10 seconds, followed by incubation for 15 minutes at RT, protected from direct exposure to light. VersaLyse™ lysing solution (2 mL) was added to the tube, vortexed for 10 seconds, and incubated for another 15 minutes at RT. After incubation, the tube was centrifuged at 300 x g for 10 minutes and the supernatant carefully aspirated, taking care not to disturb the cell pellet at the bottom of the tube. The cell pellet was resuspended in the remaining supernatant and 3 mL of sterile PBS was added to the tube. The tube was centrifuged again at 300 x g for 10 minutes after which the supernatant was carefully aspirated and discarded. The cell pellet was resuspended in 250 µL of sterile PBS. Next, 10 µL of 7-AAD was added to the tube. 7-Aminoactinomycin D is a viability dye that allows for the identification of viable cells (cells that stain negative for 7-AAD) during sample analysis. Just before acquisition, 100 µL of Flow-count™ fluorospheres (Beckman Coulter, Miami, FL, USA) was added to allow for enumeration of the cell populations of interest. The tube was analysed using the CytoFLEX flow cytometer. Samples with viability greater than 75% were further processed, i.e., rest of experiments were performed. The colour compensation matrix (*section 4.2.3.1)* and standardised settings (*section 4.2.3.2.)* were applied before acquisition of the sample.

4.2.4. Isolation and enumeration of peripheral blood mononuclear cells

The isolation and enumeration of peripheral blood mononuclear cells (PBMNCs) is described in sections *3.2.3.2 and 3.2.3.3* respectively, in Chapter 3. Table 4.2 denotes the distribution of the major cell populations within peripheral blood. Neutrophils are the major cell population in peripheral blood contributing to >50% of all nucleated cells in the peripheral blood (Table 4.2). The total frequency of MDSCs has been reported to average in the range of 0.2-0.6%[32].

Table 4.2: Reference range percentages for immune cell populations in peripheral blood[33].

Cell population	Average frequency (%)
Total $CD3^+$ T cells	22.5±3.8
$CD4^+$ T cells	14.6±3.5
$CD8^+$ T cells	6.8±1.3
$CD16^+$ Neutrophils	53.8±6.1
$CD14^+$ Monocytes	8.4±1.3
$CD19^+$ B cells	5.2±2.3
$CD56^+$ NK cells	4.4±2.4
$CD3^+CD56^+$NK T cells	0.8±0.7

CD, Cluster of differentiation; NK, Natural killer.

4.2.4.1. Myeloid-derived suppressor cell frequency and absolute counts

4.2.4.1.1. Colour compensation setup for myeloid derived suppressor cells panel

A colour compensation matrix was generated using VersaComp™ Antibody Capture beads (as outlined in *section 3.2.4.4.1 Colour compensation setup for haematopoietic stem and progenitor cell panel; Chapter 3*). Ten flow cytometry tubes were prepared as listed in Table 4.3.

It is important to note that the phycoerythrin-cyanine5.5 (PC5.5) channel was used as a dump channel. This means that positive staining of all the markers in tube 5 (Table 4.3) were excluded from downstream analyses as MDSCs are reported to be negative for these markers. These markers should thus be excluded from the phenotypic assessment. In addition, the viability dye, 7-AAD, is also detected in the PC5.5 detector channel [excitation: 488 nanometres (nm) and emission detector: 690/50 bandpass]. Positive staining for 7-AAD indicates dead cells and thus formed part of the events that needed to be excluded from downstream analysis.

Table 4.3: Panel of fluorochrome-conjugated monoclonal antibodies used to set up the colour compensation matrix for the myeloid derived suppressor cell panel.

VersaComp™ Colour Compensation Tubes	Marker	Fluorochrome
Tube 1	Unstained	
Tube 2	CD45	FITC
Tube 3	CD34	PE
Tube 4	CD38	ECD
Tube 5	CD3 CD16 CD19 CD20 CD56 7-AAD	PC5.5
Tube 6	CD33	APC
Tube 7	CD14	APC-AF700
Tube 8	CD11b	APC-AF750
Tube 9	HLA-DR	Pacific Blue
Tube 10	CD15	KO

7-AAD, 7-Aminoactinomycin-D; APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FITC, Fluorescein isothiocyanate; HLA-DR, Human Leukocyte Antigen- DR; PE, Phycoerythrin; PC, Phycoerythrin-cyanine.

4.2.4.1.2. Standardisation setup for myeloid derived suppressor cell panel

An application-specific standardisation template was generated using Flow-Set™ Fluorospheres (outlined in *3.2.4.4.2 Standardisation setup for the haematopoietic stem and progenitor cell panel; Chapter 3*) with one-parameter histograms created in alignment with the mABs listed in Table 4.4. The standardised instrument settings (gains) were applied to the MDSC panel prior to sample acquisition using the CytoFLEX flow cytometer.

4.2.4.1.3. Sample preparation for the MDSC panel

To prepare the sample for the MDSC panel, the same staining approach as described in *Chapter 3: Functionality of CD34⁺ Haematopoietic stem and progenitor cells* (*section 3.2.4.4.3 Sample preparation and acquisition for the haematopoietic stem and progenitor cell panel*) was used, using the mABs listed in Table 4.4.

Flow-count™ fluorospheres (Beckman Coulter, Miami, USA) were added to the cell suspension stained with mABs as outlined in Table 4.4 at a ratio of 1:1 (Volume of cell suspension aliquoted: Flow-count™ fluorospheres). Analysis of a minimum of 1 x 10^6 PBMNCs was done in an attempt to ensure adequate enumeration of the MDSC sub-populations. The frequency (%) and absolute counts (cell/µL) of the MDSC sub-populations were obtained using the CytoFLEX flow cytometer.

Myeloid derived suppressor cells are viewed as rare cells. Thus, to ensure optimal detection of MDSCs and to ensure thorough interrogation of any MDSC sub-populations present, the entire cell suspension volume was acquired. There needed to be at least 25 – 50 events in a cell cluster before a cell population could be identified with confidence.

Table 4.4: The panel of fluorochrome-conjugated monoclonal antibodies and their volume used to enumerate the myeloid derived suppressor cells sub-populations.

Marker		**Fluorochrome**	**Volume of mAB (µL)/tube**
StemKit	CD45	FITC	10
	CD34	PE	
	CD15	KO	10
	CD38	ECD	5
	CD19	PC5.5	5
	CD20	PC5.5	5
	CD33	APC	5
	CD14	APC AF700	5
	CD11b	APC-AF750	5
	HLA-DR	Pacific Blue	5
	CD10	BV660	5
	CD3	PC5.5	2.5
	CD56	PC5.5	2.5
	CD16	PC7	1.5

APC, Allophycocyanin; APC-AF, Allophycocyanin-Alexa fluor; BV, Brilliant violet; CD, Cluster of differentiation; ECD, R-Phycoerythrin-Texas red®-X; FITC, Fluorescein isothiocyanate; HLA-DR, Human Leukocyte Antigen- DR; KO, Krome orange; PE, Phycoerythrin; PC, Phycoerythrin-cyanine. Volumes were determined through mAB titration experiments, performed by Dr Durandt, my supervisor. This was done before the start of the project.

4.2.4.1.4. Gating strategy for the MDSC panel

To outline the MDSC sub-populations ($Lin^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{low/-}/CD14^{+}$ or $CD15^{+}$; Table 2.4, Chapter 2) for downstream analysis, an ungated forward scatter (FSC) vs side scatter (SSC) plot was generated to identify the leukocytes (Figure 4.1A). Next, the viable cells and lineage-negative (Lin^{-}) cells were selected (Figure 4.1B) using a FSC vs PC5.5 (7-AAD and Lin^{-}) plot gated on leukocytes ("Leukocytes"), as both mature and immature MDSCs have a Lin^{-} phenotype[34].

As mentioned before, the PC5.5 (7-AAD and Lin^{+}) region served as a dump region to exclude unwanted cells (Lin^{+}) and dead cells (7-AAD positive) from the analysis. This was followed by the inclusion of expression of the CD16 using a FSC vs CD16-Phycoerythrin n-Cyanine (PC)-7 plot (Figure 4.1C). The expression of CD16 is helpful in distinguishing between the major MDSCs sub-populations as M-MDSCs highly express CD16 while PMN-MDSCs show intermediate expression of CD16[22].

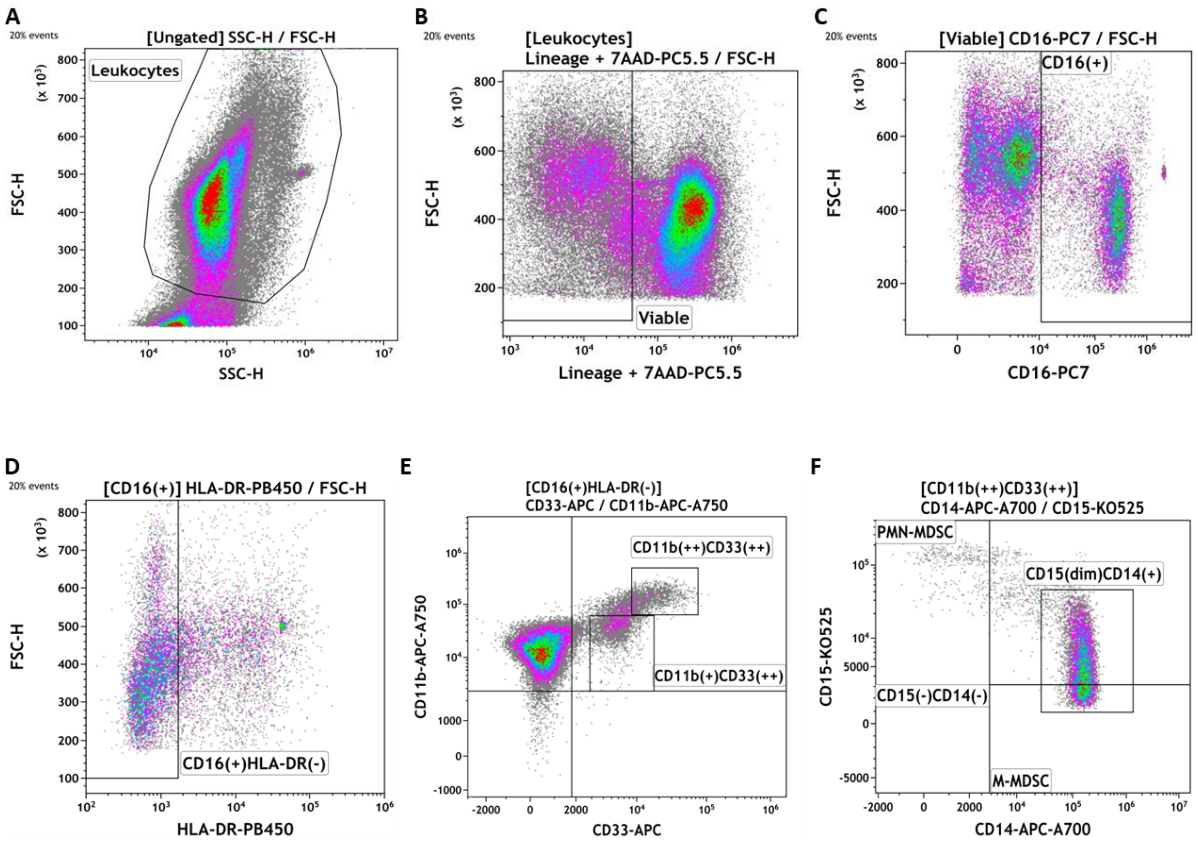
Following a sequential gating strategy that select for the phenotype reported for MDSCs, cells not expressing and/or weakly express human leukocyte antigen-DR (HLA-DR) were selected using an FSC vs HLA-DR-PB450 plot, gated on CD16-positive cells ("CD16(+)"; Figure 4.1D). MDSCs also express myeloid markers, CD11b and CD33[35], therefore, a CD11b-Allophycocyanin-Alexa fluor (APC-AF)-750 vs CD33-Allophycocyanin (APC) plot was generated, gated on "CD16(+)HLA-DR(-)" (Figure 4.1E), producing two distinct sub-populations – $Lin^{-}CD16^{+}HLA\text{-}DR^{low/-}CD11b^{++}CD33^{++}$ (shortened to $CD11b^{++}CD33^{++}$ from here-on) and $Lin^{-}CD16^{+}HLA\text{-}DR^{low/-}CD11b^{+}CD33^{+}$ (shortened to $CD11b^{+}CD33^{++}$ from here-on).

Two CD15-Krome orange (KO)-525 vs CD14-APC-AF700 plots were generated, gated on $CD11b^{++}CD33^{++}$ (Figure 4.1F) and $CD11b^{+}CD33^{++}$ (Figure 4.1G), respectively. This was used to observe the distribution of MDSC sub-populations between the two major phenotypes as MDSC sub-populations are classified based on their expression of CD14 and CD15. As mentioned earlier, PMN-MDSCs express the $CD15^{+}CD14^{-}$ phenotype whilst M-MDSCs expresses $CD15^{-}CD14^{+}$ and i-MDSCs, $CD15^{-}CD14^{-}$. As such, the frequency of the MDSC sub-populations present within the PBMNCs was determined using equation 4.1 below.

Equation 4.1: Determination of the true frequency of myeloid derived suppressor cell sub-populations present within peripheral blood mononuclear cells.

$$True\ frequency\ of\ MDSCs = \frac{Number\ of\ events\ for\ the\ respective\ MDSC\ sub-population}{Total\ number\ of\ \ events\ \ gated\ on\ the\ viable\ leukocyte\ population} x100$$

Low expression levels of CD15 were observed, as shown in Figure 4.1F, for the cells that expressed the $CD11b^{++}CD33^{++}$ phenotype. As these $CD14^{+}CD15^{-/dim}$ appeared as a uniform cluster, a region was created to encompass this population and the cells were phenotypically defined as $CD15^{-/dim}CD14^{+}$ (Figure 4.1F).



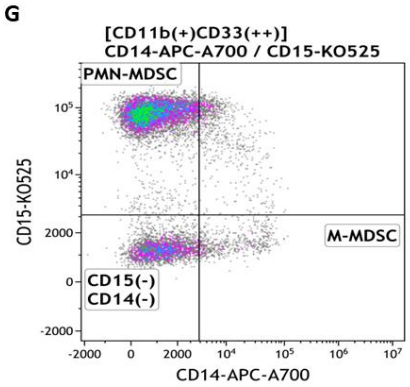


Figure 4.1: Phenotypic characterisation of myeloid derived suppressor cells sub-populations. Density plots were generated using Kaluza analysis version 2.1 software and compiled using Microsoft PowerPoint by T Mashingaidze. 7-AAD, 7-Aminoactinomycin-D; APC, Allophycocyanin; APC-A, Allophycocyanin-Alexa fluor; A, Area; CD, Cluster of differentiation; FSC, Forward scatter; H; Height; HLA-DR, Human Leukocyte Antigen – DR; KO, Krome orange; M-MDSC, Myeloid derived suppressor cells; PC, Phycoerythrin-cyanine; SSC, Side scatter.

4.2.5. Statistical analysis

The data was analysed using the Kaluza analysis software version 2.1 (Beckman Coulter). GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA, SA) was used to perform non-parametric Mann-Whitney U-tests to compare the continuous variables between the treatment-naïve, HIV-positive, and HIV-negative (control) groups. Data were presented as the median and interquartile range (IQR). All p-values were calculated as exact figures to account for ties in values, rather than approximating the p-value. Significance was reported as *, **, ***, and ****, indicating p-values of <0.05, <0.01, <0.001, and <0.0001, respectively.

A non-parametric Spearman's rank correlation test was performed to evaluate the relationship between the respective MDSC cell sub-populations and CD4 counts. The values were denoted as significantly different when $p<0.05$ (indicating two tailed P value).

4.3. Results

4.3.1. Enumeration of immune cell populations

The frequency (%) and absolute counts (cells/µL) of each population were compared between the treatment-naïve, HIV-positive and the HIV-negative (control) group. This was performed to obtain the $CD4^+$ T cell count of the HIV-positive group and further assess the impact of HIV-

1 on the immune system, i.e., whether there was an increase or decrease or negligible change in the immune populations present in peripheral blood between the two study groups.

The median (IQR) frequency (%) and absolute counts (cells/µL) of the various immune cell populations are reported in Tables 4.5A and B, respectively. The frequency is reported as a percentage of viable cells as indicated in Table 4.5A, whilst absolute cell count reports the exact cell count present within the whole blood sample. The data demonstrated no significant difference in frequency ($p=0.98$) and absolute count ($p=0.61$) of total $CD3^+$ T cells between the HIV-positive and -negative (control) groups. However, both the frequency ($p<0.0001$) and absolute count ($p=0.0009$) of the $CD4^+$ T cells was significantly lower in the HIV-positive group compared to the HIV-negative (control) group. The frequency of $CD8^+$ T cells was significantly higher ($p<0.0001$) in the HIV-positive group compared to the control group, while the absolute count was not significantly different ($p=0.17$) between the two groups.

Additionally, there were no significant differences between the HIV-positive and -negative (control) groups in terms of both frequency ($p=0.18$) and absolute count ($p=0.24$) of total $CD19^+$ B cells, as well as for the total $CD14^+$ monocyte frequency ($p=0.98$) and absolute count ($p=0.68$). Furthermore, there was no significant difference in the total $CD16^+$ neutrophils with regard to frequency ($p=0.52$) and absolute count ($p=0.78$) between the HIV-positive and -negative (control) groups.

Classical monocytes ($CD14^+CD16^-$) showed no significant differences in both frequency ($p=0.74$) and absolute cell count ($p=0.89$) between the two groups. However, intermediate monocytes ($CD14^+CD16^+$) were significantly lower in the HIV-positive group compared to the IHV-negative (control) group, both in frequency ($p=0.011$) and absolute cell count ($p=0.036$). Conversely, the non-classical monocytes ($CD14^{dim}CD16^+$) showed no significant differences between the HIV-positive and -negative (control) groups in frequency ($p=0.32$) and absolute cell count ($p=0.25$).

The frequency ($p=0.76$) and absolute cell count ($p=0.15$) of the total $CD56^+$ NK cells were not significantly different between the HIV-positive and -negative (control) groups. Within the NK population, the cytokine producing NK cells were significantly lower in the HIV-positive group than the HIV-negative (control) group for both frequency ($p=0.032$) and absolute count

(p=0.0066). However, the cytotoxic NK cells were not significantly different between the HIV-positive and -negative (control) groups in frequency (p=0.61) and absolute cell count (p=0.36).

Lastly, the total $CD3^+CD56^+$ NKT cells were not significantly different between the HIV-positive and -negative (control) groups for both frequency (p=0.93) and absolute count (p=0.43). Both the frequency (p=0.010) and absolute count (p=0.027) of the $CD4^+$ NKT sub-population was significantly lower in the HIV-positive group when compared to the HIV-negative (control) group. However, the $CD8^+$ NKT sub-population showed no significant differences between the HIV-positive and -negative groups in both frequency (p=0.13) and absolute counts (p=0.67).

Table 4.5A: Median (Interquartile range) of frequency of various immune cell populations present in peripheral blood, obtained using flow cytometric analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group.

	Expressed as a percentage of viable cells	**Median (IQR)**		
		HIV-positive (%) n=13	**Control (%) n=25**	**p-value**
Total $CD3^+$ T cells	Lymphocytes	73.90 (61.24-80.68)	73.86 (66.44-76.66)	0.98
$CD4^+$ T cells	Total $CD3^+$ T cells	20.90 (10.21-40.10)	56.49 (50.57-65.49)	<0.0001
$CD8^+$ T cells	Total $CD3^+$ T cells	67.71 (52.89-82.28)	35.89 (26.88-39.99)	<0.0001
Total $CD19^+$ B cells	Lymphocytes	6.64 (3.16-9.46)	8.11 (5.01-11.18)	0.18
Total $CD14^+$ Monocytes	Leukocytes	7.85 (6.21-9.80)	8.40 (6.29-9.76)	0.98
$CD14^+CD16^-$ Classical monocytes	Total $CD14^+$ Monocytes	56.10 (20.91-77.33)	50.49 (38.35-68.71)	0.74
$CD14^+CD16^+$ Intermediate monocytes	Total $CD14^+$ Monocytes	4.16 (1.27-17.29)	19.18 (8.42-35.63)	0.011
$CD14^{dim}CD16^+$ Non-classical monocytes	Total $CD14^+$ Monocytes	8.46 (5.42-15.38)	9.85 (8.11-18.40)	0.32
Total $CD16^+$ Neutrophils	Leukocytes	29.83 (9.78-35.76)	29.20 (19.07-38.61)	0.52

Table 4.5A continues.

Total CD56$^+$ NK cells	Lymphocytes	11.57 (4.97-19.28)	12.58 (7.64-17.20)	0.76
Cytokine producing NK cells	Total CD56$^+$ NK cells	1.14 (0.42-2.15)	1.89 (1.06-7.89)	0.032
Cytotoxic NK cells	Total CD56$^+$ NK cells	86.82 (63.99-91.96)	86.59 (75.12-94.77)	0.61
Total CD3$^+$CD56$^+$ NKT cells	Total CD3$^+$ T cells	9.88 (5.33-12.23)	8.62 (5.70-14.94)	0.93
CD4$^+$ NKT cells	Total CD3$^+$CD56$^+$ NKT cells	2.35 (1.12-9.27)	8.20 (5.10-12.78)	0.010
CD8$^+$ NKT cells	Total CD3$^+$CD56$^+$ NKT cells	73.12 (65.85-84.88)	67.27 (52.51-78.15)	0.13

CD, Cluster of differentiation; HIV, Human immunodeficiency virus; IQR, Interquartile range; NK, Natural killer; NKT, Natural killer T cells.

Table 4.5B: Median (Interquartile range) of absolute cell counts of various immune cell populations present in peripheral blood, obtained using flow cytometric analysis in the treatment-naïve, HIV-positive group and the HIV-negative (control) group.

	Expressed as a percentage of	**Median (IQR)**		
		HIV-positive (cells/µL) n=13	**Control (cells/µL) n=25**	**p-value**
Total CD3$^+$ T cells	Lymphocytes	772.00 (446.00-2021.00)	995.00 (818.00-1214.00)	0.61
CD4$^+$ T cells	Total CD3$^+$ T cells	225.00 (44.5-477.00)	587.00 (389.00-679.50)	0.0009
CD8$^+$ T cells	Total CD3$^+$ T cells	482.00 (206.50-1379)	321.0 (227.00-522.00)	0.17
Total CD19$^+$ B cells	Lymphocytes	89.00 (35.00-112.00)	105.00 (62.00-195.00)	0.24
Total CD14$^+$ Monocytes	Leukocytes	302.00 (261-555.50)	375.00 (261.50-505.0)	0.68
CD14$^+$CD16$^-$ Classical monocytes	Total CD14$^+$ Monocytes	215.00 (58.00-394.50)	224.00 (107.00-301.50)	0.89
CD14$^+$CD16$^+$ Intermediate monocytes	Total CD14$^+$ Monocytes	19.00 (4.00-49.00)	93.00 (20.00-171.50)	0.036
CD14^{dim}CD16$^+$ Non-classical monocytes	Total CD14$^+$ Monocytes	39.00 (13.00 -54.50)	45.00 (22.0-84.50)	0.25

Table 4.5B continues.

Total CD16$^+$ Neutrophils	Leukocytes	2594.00 (1242-4530)	2615.00 (1784.00-3183.00)	0.78
Total CD56$^+$ NK cells	Lymphocytes	100.00 (74.0-178.0)	187.00 (110.00-282.00)	0.15
Cytokine producing NK cells	Total CD56$^+$ NK cells	1.00 (0.00-3.00)	4.00 (2.00-6.50)	0.0066
Cytotoxic NK cells	Total CD56$^+$ NK cells	83.00 (56.0-161.0)	147.00 (56.50-265.00)	0.36
Total CD3$^+$CD56$^+$ NK T cells	Lymphocytes	48.00 (36.50-169.50)	92.0 (42.00-178.00)	0.43
CD4$^+$ NK T cells	Total CD3$^+$CD56$^+$ NK T cells	1.00 (0.500-9.00)	5.00 (3.00-11.50)	0.027
CD8$^+$ NK T cells	Total CD3$^+$CD56$^+$ NK T cells	39.00 (29.00-95.00)	62.00 (23.50-124.50)	0.67

CD, Cluster of differentiation; HIV, Human immunodeficiency virus; IQR, Interquartile range; NK, Natural killer; NKT, Natural killer T cells.

Next, the CD4/CD8 ratio for the HIV-positive and HIV-negative (control) groups were determined. The median (IQR) values of the CD4/CD8 ratio were as follows: HIV-positive group [0.30 (0.13-0.76)] and HIV-negative (control) group [1.47 (1.30-2.35)] (Figure 4.2). The HIV-positive group was significantly lower than the HIV-negative (control) group ($p<0.0001$). In comparison to the HIV-negative (control) group that demonstrated a wide distribution of CD4/CD8 ratios (≥1), the HIV-positive group exhibited a narrower distribution, with the majority of the values below 1 (≤1).

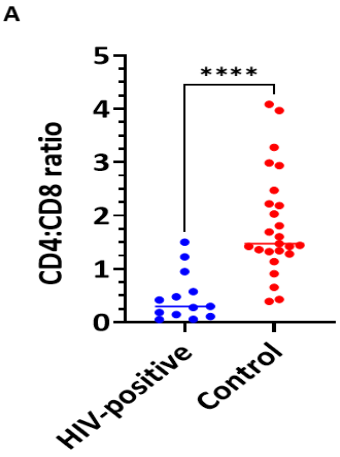


Figure 4.2: Scatter plot displaying the distribution of the CD4/CD8 ratios in HIV-positive and HIV-negative (control) groups. The median value is represented by the horizontal line. The CD4/CD8 ratio was calculated for each patient enrolled and is represented by a singular dot within the respective group. Control refers to the HIV-negative (control) group. CD, Cluster of differentiation; HIV, Human immunodeficiency virus.

4.3.2. Enumeration of myeloid derived suppressor cells populations

As previously mentioned, MDSCs are commonly found in individuals with pathological conditions associated with chronic inflammation or stress, exerting their immunosuppressive activity. Low levels of MDSCs can also be found in healthy individuals[32, 36].

Table 4.6 shows the median (IQR) values of the frequency of MDSC sub-populations present within the PBMNC fraction. Within both the M-MDSC and the i-MDSCs, the frequency of the HIV-positive group was significantly lower than the HIV-negative (control) group (p=0.030 and p=0.047) respectively. The PMN sub-population showed no statistically significant differences between the HIV-positive and -negative (control) groups. In both the HIV-positive and HIV-negative (control) groups, the PMN-MDSCs had the highest frequency, with the M-MDSC for the HIV-positive group reporting equal values and the M-MDSC for the HIV-negative (control) group (Table 4.6).

Table 4.6: Median (interquartile range) of the frequency of myeloid derived suppressor cells sub-populations, expressed as percentage of peripheral blood mononuclear cells present within peripheral blood mononuclear cells isolated from the treatment-naïve, HIV-positive, group and the HIV-negative (control) group, respectively.

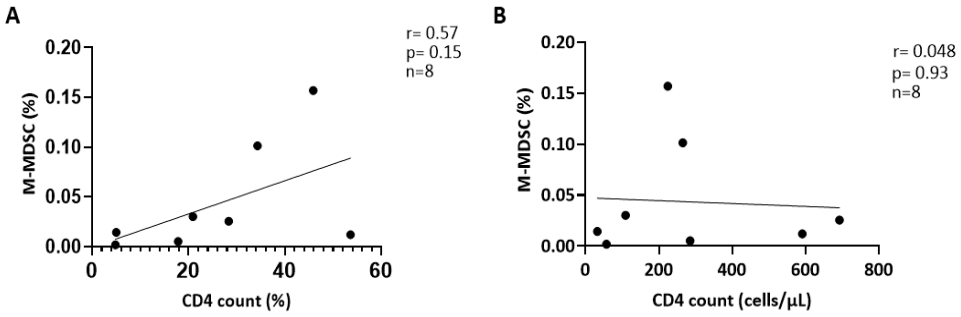
	Median(IQR)		
	HIV-positive (%) n=8	**Control (%) n=18**	**p-value**
M-MDSC	0.020 (0.0070-0.084)	0.077 (0.042-0.33)	0.030
PMN-MDSC	0.66 (0.19-1.87)	2.00 (0.30-3.59)	0.16
i-MDSC	0.020 (0.00-0.037)	0.047 (0.0072-0.095)	0.047

Control refers to the HIV-negative (control) group. HIV, Human immunodeficiency virus; i, Immature; IQR, interquartile range; MDSC, myeloid derived suppressor cells; M, myeloid; PMN, polymorphonuclear.

4.3.3. Myeloid derived suppressor cells and human immunodeficiency virus disease progression

The CD4 count is considered the superior disease progression marker for HIV, preferred over viral load (VL)[12]. Therefore, MDSC sub-populations were correlated with the CD4 count at baseline to assess if there is any association between MDSC frequency and disease progression.

Figure 4.3 illustrates the correlations between the percentage of MDSCs and both the CD4 frequency (Figure 4.3A, C, E) and absolute count (Figure 4.3B, D, F) within the PBMNC fraction of the HIV-positive group. No significance was observed for the parameters tested (Figure 4.3A-F). No association between MDSC sub-populations frequency and disease progression could thus be established in this study.



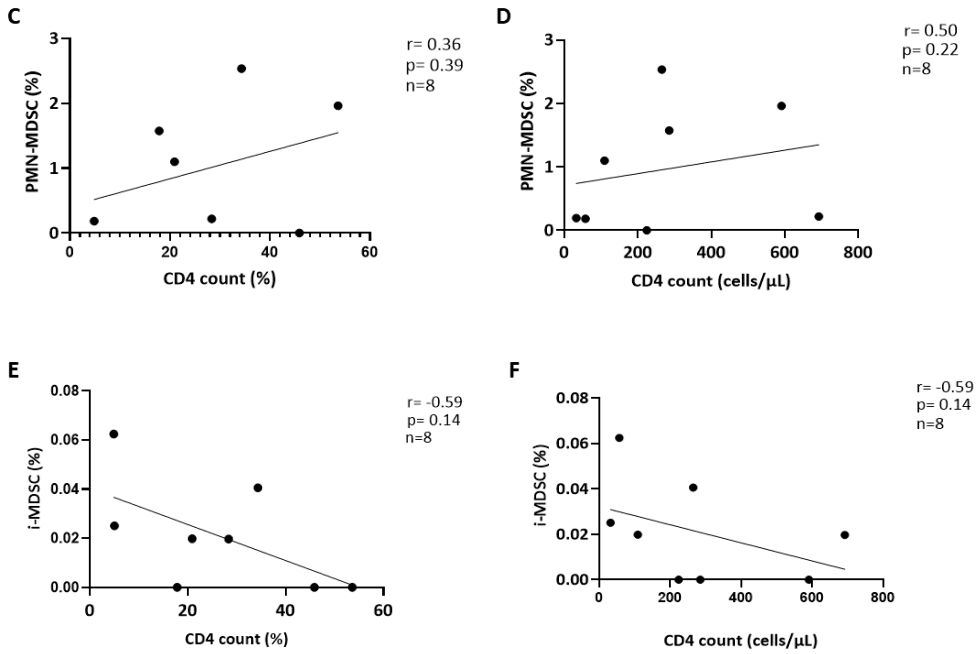


Figure 4.3: Correlation analysis between the frequency of myeloid derived suppressor cell sub-populations and the frequency (left) and absolute count (right) of the CD4⁺ T cells present within the Treatment-naïve, HIV-positive group. Correlations between M-MDSC (A-B), PMN-MDSC (C-D), and iMDSC (E-F) and CD4 count, and frequency are shown. CD, Cluster of differentiation; i, Immature; IQR, Interquartile range; MDSC, Myeloid derived suppressor cells; M, Myeloid; PMN, Polymorphonuclear.

4.4. Discussion

It is widely established that the presence of HIV has detrimental effects on the immune system. This is observed by the progressive depletion of CD4⁺ T cells, chronic immune activation, and inflammation, established hallmarks of HIV that promote an immunodeficient environment[37-39]. This chapter aimed to elucidate the impact of HIV infection on the frequency of various immune cell populations and further investigate the associations between MDSC frequency and disease progression (CD4 absolute counts) in a treatment-naïve, HIV-positive cohort.

The main findings of this study show the impact of HIV on major immune effector cell populations. Tables 4.5A and B indicate a significant decrease in the CD4⁺ T cells and a significant increase in the frequency of CD8⁺ T cells in the treatment-naïve HIV-positive group, compared to the HIV-negative (control) group. No significant difference in the frequency of

$CD56^{+}$ NK cells between the HIV-positive group and the HIV-negative (control) group was observed. However, the $CD56^{++}$ cytokine producing-NK cells and the $CD4^{+}$ NK T cell sub-populations both presented with significantly lower frequencies in the treatment-naïve HIV-positive group when compared to the HIV-negative (control) group. Regarding the MDSCs, PMN-MDSCs presented with the highest frequencies within the treatment-naïve HIV-positive group followed by i-MDSCs and lastly, M-MDSCs. No significant correlations between the MDSC sub-populations and the CD4 count were observed.

As a consequence of HIV infection, T cell homeostasis becomes dysregulated through decreased thymopoiesis and an increase in T cell destruction[40]. This data is in alignment with Guo and colleagues (2023), who observed similar findings with respect to the $CD4^{+}$, $CD8^{+}$ T cells, and $CD56^{+}$ NK cells when comparing an treatment-naïve HIV-positive cohort to HIV-negative controls[41].

The severity of the decrease observed in the $CD4^{+}$ T cell population is not only due to the virus exerting its cytopathic effects on the HIV-infected $CD4^{+}$ T cells but also because of its effect on the uninfected $CD4^{+}$ T 'by-stander' cells (T cells activated independently of antigen recognition)[42-44]. The virus triggers cell death in these 'by-stander' cells through either the cytotoxic effects of viral proteins or the HIV-induced pro-inflammatory environment (pyroptotic death) and/or the over-expression of death ligands by other immune cells[43, 45].

Nabatanzi and colleagues (2019) reported that both $CD56^{++}$ cytokine producing- and $CD56^{dim}$ cytotoxic-NK cells were lower in HIV-positive individuals when compared to HIV-negative individuals[46]. Notably, Nabatanzi and colleagues (2020) investigated a cohort of non-pregnant, treatment-adherent HIV-positive women with a median age [interquartile range(IQR)] of 40 (38-46) years whilst the present study investigated pregnant, treatment-naïve individuals with a lower median (IQR) age of 33 (19-46) years. Despite this the data reported in both studies are similar. In the present study, we observed that the NK sub-population ($CD56^{++}$ cytokine producing-NK cells) as well as the $CD4^{+}$ NK T cells were lower in the treatment-naïve, HIV-positive group when compared to both the frequencies and absolute cell counts observed in the HIV-negative (control) group. Furthermore, the $CD56^{dim}$ cytotoxic-NK cell sub-population was lower in the treatment-naïve, HIV-positive group when compared to the absolute cell counts observed in the HIV-negative (control) group.

Natural killer T cells are a subpopulation of T cells that express both T and NK cell markers and further secrete large quantities of T helper (Th)-1 and-2 cytokines that influence both innate and adaptive immune systems. In addition, NKT cells have cytotoxic activity and restrict viral replication during HIV infection[47, 48].

De Almeida and colleagues (2023) reported similar findings to the present study, as the percentage of total $CD3^+CD56^+$ NKT cells in the peripheral blood of the HIV-positive cohort was lower than that of the HIV-negative cohort[49]. In contrast to this present study, de Almeida and colleagues (2023) investigated a heterogenous HIV-positive group of male and female participants who were either treatment-adherent or treatment-naïve. The authors hypothesised that the lower percentage of $CD3^+CD56^+$ NKT cells was either a result of direct HIV infection of NKT cells or decreased production of NKT cell survival cytokines and relocation of NKT cells to other tissues (e.g. cerebrospinal fluid)[49]. Taken together, the data demonstrate HIV-mediated immune cell dysregulation through the reduction of the frequencies of lymphocyte subpopulations such as NKT cells.

Our results also demonstrated a significant decrease in the $CD14^+CD16^+$ intermediate monocyte frequencies between the treatment-naïve, HIV-positive and the HIV-negative (control) group. However, analysis of both $CD14^+CD16^-$ classical monocytes and $CD14^{dim}CD16^+$ non-classical monocytes did not show any significant differences between the same groups. When compared with literature, Prabhu and colleagues (2019), showed that the frequencies of $CD14^+CD16^+$ intermediate monocytes were higher in the ART-naïve individuals compared to HIV-negative controls[50]. The authors also noted that the ART-naïve group presented with significantly lower $CD14^+CD16^-$ classical monocytes in comparison to the HIV-negative controls. No differences were observed between groups for $CD14^{dim}CD16^+$ non-classical monocytes[50]. As such, the reason for the decrease in the $CD14^+CD16^+$ intermediate monocyte subsets observed in this study is unclear and needs further investigation.

As a consequence of infection, MDSCs undergo expansion that allows for HIV disease progression[51]. The immunosuppressive abilities of MDSCs on $CD4^+$ T helper cells impact their functionality and promote T cell exhaustion[52]. The data obtained in this study demonstrates that within the HIV-positive group, the PMN-MDSCs had the highest frequency, with equal frequencies of the M-MDSCs and i-MDSCs (Table 4.6). Similarly, within the HIV-negative

(control) group, the PMN-MDSCs had the highest frequency, however, but unlike the HIV-positive group, the M-MDSC frequency was higher than the i-MDSCs (Table 4.6). The data are in alignment with that of Zhang and colleagues (2017) as well as Agrati and colleagues (2019) who reported increased PMN-MDSCs levels in a treatment-naïve, HIV-positive cohort when compared to HIV-negative controls[24, 53]. This study demonstrated significant differences between the frequencies of M-MDSCs between the two groups. This result contradicted the findings by Li and colleagues (2023) who reported no significant differences in the frequency of M-MDSCs between treatment-naïve, HIV-positive individuals and HIV-negative controls[11].

In the present study, MDSC sub-populations were correlated with CD4 count (Figure 4.2) and the percentages of M-, PMN-, and i-MDSCs showed no correlation to the CD4 count (cells/µL). Both Li and colleagues (2023) and Agrati and colleagues (2019) reported similar findings with the percentage of M- and PMN-MDSCs respectively with the CD4 count (cells/µL) with no correlation being reported within the treatment-naïve , HIV-positive group[11, 24].

Notably, there is very little data pertaining to i-MDSCs and treatment-naïve, HIV-positive cohort. As such, this study hypothesised that the increased frequencies of i-MDSCs (Table 4.6) observed are as a result of the sustained pro-inflammatory conditions induced by HIV infection. This results in skewed differentiation of the CMPs, to differentiate into i-MDSC, that accumulate and promote HIV-mediated suppression of T cell activity.

Overall, the data showed that the presence of HIV results in immune dysfunction that impacts the frequency of immune cell populations. More particularly those of lymphoid origin, as they are instrumental in fighting infection by stimulating innate and adaptive immune systems[54]. The resulting immune responses are specific, accelerate antigen clearance, and promote formation of memory compartments[54-56]. As mentioned earlier, the lymphocyte precursors, CLPs reside in the BM and the presence of HIV in the BM is detrimental to steady-state haematopoiesis and the cell signalling required for the maintenance of immune cell frequencies[2, 57, 58]. Agrati and colleagues (2019) demonstrated that PMN-MDSCs interfere with the $CD34^+$ progenitor cell activity and subsequent T cell commitment *in vitro*[24]. As such, there is an increase in the rate of depletion of the immune cells that cannot be replenished at the same rate at which the depletion occurs.

In conclusion, the differences observed in the frequency and cell counts of immune cells, along with the increase in i-MDSCs frequency, are indicative of HIV-mediated T cell dysfunction and inflammation.

A limitation of this study was the absence of the VLs and cytokine analysis, as this would aid in obtaining a better understanding of the relationship between MDSCs and disease progression. Furthermore, the participants in the study were pregnant, and pregnancy itself induces its own physiological changes onto the immune system. Additionally, limitations of the MDSC analyses included the small study population. The limited number of events acquired may also have a negative impact on the accuracy of the results. Myeloid derived suppressor cells are viewed as rare cell populations, which necessitate the acquisition of a large number of cells (events) to accurately detect these rare cell populations. On average, 100 000 events were acquired, which in hindsight might be insufficient to accurately detect these cells. A larger number of study participants, especially in the treatment-naïve cohort, are required to draw concise conclusions as such the results should therefore be interpreted with caution. Overall, as the immune system is a component of the bigger haematopoietic system, the dysregulation can be linked to HIV-mediated haematopoietic dysregulation.

4.5. References

1. Charles A, Janeway J, Travers P, Walport M, Shlomchik M. Immunobiology: the immune system in health and disease [Internet]. New York:Garland Publishing Inc; 2001 [cited: 2023/03/10]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK10757/

2. Durandt C, Potgieter JC, Mellet J, Herd C, Khoosal R, Nel JG*, et al.* HIV and haematopoiesis. S. Afr. Med. J. 2019; 109(8b):40-5.

3. Balasubramaniam M, Pandhare J, Dash C. Immune Control of HIV. J Life Sci (Westlake Village). 2019; 1(1):4-37.

4. Nasi A, Chiodi F. Mechanisms regulating expansion of CD8+ T cells during HIV-1 infection. J Intern Med. 2018; 283(3):257-67.

5. Konadu KA, Anderson JS, Huang MB, Ali SA, Powell MD, Villinger F*, et al.* Hallmarks of HIV-1 pathogenesis are modulated by Nef's Secretion Modification Region. J. AIDS Clin. Res. 2015; 6(7):476.

6. Aavani P, Allen LJS. The role of CD4 T cells in immune system activation and viral reproduction in a simple model for HIV infection. Appl Math Model. 2019; 75:210-22.

7. Kervevan J, Chakrabarti LA. Role of CD4+ T Cells in the Control of Viral Infections: Recent Advances and Open Questions. Int. J. Mol. Sci. 2021; 22(2):523.

8. Cenerenti M, Saillard M, Romero P, Jandus C. The Era of Cytotoxic CD4 T Cells. Front. Immunol. 2022; 13(867189):1-14.

9. Wang S, Zhang Q, Hui H, Agrawal K, Karris MAY, Rana TM. An atlas of immune cell exhaustion in HIV-infected individuals revealed by single-cell transcriptomics. Emerg Microbes Infect. 2020; 9(1):2333-47.

10. Wang Y, Zhang Y, Tang T, Zhao W, Fu S, Zhang Z*, et al.* Natural killer cell counts in primary HIV infection predicts disease progression and immune restoration after treatment. Virology. 2020; 550:89-98.

11. Li Z, Yan P, Wang R, Lu X, Zhang Y, Su B*, et al.* Persistent T cell proliferation and MDSCs expansion precede incomplete CD4+ T cell recovery in people with acute HIV-1 infection with early ART. Heliyon. 2023; 9(5):e15590.

12. Dessie ZG, Zewotir T, Mwambi H, North D. Modelling of viral load dynamics and CD4 cell count progression in an antiretroviral naive cohort: using a joint linear mixed and multistate Markov model. BMC Infect. Dis. 2020; 20(246):1-14.

13. Centers for Disease Control and Prevention. Monitoring selected national HIV prevention and care objectives by using HIV surveillance data—United States and 6 dependent areas, 2019. HIV Surveillance Supplemental Report. Atlanta, Georgia, USA. 2021; Document number: 2. [updated: May 2021. cited: 2023/11/30.] Available from: https://www.cdc.gov/hiv/library/reports/hiv-surveillance/vol-26-no-2/content/technical-notes.html#print

14. Shoko C, Chikobvu D, Bessong PO. A Markov model to estimate mortality due to HIV/AIDS using viral load levels-based states and CD4 cell counts: a principal component analysis approach. Infect Dis Ther. 2018; 7(4):487-71.

15. Belay AS, Manaye GA, Kebede KM, Abateneh DD. Predictors of Current CD4+ T-Cell Count Among Women of Reproductive Age on Antiretroviral Therapy in Public Hospitals, Southwest Ethiopia. HIV AIDS (Auckl.). 2021; 13:667-79.

16. Zhang W, Ruan L. Recent advances in poor HIV immune reconstitution: what will the future look like? Front. Microbiol. 2023; 14(1236460):1-14.

17. Martínez-Sanz J, Díaz-Álvarez J, Rosas M, Ron R, Iribarren JA, Bernal E*, et al.* Expanding HIV clinical monitoring: the role of CD4, CD8, and CD4/CD8 ratio in predicting non-AIDS events. eBioMedicine. 2023; 95(104773):1-8.

18. Maartens G. HIV infection: epidemiology, pathogenesis, treatment, and prevention. The Lancet. 2014; 384(9939):258.

19. McBride JA, Striker R. Imbalance in the game of T cells: What can the CD4/CD8 T-cell ratio tell us about HIV and health? PLoS Pathog. 2017; 13(11):e1006624.

20. Mussini C, Lorenzini P, Cozzi-Lepri A, Lapadula G, Marchetti G, Nicastri E*, et al.* CD4/CD8 ratio normalisation and non-AIDS-related events in individuals with HIV who achieve viral load suppression with antiretroviral therapy: an observational cohort study. Lancet HIV. 2015; 2(3):e98-106.

21. Ma H, Xia CQ. Phenotypic and Functional Diversities of Myeloid-Derived Suppressor Cells in Autoimmune Diseases. Mediators Inflamm. 2018; 2018(4316584):1-8.

22. Ademe M. Paradoxes in the phenotype, frequency and roles of myeloid-derived suppressor cells during HIV infection. HIV AIDS (Auckl.). 2020; 12:151-6.

23. Grützner EM, Hoffmann T, Wolf E, Gersbacher E, Neizert A, Stirner R*, et al.* Treatment intensification in HIV-infected patients is associated with reduced frequencies of regulatory T cells. Front. Immunol. 2018; 9(811):1-13.

24. Agrati C, Tumino N, Bordoni V, Pinnetti C, Sabatini A, Amendola A*, et al.* Myeloid Derived Suppressor Cells Expansion Persists After Early ART and May Affect CD4 T Cell Recovery. Front. Immunol. 2019; 10(1886):1-10.

25. Singh R, Chakraborty M, Gautam A, Roy SK, Halder I, Barber J*, et al.* Residual immune activation in HIV-Infected individuals expands monocytic-myeloid derived suppressor cells. Cell. Immunol. 2021; 362(104304):1-8.

26. Vollbrecht T, Stirner R, Tufman A, Roider J, Huber RM, Bogner JR*, et al.* Chronic progressive HIV-1 infection is associated with elevated levels of myeloid-derived suppressor cells. AIDS. 2012; 26(12):F31-F7.

27. Garg A, Spector SA. HIV Type 1 gp120–Induced Expansion of Myeloid Derived Suppressor Cells Is Dependent on Interleukin 6 and Suppresses Immunity. J. Infect. Dis. 2013; 209(3):441-51.

28. Lei AH, Yang Q, Cai WP, Liu YF, Lan Y, Qin AP*, et al.* Clinical Significance of Myeloid-Derived Suppressor Cells in Human Immunodeficiency Virus-1/ Hepatitis C Virus-coinfected Patients. Scand. J. Immunol. 2016; 83(6):438-44.

29. Medina E, Hartl D. Myeloid-Derived Suppressor Cells in Infection: A General Overview. J. Innate Immun. 2018; 10(5-6):407-13.

30. Zhao Y, Wu T, Shao S, Shi B, Zhao Y. Phenotype, development, and biological function of myeloid-derived suppressor cells. Oncoimmunology. 2016; 5(2):e1004983.

31. Morawski PA, Motley SJ, Campbell DJ. Rapid Light-Dependent Degradation of Fluorescent Dyes in Formulated Serum-Free Media. Immunohorizons. 2019; 3(12):585-92.

32. Apodaca MC, Wright AE, Riggins AM, Harris WP, Yeung RS, Yu L*, et al.* Characterization of a whole blood assay for quantifying myeloid-derived suppressor cells. J Immunother Cancer. 2019; 7(1):230.

33. Miltenyi Biotec technologies. MACS Handbook.Rheinisch-Bergischer Kreis, Germany; Available from: https://www.miltenyibiotec.com/DE-en/resources/macs-handbook/human-cells-and-organs/human-cell-sources/blood-human.html

34. Cole KE, Ly QP, Hollingsworth MA, Cox JL, Padussis JC, Foster JM*, et al.* Human splenic myeloid derived suppressor cells: Phenotypic and clustering analysis. Cell. Immunol. 2021; 363(104317):1-14.

35. Wang L, Zhao J, Ren JP, Wu XY, Morrison ZD, Elgazzar MA*, et al.* Expansion of myeloid-derived suppressor cells promotes differentiation of regulatory T cells in HIV-1^{+} individuals. AIDS. 2016; 30(10):1521-31.

36. Wang S, Zhao X, Wu S, Cui D, Xu Z. Myeloid-derived suppressor cells: key immunosuppressive regulators and therapeutic targets in hematological malignancies. Biomarker Res. 2023; 11(1):34.

37. Khanal S, Tang Q, Cao D, Zhao J, Nguyen LN, Oyedeji OS*, et al.* Telomere and ATM Dynamics in CD4 T-Cell Depletion in Active and Virus-Suppressed HIV Infections. J. Virol. 2020; 94(22):e01061-20.

38. Akiyama H, Gummuluru S. HIV-1 Persistence and Chronic Induction of Innate Immune Responses in Macrophages. Viruses. 2020; 12(7):711.

39. Lv T, Cao W, Li T. HIV-Related Immune Activation and Inflammation: Current Understanding and Strategies. J. Immunol. Res. 2021; 2021(7316456):1-13.

40. Dos Santos Guedes MC, Carvalho-Silva WHV, Andrade-Santos JL, Brelaz-de-Castro MCA, Souto FO, Guimarães RL. Thymic Exhaustion and Increased Immune Activation Are the Main Mechanisms Involved in Impaired Immunological Recovery of HIV-Positive Patients under ART. Viruses. 2023; 15(2):440.

41. Guo X-Y, Qu M-M, Wang X, Wang Z-R, Song J-W, Yang B-P*, et al.* Characteristics of blood immune cell profile and their correlation with disease progression in patients infected with HIV-1. BMC Infect. Dis. 2023; 23(1):893.

42. Doitsh G, Greene WC. Dissecting How CD4 T Cells Are Lost During HIV Infection. Cell Host Microbe. 2016; 19(3):280-91.

43. Cummins NW, Badley AD. Making sense of how HIV kills infected CD4 T cells: implications for HIV cure. Mol Cell Ther. 2014; 2(20):1-7.

44. Shim CH, Cho S, Shin YM, Choi JM. Emerging role of bystander T cell activation in autoimmune diseases. BMB Rep. 2022; 55(2):57-64.

45. Doitsh G, Galloway NLK, Geng X, Yang Z, Monroe KM, Zepeda O*, et al.* Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. Nature. 2014; 505(7484):509-14.

46. Nabatanzi R, Bayigga L, Cose S, Rowland-Jones S, Canderan G, Joloba M*, et al.* Aberrant natural killer (NK) cell activation and dysfunction among ART-treated HIV-infected adults in an African cohort. Clin. Immunol. 2019; 201(4):55-60.

47. Littwitz-Salomon E, Schimmer S, Dittmer U. Natural killer T cells contribute to the control of acute retroviral infection. Retrovirology. 2017; 14(1):5.

48. Khan MA, Khan A. Role of NKT Cells during Viral Infection and the Development of NKT Cell-Based Nanovaccines. Vaccines. 2021; 9(9):949.

49. de Almeida SM, Beltrame MP, Tang B, Rotta I, Justus JLP, Schluga Y*, et al.* CD3+CD56+ and CD3−CD56+ lymphocytes in the cerebrospinal fluid of persons with HIV-1 subtypes B and C. J. Neuroimmunol. 2023; 377(578067):1-13.

50. Prabhu VM, Singh AK, Padwal V, Nagar V, Patil P, Patel V. Monocyte Based Correlates of Immune Activation and Viremia in HIV-Infected Long-Term Non-Progressors. Front. Immunol. 2019; 10(2849):1-15.

51. Yaseen MM, Abuharfeil NM, Darmani H. Myeloid-derived suppressor cells and the pathogenesis of human immunodeficiency virus infection. Open Biol. 2021; 11(11):210216.

52. Green WR, O'Connor MA. HIV vaccines: Unmasking myeloid derived suppressor cells. eBioMedicine. 2020; 61(103063):1-2.

53. Zhang ZN, Yi N, Zhang TW, Zhang LL, Wu X, Liu M*, et al.* Myeloid-Derived Suppressor Cells Associated With Disease Progression in Primary HIV Infection: PD-L1 Blockade Attenuates Inhibition. J. Acquir. Immune Defic. Syndr. 2017; 76(2):200-8.

54. Davis BP, Rothenberg ME. Inflammatory and Effector Cells/Cell Migration. In: Leung DYM, Szefler SJ, Bonilla FA, Akdis CA, Sampson HA, editors. Pediatric Allergy: Principles and Practice (Third Edition). London: Elsevier; 2016. p. 41-53.e4.

55. Corsini E, Iulini M. Immune system. In: Wexler P, editor. Encyclopedia of Toxicology (Fourth Edition). Oxford: Academic Press; 2024. p. 471-95.

56. Al-Shura AN. Lymphocytes. In: Al-Shura AN, editor. Advanced Hematology in Integrated Cardiovascular Chinese Medicine. 1st ed. Cambridge, Massachusetts: Academic Press; 2020. p. 41-6.

57. Herd CL, Mellet J, Mashingaidze T, Durandt C, Pepper MS. Consequences of HIV infection in the bone marrow niche. Front. Immunol. 2023; 14(1163012):1-17.

58. Nasiri K, Mohammadzadehsaliani S, Kheradjoo H, Shabestari AM, Eshaghizadeh P, Pakmehr A*, et al.* Spotlight on the impact of viral infections on Hematopoietic Stem Cells (HSCs) with a focus on COVID-19 effects. J Cell Commun Signal. 2023; 21(1):103.

Chapter 5: Associations of circulating haematopoietic stem and progenitor cells with the haematological and immunological parameters in treatment-naïve, HIV-positive pregnant women

5.1. Introduction

Infection with the human immunodeficiency virus (HIV) can significantly affect physiological systems other than the immune system, such as the haematopoietic system, which is responsible for the production of all blood cell lineages in the bone marrow (BM)[1]. Haematopoietic stem and progenitor cells (HSPCs) are at the apex of haematopoiesis, serving as the origin of all blood cell lineages. Haematopoietic stem and progenitor cells are predominantly quiescent to maintain their self-renewal capacity[2, 3]. However, in response to stressors such as HIV infection, HSPCs exit quiescence, entering into a proliferative state leading to aberrant haematopoiesis and subsequently, a range of haematological abnormalities[4-6].

The most commonly observed HIV-associated haematological abnormalities in individuals with HIV infection are cytopenias, including anaemia, thrombocytopenia, and/or leucopenia[7]. These cytopenias are frequently linked with decreased cluster of differentiation (CD)4^+ T cell counts, disease progression, and mortality in people living with HIV (PLWH)[8, 9]. The occurrence of these peripheral blood cytopenias is multifactorial and the potential causative factors may be as a result of both direct and indirect effects of the virus on HSPCs[10, 11].

Notably, HSPCs are tightly regulated in the BM microenvironment, as such an indirect effect of the virus includes HIV-mediated dysregulation of HSPC function by disrupting BM homeostasis[11]. As a consequence, HSPCs experience impaired functionality, manifesting as significant reductions in blood cell lineages, dysplastic changes, and/or megaloblastic abnormalities[8]. Direct effects include the potential infection of HSPCs with HIV, as these cells express low levels of CD4 receptors and C-X-C chemokine receptor type-4 (CXCR4), that makes them susceptible to infection with HIV[12]. However, the question of whether HSPCs are susceptible to HIV infection is still a topic of debate within the scientific community. Lastly, secondary infections, side-effects of prophylactic drugs, and nutritional deficiencies further contribute to the occurrence of haematological abnormalities[6, 7].

Anaemia has been reported as the most prevalent cytopenia among PLWH, with rates of up to 36.6% reported in treatment-naïve, HIV-positive cohorts[7, 9, 13]. The occurrence of anaemia in PLWH is multifactorial and is primarily characterised by decreased haemoglobin (HGB) concentrations and/or red blood cell (RBC) counts falling below defined thresholds[14]. However, HGB is primarily used to diagnose anaemia[15]. In addition to the HGB and RBC values, haematocrit (HCT), which measures the percentage of RBCs in whole blood, is also used as an indicator of anaemia[16]. A decrease in RBC numbers is often as a result of underlying pathological mechanisms such as abnormal RBC maturation, increased RBC destruction, decreased RBC production, and/or blood loss[10, 17, 18]. Upon confirmation of anaemia, the type of anaemia may be further identified by the mean corpuscular volume (MCV), which measures the average size and volume of RBCs[19]. Anaemia is classified as microcytic [MCV <80 femtolitres (fL)], macrocytic [MCV >100 fL], and normocytic [MCV ranging between 80-100 fL] [19, 20].

Following anaemia, thrombocytopenia is the second most prevalent cytopenia in HIV infection. Immune thrombocytopenic purpura (ITP) stands as the leading cause of thrombocytopenia in HIV infection and may occur at any stage of disease progression[21, 22]. The primary mechanism underlying ITP involves increased anti-platelet antibodies which contribute to the formation of immune complexes, leading to increased platelet destruction[23-25]. Alternatively, thrombotic thrombocytopenic purpura (TTP) results in a severe decrease in platelet counts[26]. Infection with HIV increases the likelihood of TTP occurring (approximately 40-fold), and is associated with lower CD4 counts, bicytopenia (thrombocytopenia and anaemia) and various comorbidities[27]. Thrombotic thrombocytopenic purpura occurs as a result of increased auto-antibodies that significantly reduce the a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13) enzymatic activity[26]. The auto-antibodies are typically produced in response to a trigger such as HIV-infection, autoimmune diseases and pregnancy[26]. Furthermore, various literature suggests that infection of megakaryocytes with HIV may also lead to decreased platelet production (ineffective thrombopoiesis)[23, 24, 28]. Thrombocytopenia is often asymptomatic[13, 29]. More severe thrombocytopenia is associated with multiple bleeding abnormalities[29]. A systematic review by Getawa *et al.* reported a prevalence of up to 41% of thrombocytopenia in a treatment-naïve, HIV-positive cohort[22].

Leukopenia occurs when the white blood cell count (WBC) becomes abnormally low, with neutropenia being the most commonly observed form[5, 30]. Neutropenia is commonly associated with pancytopenia and late-stage HIV infection[31, 32]. As such, neutropenia is often associated with lower $CD4^+$ T cell counts and higher viral loads [33]. A prevalence of up to 15.6% neutropenia has been reported in treatment-naïve, HIV-positive individuals[7, 21]. Since neutrophils comprise up to 70% of WBCs, neutropenia in conjunction with reduced CD4 counts increases the likelihood of opportunistic infections and associated complications (i.e., septic shock)[34, 35]. Several mechanisms contribute to the onset of neutropenia, including suppression of granulocyte-monocyte progenitor (GMP) cells by HIV proteins, HIV infection of BM stromal cells, and/or decreased production of granulocyte colony-stimulating factor (G-CSF)[33, 36]. In addition, auto-immune disorders, secondary infections, malignancies, and myelosuppressive drugs can all contribute to increased neutrophil clearance[5, 36].

The aim of this chapter was to investigate the impact of HIV on the haematopoietic system and its implication on the haematological profile on treatment-naïve, HIV-positive pregnant women.

5.2. Methodology

5.2.1. Study design

A comparative cross-sectional study was performed. Individuals were enrolled between November 2020 and November 2022 from the Antenatal Clinic (ANC) at Dr George Mukhari Academic Hospital (DGMAH) in Ga-Rankuwa, Pretoria, South Africa. All participants provided written informed consent.

Approximately 15 millilitres (mL) of peripheral blood were collected from each of the study participants and the collected blood was processed on the day of collection. A full blood count (FBC) with white cell differential, basic immune profile, and the determination of the frequency of circulating haematopoietic stem and progenitor cells (HSPCs) were performed. Bio-demographic and clinical data, including age, gender, ethnicity, and gestational period were also collected.

For the purpose of this chapter, “treatment-naïve HIV-positive,” (study group 1) refers to individuals who were diagnosed with HIV at time of enrolment (n=13) and/or have not

received combined antiretroviral-treatment (cART) for at least six months prior to enrolment in the study (defaulted; n = 0). Exclusion criteria for this cohort includes individuals currently on cART and those who have defaulted from cART less than six months ago.

Study group 2 consisted of women from the same clinic who tested negative for HIV (inclusion criteria). Furthermore, individuals were not excluded based on the presence of peripheral blood cytopenias in the HIV-negative (control) group. Therefore, resulting in an increase in the number of individuals enrolled as controls (n = 53). Individuals younger than 18 years of age, regardless of HIV-status, were excluded from the study.

5.2.2. Ethics approval

The research study obtained ethics approval from the Faculty of Health Sciences Research Ethics Committee (protocol number: 738/2020), University of Pretoria, and Sefako Makgatho Health Sciences University Research Ethics Committee (reference number: SMUREC/M/86/2020:IR). Approval was also obtained from the National Health Laboratory Services (NHLS), DGMAH (reference number: PR2010458). This study adhered to the principles outlined in the Declaration of Helsinki.

5.2.3. Full blood count and differential

A FBC with a differential white cell count was performed at the NHLS, Dr George Mukhari Tertiary Laboratory (DGMTL) on all blood samples collected. The Advia®2120i automated haematology analyser (Siemens AG, Munich, Germany) was used to obtain the automated FBC with differential. The differential was reviewed and verified on peripheral blood smear by a haematologist. Each collected sample was assigned a unique NHLS barcode identification code to ensure donor anonymity. Cytopenias were defined based on reference ranges obtained from the NHLS DGMTL. The definitions used to define the cytopenia and the severity thereof are listed in Table 5.1.

Table 5.1: Defining criteria for various cytopenias.

Cytopenia	Parameter	Criteria	Mild	Moderate	Severe	Reference
Anaemia	HGB (g/dL)	<11.6	10.0 - 11.5	7.0 - 9.9	<7.0	[17, 37]
Thrombocytopenia	Platelets ($x10^9$/L)	<186	100 - 185	50 - 99	<50	[6, 37]
Neutropenia	Neutrophils ($x10^9$/L)	<1.5	1 - 1.5	1 - 0.5	0.5 - 0.1	[36, 37]

dL, decilitre; g, gram; HGB, haemoglobin; L, Litre.

5.2.4. Enumeration of $CD4^+$ T helper and $CD8^+$ cytotoxic cells

The DuraClone Basic Immunophenotyping kit (Beckman Coulter, Miami, FL, USA) was used to determine the frequency (%) and absolute counts [cells per microlitre (cells/µL)] of $CD4^+$ T helper and $CD8^+$ cytotoxic cells.

The sample was prepared according to section *4.2.3.3* in Chapter 4. A compensation matrix and an application specific standardisation template generated in sections *4.2.3.1* and *4.2.3.2* respectively in Chapter 4 were applied prior to sample acquisition.

The resulting frequencies and absolute counts were compared between treatment-naïve, HIV-positive and HIV-negative (control) patients. The assay was performed according to the manufacturer's instructions on a CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA).

5.2.5. Isolation of peripheral blood mononuclear cells

The isolation and enumeration of peripheral blood mononuclear cells (PBMNCs) was performed as described in sections *3.2.3.2 and 3.2.3.3* respectively, in Chapter 3.

5.2.6. Circulating haematopoietic stem and progenitor cell frequency

Circulating haematopoietic stem and progenitor cells (HSPCs) are present in low frequencies in peripheral blood. As such, PBMNCs were isolated to enrich for circulating HSPCs and used for the enumeration of circulating HSPCs. The sample was prepared as outlined in section *3.2.3.4.2* in Chapter 3.

A colour compensation matrix and an application specific standardisation template generated in sections *3.2.4.4.1* and *3.2.4.4.2,* respectively in Chapter 3 were applied prior to sample acquisition.

The frequency (%) and absolute count (cells/µL) of circulating HSPCs was determined using either the BD FACSAria™ Fusion cell sorter (BD Biosciences, New Jersey, NJ, USA) or the CytoFLEX flow cytometer (Beckman Coulter). The resulting $Lin^{-}CD34^{++}$ HSPCs frequencies were compared between treatment-naïve, HIV-positive and HIV-negative (control) groups.

5.3. Statistical analysis

GraphPad Prism 9 (GraphPad Software Inc., La Jolle, CA, USA) software was used for data visualisation and statistical analyses. A non-parametric Mann-Whitney U test was used to compare the haematological and immunological parameters between the treatment-naïve HIV-positive, and HIV-negative (control) group. Correlations were performed using the non-parametric, Spearman's r test. p-values <0.05 were classified as significant.

5.4. Results

5.4.1. General characteristics of study participants

A total of 66 pregnant women with a mean [standard deviation (SD)] age of 33.18 ± 6.45 (ranging from 19 to 47) were included in the study. In the treatment-naïve, HIV-positive cohort (n=13), 7.69% (n=1) of the women were in their first trimester, 7.69% (n=1) in the second trimester, 61.54% (n=8) in the third trimester, and data for the remaining 23.08% (n=3) was unavailable. The comorbidities observed in the treatment-naïve, HIV-positive group are outlined in section *3.2.3.3* in Chapter 3.

In the HIV-negative (control) cohort (n=53), none of the participants enrolled were in the first trimester, whilst 45.28% (n=24) of the women were in their second trimester, 43.40% (n=23) were in their third trimester, and data for the remaining 11.32% (n=6) was unavailable. Amongst the HIV-negative (control) group, 77.36% (n=41) had no comorbidities, whilst 9.43% (n=5) presented with high blood pressure, another 9.43% (n=5), asthma, 1.89% (n=1) gestational diabetes and another 1.89% (n=1) arthritis.

5.4.2. Haematological profiles of study participants

The mean values of haematological parameters from each of the respective groups are summarised in Table 5.2. As such, the HIV-positive group was compared to the HIV-negative (control) group.

The mean values of the white cell count (WCC) for the HIV-positive group were 7.00 ± 1.80 $x10^9$/L, which was significantly lower than those of the HIV-negative (control) group, which was 8.27 ± 2.48 $x10^9$/L (p=0.045).

The RBC count for the HIV-positive group was 3.75 ± 0.39 $x10^{12}$/L, significantly lower than the HIV-negative (control) group, which was 4.10 ± 0.34 $x10^{12}$/L (p=0.0031). The mean values of the HGB of the HIV-positive group were 11.03 ± 1.38 g/dL, significantly lower than those in the HIV-negative (control) group, which were 11.96 ± 1.15 g/dL (p=0.019). Similarly, the HCT value of the HIV-positive group was 0.34 ± 0.035 L/L, significantly lower than that in the HIV-negative (control) group, which was 0.37 ± 0.032 L/L (p=0.0084). Furthermore, the MCV of the HIV-positive group was 91.63 ± 9.75 fL, which was not significantly higher than that of the HIV-negative (control) group, 90.30 ± 6.27 fL (p=0.28).

The platelet (PLT) count of the HIV-positive group was 257.8 ± 65.29 $x10^9$/L, not significantly higher than those of the HIV-negative (control) group, which were 229.80 ± 57.42 $x10^9$/L (p=0.19).

The absolute neutrophil count for the HIV-positive group (4.60 ± 2.16 x 10^9/L) was significantly lower than that of the HIV-negative (control) group (5.77 ± 2.08 x 10^9/L) (p=0.015). However, the percentages of neutrophils for the HIV-positive group, 64.41 ± 10.99%, were not significantly lower when compared to those of the HIV-negative (control) group, 68.59 ± 6.69% (p=0.058).

The mean values of the absolute lymphocyte count (lymphs) of the HIV-positive group were 1.62 ± 0.60 x 10^9/L, which was not significantly lower than that of the HIV-negative (control) group, which was 1.76 ± 0.48 x 10^9/L (p=0.32). The percentage of lymphocytes was not significantly higher for the HIV-positive group, 25.01 ± 9.33%, when compared to the HIV-negative (control) group, which was 22.27 ± 5.48% (p=0.14).

Table 5.2: Comparison of haematological parameters of the respective study groups.

Parameter	Mean ± SD		p-value
	HIV-positive (n = 13)	Control (n = 53)	
WCC (10^9/L)	7.00±1.80	8.27±2.48	0.045
RBC (10^{12}/L)	3.75±0.39	4.10±0.34	0.0031
HBG (g/dL)	11.03±1.38	11.96±1.15	0.019
HCT (L/L)	0.34±0.035	0.37±0.032	0.0084
MCV (fL)	91.63±9.75	90.30±6.27	0.29
Platelets (10^9/L)	257.8±65.29	229.80±57.42	0.19
Neutrophils (10^9/L)	4.60±2.16	5.77±2.08	0.015
Neutrophils (%)	64.41±10.99	68.59±6.69	0.058
Lymphocytes (10^9/L)	1.62±0.60	1.76±0.48	0.32
Lymphocytes (%)	25.01± 9.33	22.27±5.48	0.14

dL; Decilitre; fL; Femtolitres; g, grams; HBG, haemoglobin; HIV, Human immunodeficiency virus; L, Litre; RBC, red blood cells; SD; Standard deviation; WCC, white cell count. Results are expressed as mean ± SD. Control refers to the HIV-negative (control) group. Significance: p <0.05.

Next, the absolute counts of $CD4^+$ T cells and $CD8^+$ T cells were compared between each of the groups, as summarised in Figure 5.1. With respect to the $CD4^+$ T helper cells, the mean absolute count was significantly lower (p=0.0021) in the HIV-positive group (268.00 ± 229.60 cells/µL) when compared to the mean absolute count (488.80 ± 201.10 cells/µL) for the HIV-negative (control) group. Conversely, the absolute $CD8^+$ T cell counts were significantly higher (p=0.048) between the two groups. The mean absolute $CD8^+$ T cell counts for the HIV-positive and HIV-negative (control) groups were 744.90 ± 647.30 cells/µL and 341.90 ± 202.30 cells/µL, respectively.

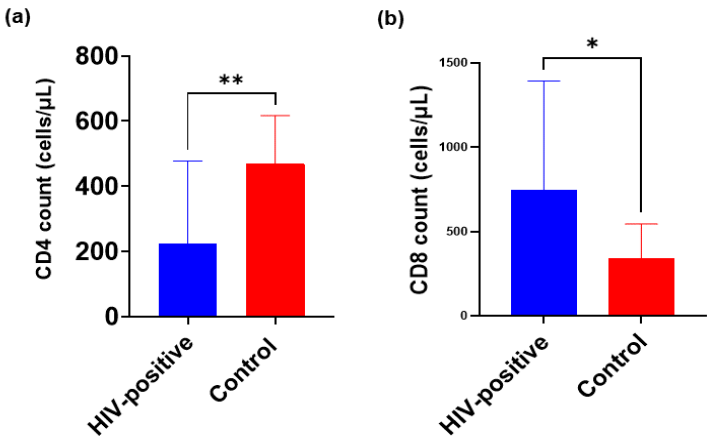


*Figure 5.1: Absolute CD4⁺ and CD8⁺ T cell count distribution in HIV-positive and control groups. Control refers to HIV-negative (control) group. Bar graph in each cluster represents the mean ± SD. * p < 0.05; ** p<0.01. CD; Cluster of differentiation; HIV, Human immunodeficiency virus.*

The distribution of CD4 counts was also investigated and is presented in Table 5.3. Low CD4 counts are defined as <200 cells/µL, intermediate CD4 counts as 200-499 cells/µL and high CD4 counts as ≥500 cells/µL.

Five individuals (38.46%) within the HIV-positive group presented with CD4 counts <200 cells/µL and another 38.46% (n=5) of individuals had CD4 cell counts ranging between 200-499 cells/µL. The CD4 counts of 30.77% (n=3) of the treatment-naïve study participants were ≥500 cells/µL. Surprisingly, 9.43% (n=5) of the HIV-negative (control) group, had CD4 counts <200 cells/µL, whilst 43.40% (n=23) had CD4 counts ranging from 200-499 cells/µL, and 47.17% (n=25) had CD4 counts ≥500 cells/µL.

Table 5.3: Distribution of CD4 counts of study groups.

	HIV-positive (n=13) n (%)			**HIV-negative (n=53) n (%)**		
CD4 count (cells/µL)	<200	200-499	≥500	<200	200-499	≥500
n (%)	5 (38.46%)	5 (38.46%)	3 (23.08%)	5 (9.43%)	23 (43.40%)	25 (47.17%)
Median (IQR)	32 (25-83)	265 (225-341)	591 (558-692)	92 (90-158)	375 (348-440)	621 (560-711)

CD; Cluster of differentiation; HIV, Human immunodeficiency virus; IQR, Interquartile range.

Next, the presence of haematological abnormalities between the HIV-positive and HIV-negative (control) groups was studied. The prevalence of cytopenias in the Treatment-naïve, HIV-positive patient group and HIV-negative (control) group are summarised in Table 5.4.

Among the HIV-positive group, anaemia was the most prevalent cytopenia (69.23%; n=9). For this group, thrombocytopenia was observed in one individual (7.69%; n=1), with no cases of bicytopenia being recorded.

The remaining individuals within the HIV-positive group did not present with any cytopenia (23.08%; n=3). Out of the individuals with anaemia, 77.78% (n=7) of cases were mild and 22.22% (n=2) were moderate. Furthermore, 55.56% (n=5) of all incidences of anaemia were normocytic, 33.33% (n=3) were macrocytic, and 11.11% (n=1) were microcytic.

Among the HIV-negative (control) group, anaemia was also the most prevalent cytopenia (30.19%; n=16), followed by thrombocytopenia (18.87%; n=10) and lastly bicytopenia (3.77%; n=2; anaemia and thrombocytopenia). The remaining individuals (47.17%; n=25) did not present with any cytopenia. Out of all the individuals with anaemia, 87.50% (n=14) of cases were mild and 12.50% (n=2) were moderate. The two individuals who presented with bicytopenia had mild anaemia. Additionally, 81.25% (n=15) of all incidences of anaemia were normocytic, 18.75% (n=3) were microcytic, and all individuals who presented with bicytopenia had normocytic anaemia. No cases of neutropenia or leukopenia were recorded in either group.

Table 5.4: Prevalence of cytopenia observed in the HIV-positive and HIV-negative (control) groups.

	HIV-positive n (%)	**Control n (%)**
Anaemia	9 (69.23%)	16 (30.19%)
Thrombocytopenia	1 (7.69%)	10 (18.87%)
Bicytopenia	0	2 (3.77%)
No cytopenias	3 (23.08%)	25 (47.17%)

Bicytopenia is indicated as a combination of anaemia and thrombocytopenia. Control refers to the HIV-negative (control) group. HIV, Human immunodeficiency virus.

Cytopenias were further examined in relation to CD4 counts within the HIV-positive group (Table 5.5A). All individuals (100%; n=3) with CD4 counts ≥500 cells/µL presented with anaemia. Among those with CD4 counts between 200-499 cells/µL, only 40% (n=2) presented with anaemia, whilst the remaining 60% (n=3) had no cytopenias. Additionally, 80% (n=4) of individuals with CD4 counts <200 cells/µL presented with anaemia, and 20% (n=1) presented with thrombocytopenia.

Within the HIV-negative (control) group (Table 5.5B), 16% (n=4) of individuals with CD4 counts ≥500 cells/µL presented with anaemia, and the same number had thrombocytopenia. The remaining 68% (n=17) had no cytopenia. Among individuals with CD4 counts ranging between 200-499 cells/µL, 43.48% (n=10) had anaemia, 17.39% (n=4) had thrombocytopenia, and 8.70% (n=2) had bicytopenia. The remaining 30.43% (n=7) had no cytopenia. Lastly, among individuals with CD4 counts <200 cells/µL, 40% (n=2) had anaemia, 40% (n=2) had thrombocytopenia, and the remaining 20% (n=1) had no cytopenia.

Except for one individual, individuals with a CD4 count < 200 cells/µL, regardless of HIV-status, presented with at least one cytopenia. Furthermore, among HIV-positive individuals, those with CD4 counts <200 cells/µL had the highest occurrence of cytopenias, whilst among the HIV-negative (control) individuals, the highest occurrence of cytopenias was observed in those with CD4 counts between 200-499 cells/µL.

Table 5.5A: Associations between varying degrees of CD4 counts and cytopenias within the HIV-positive group.

CD4 count (cells/µL)	HIV-positive (n=13)		
	Anaemia n (%)	**Thrombocytopenia n (%)**	**No cytopenia n (%)**
<200	4 (80%)	1 (20%)	0
200-499	2 (40%)	0	3 (60%)
≥500	3 (100%)	0	0

CD; Cluster of differentiation; HIV, Human immunodeficiency virus.

Table 5.5B: Associations between varying degrees of CD4 counts and cytopenias within the HIV-negative (control) group.

CD4 count (cells/µL)	HIV-negative (n=53)			
	Anaemia n (%)	Thrombocytopenia n (%)	Bicytopenia n (%)	No cytopenia n (%)
<200	2 (40%)	2 (40%)	0	1 (20%)
200-499	10 (43.47%)	4 (17.39%)	2 (8.70%)	7 (30.43%)
≥500	4 (16%)	4 (16%)	0	17 (68%)

Bicytopenia is indicated as a combination of anaemia and thrombocytopenia. CD; Cluster of differentiation; HIV, Human immunodeficiency virus.

5.4.3. Circulating haematopoietic stem and progenitor cell frequencies

Under normal physiological conditions, a small amount of HSPCs exit the BM and enter into circulation (peripheral blood)[38]. The exact function of circulating $Lin^{-}CD34^{++}$ HSPCs is yet to be determined, but it is currently hypothesised that they are involved in maintenance of the HSPC niches and tissue repair[39]. The frequencies and absolute counts of the $Lin^{-}CD34^{++}$ HSPCs in the two groups were compared.

The mean frequency observed in the HIV-positive group (0.090 ± 0.057%) was significantly lower than that of the HIV-negative (control) group (0.21 ± 0.18%) (p=0.0022) (Figure 5.2A). Similarly, the mean absolute count in the HIV-positive group (314.50 ± 224.20 cells/µL) was also significantly lower than the HIV-negative (control) group (802.60 ± 668.80 cells/µL) (p=0.0096) (Figure 5.2B).

Furthermore, the Spearman's rank correlation test was used to correlate the circulating $Lin^{-}CD34^{++}$ HSPCs with the CD4 counts for both groups to determine potential associations (Figure 5.3A-D). However, no significant associations were observed between the circulating HSPCs and the CD4 counts for either group. Furthermore, all haematological parameters were correlated with the frequency and absolute counts of circulating $Lin^{-}CD34^{++}$ HSPCs (Appendix V: supplementary figures S1-2). No clear associations pertaining to the circulating HSPCs could be identified.

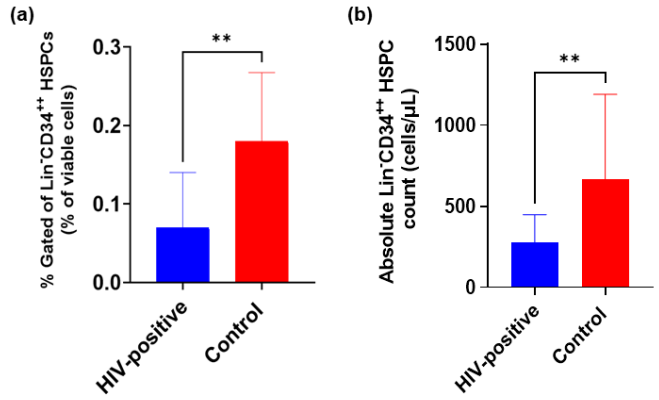


*Figure 5.2: Comparative graph of circulating Lin$^-$CD34^{++} haematopoietic stem and progenitor cells in HIV-positive and -negative (control) individuals. (A) Lin$^-$CD34^{++} haematopoietic stem and progenitor cells as a percentage of viable cells. (B) Absolute Lin$^-$CD34^{++} haematopoietic stem and progenitor cells cell counts (cells/µL). Control refers to the HIV-negative (control) group. Bar graphs represent mean ± SD values. **p<0.01. CD, Cluster of differentiation; HIV; Human immunodeficiency virus; HSPC, Haematopoietic stem and progenitor cells; Lin, Lineage.*

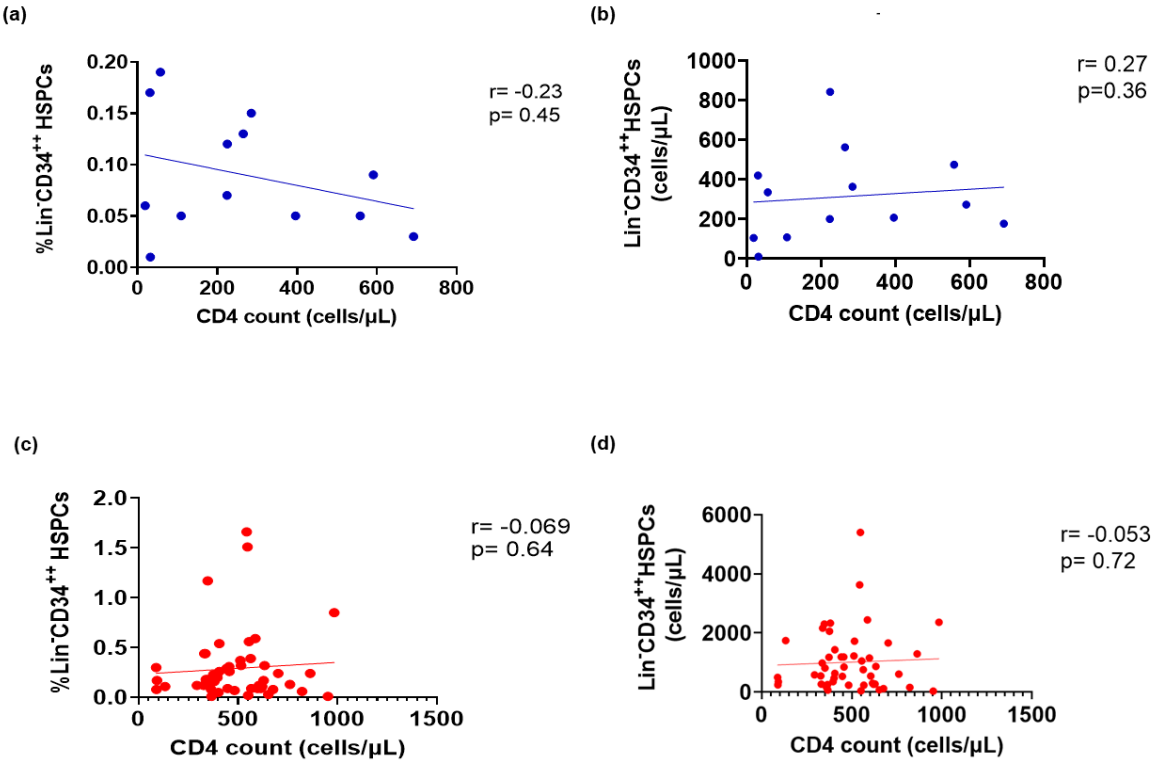


Figure 5.3: Correlation analysis between the frequency (left) and absolute counts (right) of circulating Lin$^-$CD34^{++} HSPCs and absolute CD4$^+$ T cell counts. Correlations between (A-B) HIV-positive (n=13) and (C-D) HIV-negative (control) (n=53) groups are shown. CD; Cluster of differentiation; HIV, Human immunodeficiency virus; HSPC, Haematopoietic stem and progenitor cells; Lin, Lineage.

5.5. Discussion

This study investigated the haematological and immunological parameters within a treatment-naïve, HIV-positive cohort as well as their associations with circulating $Lin^{-}CD34^{++}$ HSPC.

The CD4 count is used to monitor immune status and susceptibility to opportunistic infections during HIV infection[40]. According to the Centres of Disease Control (CDC) guidelines, 23.08% of the Treatment-naïve, HIV-positive individuals were classified as stage I (≥500 cells/µL) based solely on their CD4 counts[41]. The remaining individuals were evenly distributed between stages II (38.46%; 200-499 cells/µL) and III (38.46%; <200 cells/µL) of HIV infection[41]. Among the treatment-naïve, HIV-positive group, $CD4^{+}$ T cell counts were significantly lower, while the $CD8^{+}$ T cell count was significantly higher when compared to the HIV-negative controls (Figure 5.1). This is reflective of the progressive HIV-mediated immune destruction that occurs in the absence of treatment[42].

The HIV-mediated immune destruction is characterised by $CD4^{+}$ T cell depletion and $CD8^{+}$ T cell dysfunction[43]. The virus preferentially infects and actively eliminates $CD4^{+}$ T cells via direct or indirect mechanisms of action[44]. This is significant as $CD4^{+}$ T cells orchestrate adaptive immune responses by recruiting various effector cells to sites of infection in addition to their own direct effector functions[45]. The direct mechanism of $CD4^{+}$ T cell depletion involves the cytolytic effects exerted by the virus, while indirect mechanisms include chronic immune activation and inflammation, leading to apoptosis[44]. Furthermore, both the absolute counts and frequency (percentage) of $CD4^{+}$ T cells decrease in circulation and body reserves[44]. As such, by targeting the $CD4^{+}$ T cell population, the virus creates an immunodeficient environment with heightened susceptibility to opportunistic infections[46].

Moreover, the virus induces a state of immune dysfunction, as evidenced by the $CD8^{+}$ T cells [46]. Cluster of differentiation-8^{+} T cells play a crucial role in controlling viraemia, through their potent cytolytic and non-cytolytic activity[47]. Furthermore, during infection, naïve $CD8^{+}$ T cells are stimulated upon encountering antigens and continue to actively proliferate even in the absence of antigens[48]. The persistent expansion of naïve $CD8^{+}$ T cells into effector $CD8^{+}$ T cells is sustained by the chronic immune activation and inflammation promoted by the virus[48, 49]. Notably, the activity of $CD8^{+}$ T cells is tightly regulated; however, the HIV-induced persistent

expansion and over-activation lead to T cell exhaustion and impaired homing and retention capacity[48, 50].

T cell exhaustion is characterised by the decreased potency of $CD8^+$ T cell responses and survival, as well as the upregulation of inhibitory molecules such as programmed cell death protein 1 and its ligand[51].

Individuals with low CD4 counts are more susceptible to the development of cytopenias[52, 53]. As observed in this study (Table 5.5A), all HIV-positive individuals with CD4 counts <200 cells/µL presented with at least one cytopenia. As such, the haematological indices were assessed to monitor in the haematological parameters when the treatment-naïve HIV-positive and HIV-negative (control) groups were compared.

The mean values of the RBC, HGB, and HCT (Table 5.2) were significantly lower in the HIV-positive group than the HIV-negative (control) group. This finding was in alignment with the results reported by Malapati *et al.* and Martina *et al.*[54, 55]. Notably, the MCV mean value was non-significantly higher in the treatment-naïve HIV-positive group, which contrasted the findings of Malapati *et al.* who reported a significantly lower MCV also in a treatment-naïve, HIV-positive group compared to the HIV-negative controls[54]. Anaemia was recorded as the most prevalent cytopenia in the treatment-naïve, HIV-positive group (69.23%). This observation is in agreement with similar findings reported in various studies. In particular the prevalence of anaemia across Sub-Saharan Africa varied from 67.38% within the Mbarara region in Uganda[56]; 66.1%, in a study performed across Malawi and Mozambique[57]; 63.4%, in Dessie, Ethiopia[23]; and 66%, in Zaria, Nigeria[58]. However, other studies reported a lower prevalence of anaemia in treatment-naïve cohorts. In Limpopo South Africa, Swafo *et al.* [59] reported a prevalence of 13.0%, while Takuva *et al.* [60] reported a prevalence of 25.8% in Johannesburg, South Africa. Damtie *et al.* [5] reported a prevalence of 37.1% in Debre Tabor, Ethiopia. Due to the small number of study participants in the treatment-naïve, HIV-positive cohort, the prevalence reported in this study should be interpreted with caution.

This study observed that 80% of individuals with low CD4 counts (<200 cells/µL) presented with anaemia (Table 5.5A). This was also observed by Tamir *et al.* and Suja *et al.*, where anaemia was predominantly reported in individuals with low CD4 counts (<200 cells/µL)[23, 61].

As the onset of anaemia is multifactorial, distinguishing between the degree of anaemia severity and the morphological characteristics assists with narrowing down anaemia aetiologies[62]. The majority of anaemia cases observed were mild (77.78%) whilst the remaining cases were moderate (22.22%), similarly to the results of Damtie *et al.* where the predominate cases of anaemia were mild (76.7%) followed by moderate (22.4%) and severe (0.9%)[5]. The morphological characteristics of anaemia in this study revealed among the treatment-naïve, HIV-positive group, 55.56% of incidences of anaemia were normocytic, 33.33% was macrocytic and 11.11% was microcytic. Typically microcytic, and macrocytic anaemias are associated with nutritional deficiencies that arise due to HIV-mediated gastrointestinal dysfunctional disturbing iron, folate, and vitamin B12 absorption[56, 63, 64]. Normocytic anaemia is the most common type of anaemia in HIV infection as observed in this study. In their study, Enawgaw *et al.* reported that 48.9% of anaemia cases was normocytic[65]. Notably, anaemia of inflammation or chronic disease often presents as a mild to moderate microcytic or normocytic anaemia among HIV-positive individuals[66]. Normocytic anaemia observed in the treatment-naïve, HIV-positive cohort is typically associated with BM myelodysplasia[56, 67]. The BM myelodysplasia occurs as consequence of HIV infection, subsequently resulting in impaired erythropoiesis[56].

Notably, erythropoiesis is triggered by erythropoietin (EPO) which functions to maintain blood concentrations of HGB[68]. Interestingly, Panjeta *et al*[69] reported a significant, strong negative correlation between EPO and HGB levels. These investigators observed that higher EPO concentrations were associated with decreased HGB concentrations in the blood (anaemia). Under hypoxic conditions EPO is secreted by the kidneys to stimulate erythroid proliferation and differentiation[68]. Impaired erythropoiesis is associated with an imbalance between the erythroid proliferation and differentiation axis. The subsequent imbalance is due to a surge in erythroid proliferation with limited to no differentiation thereby giving rise to immature (anucleated) RBCs, ultimately leading to anaemia[70]. Whilst the occurrence of anaemia is multifaceted, chronic kidney disease or chronic infections resulting in a pro-inflammatory environment and/or nutritional deficiencies may perpetuate an erythroid imbalance[71, 72]. In addition, pregnancy itself is associated with a variety of physiological changes that frequently result in physiological anaemia. Furthermore, impairment due to decreased/disruptions in EPO activity, limits differentiation of erythroid precursors into mature RBCs in blood

(decreased RBCs frequency and HCT levels)[73]. Therefore, the significantly lower RBC, HGB and HCT mean values (Table 5.2) observed in this study may be associated with HIV-mediated disruption of erythropoiesis.

Thrombocytopenia has been reported as highly prevalent, as outlined in a meta-analysis by Getawa *et al,* in treatment-naïve cohorts[22]. Although thrombocytopenia is a common haematological complication in PLWH[74, 75], only one treatment-naïve, HIV-positive study participant, who also had a low CD4 count (<200 cells/µL) presented with thrombocytopenia in this study. This can be attributed to the small number of study participants in the treatment-naïve, HIV-positive group.

Thrombocytopenia was observed as the second most common (15%) unicytopenia in a treatment-naïve, HIV-positive cohort by Sari *et al.* [30]. Furthermore, thrombocytopenia has been frequently coupled with rapid loss of CD4 cells, accelerated disease progression and increased mortality[24, 76]. The non-significant difference observed for platelet counts (Table 5.2) in which the platelet counts were numerically higher in the HIV-positive group compared to the HIV-negative (control) group is a reflection that the majority of HIV-positive study participants did not present with thrombocytopenia.

The drivers of erythropoiesis and megakaryopoiesis are EPO and thrombopoietin (TPO) which are produced by the kidney and liver respectively[77]. The immune activation associated with HIV, aides in the suppression of EPO resulting in impaired erythropoiesis[63]. Impaired erythropoiesis leads to inefficient oxygenation of tissues with the subsequent dampening of the immune defence mechanisms[63]. This dampened immune mechanisms promotes an optimal environment for the proliferation of pathogens and increasing susceptibility to opportunistic infections[63].

Megakaryocyte maturation can be impaired by HIV infection, by dysregulating cell signalling during megakaryocyte-erythroid progenitor (MEP) differentiation[78]. This affects both the megakaryocyte lineage and erythroid lineages as both RBCs and platelets maintain the same differentiation pathway up until the MEPs where they part ways[11, 28]. During HIV infection, mature megakaryocytes internalise the virus and potentially act as carriers[79]. The HIV-infected megakaryocytes further produce infected platelets that have a truncated life span[28, 80].

Whilst there were no cases of leukopenia nor neutropenia observed in this study, various studies reported noteworthy prevalence of both leukopenia and neutropenia. Princy *et al.*, reported a prevalence of 12.7% and 8.2% for leukopenia and neutropenia respectively[78]. In another study, Tilahun *et al.* reported a prevalence of leukopenia (26.7%) and neutropenia (16.5%) respectively[79]. Both these studies were done in treatment-naïve cohorts[81, 82]. Furthermore, in the same studies, thrombocytopenia was less prevalent than leukopenia and neutropenia, at 25.9% and 11.3% respectively[81, 82]. Interestingly, in this study both the WCC and absolute neutrophil count were significantly lower in the HIV-positive group than the HIV-negative (control) group (Table 5.2).

It is important to note that besides HIV infection, pregnancy also produces various physiological changes that impact an individual's haematological profile, together with the inclination to malabsorption, supporting the onset of cytopenias[83]. During pregnancy, both maternal and foetal nutritional requirements become considerably higher, thus increasing the likelihood of anaemia[84]. The incidence of anaemia during pregnancy steadily increase from the first to the third trimester[85]. Iron, folate, and vitamin B12 deficiencies are some of the factors that contribute to the onset of anaemia in pregnancy[85]. Microcytic anaemia are associated with an iron deficiency, whilst macrocytic anaemias are associated with folate and vitamin B12 deficiencies[85]. Notably, physiological anaemia is common in pregnancy. This is when the increase in RBC volume does not keep up with the increase in plasma volume. Plasma volumes may expand up to 60% during pregnancy, while the percentage expansion of RBC volume only expands up to 50%[85]. The HIV-negative (control) group reported a prevalence of 30.19% representative of their nutrition deficiencies or physiological anaemia.

Thrombocytopenia during pregnancy is a benign and common diagnosis, with approximately 70-80% of all incidences being classified as gestational thrombocytopenia (defined as PLTs $<150 \times 10^9/L$)[86, 87]. Low PLT counts can be observed from the first trimester with a progressive depletion throughout pregnancy, reaching nadir at delivery[88]. Whilst gestational thrombocytopenia may occur at any trimester it is most commonly observed during mid-to late second trimester and third trimester[86]. The occurrence of gestational thrombocytopenia is hypothesised to be due to different pathophysiological conditions some of which are exclusive to pregnancy[86]. The pathophysiological conditions exclusive to pregnancy include haemodilution, increased PLT consumption as well as placental dysfunction[86, 88, 89]. Usually

platelet counts <100 x 10^9/L exclude gestational thrombocytopenia and requires further investigations into the aetiology[90]. Among the HIV-negative (control) group, the prevalence of thrombocytopenia was 18.87%, and is likely due to the manifestation of gestational thrombocytopenia.

Taken together, the differences observed within the haematological and immunological profile between the treatment-naïve, HIV-positive and HIV-negative (control) cohorts is hypothesised to be due to ongoing HIV-mediated disruption of haematopoiesis. This hypothesis is strengthened by the significantly lower frequencies and absolute counts of the circulating Lin^-CD34^{++} HSPCs in the HIV-positive group when compared to the HIV-negative (control) cohort (Figure 5.2). We observed in this study that HIV infection results in the reduction of circulating Lin^-CD34^{++} HSPC. No associations could be determined between the circulating Lin^-CD34^{++} HSPCs and the CD4 count (Figure 5.3). The small number of treatment-naïve HIV-positive study participants may have contributed to the lack of association observed in this study.

The decrease in circulating Lin^-CD34^{++} HSPCs may be indicative of HIV-mediated impaired haematopoiesis, which commonly manifests as impaired erythropoiesis (anaemia) and impaired megakaryopoiesis (thrombocytopenia). Gibellini *et al.*[77] suggested that the occurrence of peripheral blood cytopenias is not limited to a single mechanism of action regarding HIV interaction with HSPCs. Rather, cytopenias occur due to various HIV-mediated mechanisms such as (i) impaired HSPC functionality (differentiation and self-renewal) (ii) inhibition of HSPC differentiation into specific blood cell lineages (iii) impaired BM-derived stromal cell function, and (iv) disruption of the cytokine milieu in the BM[77].

The limitations of this study include the small population of the treatment-naïve, HIV-positive group as well as the absence of the viral load data. Furthermore, the results of the treatment-naïve, HIV-positive group are confounded by pregnancy which are also associated with the manifestation of cytopenias. Furthermore, participants should have been matched for gestational weeks, as the stage of pregnancy may have had a stronger influence on the results observed. Lastly, an assessment of the C-reactive protein levels would have been useful to determine the presence of any underlying infections within the HIV-negative (control) group that could have contributed to the results obtained.

5.6. Conclusion

Anaemia and thrombocytopenia were the most observed peripheral blood cytopenias in this study. Furthermore, HIV-associated reduction of circulating $Lin^{-}CD34^{++}$ HSPC was observed which may be reflective of impaired haematopoiesis. This suggested that the HIV-mediated impaired haematopoiesis presents as peripheral blood cytopenias, such as anaemia and thrombocytopenia among the treatment-naïve HIV-positive individuals.

5.7. References

1. Reichard A, Wanner N, Farha S, Asosingh K. Hematopoietic stem cells and extramedullary hematopoiesis in the lungs. Cytometry A. 2023; 103(12):967-77.

2. Takayama N, Murison A, Takayanagi S-i, Arlidge C, Zhou S, Garcia-Prat L*, et al.* The Transition from Quiescent to Activated States in Human Hematopoietic Stem Cells Is Governed by Dynamic 3D Genome Reorganization. Cell Stem Cell. 2021; 28(3):488-501.e10.

3. Luis TC, Barkas N, Carrelha J, Giustacchini A, Mazzi S, Norfo R*, et al.* Perivascular niche cells sense thrombocytopenia and activate hematopoietic stem cells in an IL-1 dependent manner. Nat Commun. 2023; 14(1):6062.

4. Chen Z, Guo Q, Song G, Hou Y. Molecular regulation of hematopoietic stem cell quiescence. Cell. Mol. Life Sci. 2022; 79(4):218.

5. Damtie S, Workineh L, Kiros T, Eyayu T, Tiruneh T. Hematological abnormalities of adult HIV-infected patients before and after initiation of highly active antiretroviral treatment at Debre Tabor Comprehensive Specialized Hospital, northcentral Ethiopia: a cross-sectional study. HIV AIDS (Auckl.). 2021; 2021(13):477-84.

6. Gebreweld A, Fiseha T, Girma N, Haileslasie H, Gebretsadik D. Prevalence of cytopenia and its associated factors among HIV infected adults on highly active antiretroviral therapy at Mehal Meda Hospital, North Shewa Zone, Ethiopia. PLoS One. 2020; 15(9):e0239215.

7. Duguma N, Tesfaye Kiya G, Adissu Maleko W, Bimerew LG. Hematological parameters abnormalities and associated factors in HIV-positive adults before and after highly active antiretroviral treatment in Goba Referral Hospital, southeast Ethiopia: A cross-sectional study. SAGE Open Med. 2021; 9(20503121211020175):1-12.

8. Marchionatti A, Parisi MM. Anemia and thrombocytopenia in people living with HIV/AIDS: a narrative literature review. Int Health. 2021; 13(2):98-109.

9. Fiseha T, Ebrahim H. Prevalence and Predictors of Cytopenias in HIV-Infected Adults at Initiation of Antiretroviral Therapy in Mehal Meda Hospital, Central Ethiopia. J. Blood Med. 2022; 13:201-11.

10. Durandt C, Potgieter JC, Mellet J, Herd C, Khoosal R, Nel JG*, et al.* HIV and haematopoiesis. S. Afr. Med. J. 2019; 109(8b):40-5.

11. Herd CL, Mellet J, Mashingaidze T, Durandt C, Pepper MS. Consequences of HIV infection in the bone marrow niche. Front. Immunol. 2023; 14(1163012):1-17.

12. Sebastian NT, Zaikos TD, Terry V, Taschuk F, McNamara LA, Onafuwa-Nuga A*, et al.* CD4 is expressed on a heterogeneous subset of hematopoietic progenitors, which persistently

harbor CXCR4 and CCR5-tropic HIV proviral genomes in vivo. PLoS Pathog. 2017; 13(7):e1006509.

13. Cao G, Wang Y, Wu Y, Jing W, Liu J, Liu M. Prevalence of anemia among people living with HIV: A systematic review and meta-analysis. EClinicalMedicine. 2022; 44(101283):1-15.

14. Chaparro CM, Suchdev PS. Anemia epidemiology, pathophysiology, and etiology in low- and middle-income countries. Ann. N. Y. Acad. Sci. 2019; 1450(1):15-31.

15. Buttarello M. Laboratory diagnosis of anemia: are the old and new red cell parameters useful in classification and treatment, how? Int. J. Lab. Hematol. 2016; 38(S1):123-32.

16. Ware AD. The complete blood count and white blood cell differential. In: Clarke W, Marzinke MA, editors. Contemporary Practice in Clinical Chemistry (Fourth Edition). Cambridge, Massachusetts: Academic Press; 2020. p. 429-44.

17. Pasricha S-R, Colman K, Centeno-Tablante E, Garcia-Casal M-N, Peña-Rosas J-P. Revisiting WHO haemoglobin thresholds to define anaemia in clinical medicine and public health. Lancet Haematol. 2018; 5(2):e60-e2.

18. Turner J, Parsi M, Badireddy M. Anemia [Internet]. StatPearls StatPearls Publishing; 2022. [cited: 2024/01/24]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK499994/

19. Schop A, Stouten K, Riedl JA, van Houten RJ, Leening MJG, Bindels PJE*, et al.* The accuracy of mean corpuscular volume guided anaemia classification in primary care. Fam. Pract. 2021; 38(6):735-9.

20. Hughes CA. Anemia Assessment. In: Mahmoud SH, editor. Patient Assessment in Clinical Pharmacy: A Comprehensive Guide. Cham: Springer International Publishing; 2019. p. 415-21.

21. Talargia F, Getacher L. Thrombocytopenia and Associated Factors Among HIV Infected Patients in Pre-and Post-Anti-Retroviral Therapy, North East Ethiopia. J. Blood Med. 2021; 12:741-8.

22. Getawa S, Aynalem M, Bayleyegn B, Adane T. The global prevalence of thrombocytopenia among HIV-infected adults: A systematic review and meta-analysis. Int. J. Infect. Dis. 2021; 105:495-504.

23. Tamir Z, Seid A, Haileslassie H. Magnitude and associated factors of cytopenias among antiretroviral therapy naïve Human Immunodeficiency Virus infected adults in Dessie, Northeast Ethiopia. PLoS One. 2019; 14(2):e0211708.

24. Ayanaw MA, Yabeyu AB, Lenjiso G, Kifle ZD. Prevalence and predictors of thrombocytopenia among HAART naive HIV positive patients at Ambo University Referral Hospital. Clin Epidemiol Glob Health. 2022; 15(101049):1-4.

25. Sun S, Urbanus RT, ten Cate H, de Groot PG, de Laat B, Heemskerk JWM*, et al.* Platelet Activation Mechanisms and Consequences of Immune Thrombocytopenia. Cells. 2021; 10(12):3386.

26. Louw S, Jacobson BF, Wiggill TM, Chapanduka Z, Sarah Mayne E. HIV-associated thrombotic thrombocytopenic purpura (HIV-TTP): A practical guide and review of the literature. HIV Med. 2022; 23(10):1033-40.

27. Louw S, Gounden R, Mayne ES. Thrombotic thrombocytopenic purpura (TTP)-like syndrome in the HIV era. Thrombosis Journal. 2018; 16(1):35.

28. Pretorius E. Platelets in HIV: A Guardian of Host Defence or Transient Reservoir of the Virus? Front. Immunol. 2021; 12(649465):1-12.

29. Woldeamanuel GG, Wondimu DH. Prevalence of thrombocytopenia before and after initiation of HAART among HIV infected patients at black lion specialized hospital, Addis Ababa, Ethiopia: a cross sectional study. BMC Hematol. 2018; 18:9.

30. Sarı Ö, Tekin S, Üre Ü, Kapmaz M. Cytopenias in Treatment-naive HIV Patients: A Comparison of Turkey and Somalia. Bagcilar Med Bull. 2022; 7(3):279-87.

31. Shi X, Sims MD, Hanna MM, Xie M, Gulick PG, Zheng YH*, et al.* Neutropenia during HIV infection: adverse consequences and remedies. Int. Rev. Immunol. 2014; 33(6):511-36.

32. Fan L, Han J, Xiao J, Dai G, Hao Y, Yang D*, et al.* The stage-specific impairment of granulopoiesis in people living with HIV/AIDS (PLWHA) with neutropenia. J Leukoc Biol 2020; 107(4):635-47.

33. Hensley-McBain T, Klatt NR. The Dual Role of Neutrophils in HIV Infection. Curr. HIV/AIDS Rep. 2018; 15(1):1-10.

34. Iuliano AD, Weidle PJ, Brooks JT, Masaba R, Girde S, Ndivo R*, et al.* Neutropenia in HIV-Infected Kenyan Women Receiving Triple Antiretroviral Prophylaxis to Prevent Mother-to-Child HIV Transmission Is Not Associated with Serious Clinical Sequelae. J Int Assoc Provid AIDS Care. 2015; 14(3):261-8.

35. Wan J, Ren Y, Yang X, Li X, Xia L, Lu N. The Role of Neutrophils and Neutrophil Extracellular Traps in Acute Pancreatitis. Front Cell Dev Biol 2021; 8(565758):1-8.

36. Rupa-Matysek J, Gil L, Mozer-Lisewska I, Brzeźniakiewicz-Janus K. Neutropenia – there are always two sides to a story. Acta Haematol. Pol. 2020; 51(3):133-41.

37. Lawrie D, Coetzee LM, Becker P, Mahlangu J, Stevens W, Glencross DK. Local reference ranges for full blood count and CD4 lymphocyte count testing. S. Afr. Med. J. 2009; 99(4):243-8.

38. Mende N, Laurenti E. Hematopoietic stem and progenitor cells outside the bone marrow: where, when, and why. Exp. Hematol. 2021; 104:9-16.

39. Fadini GP, Mehta A, Dhindsa DS, Bonora BM, Sreejit G, Nagareddy P*, et al.* Circulating stem cells and cardiovascular outcomes: from basic science to the clinic. Eur. Heart J. 2020; 41(44):4271-82.

40. Bekolo CE, Ndeso SA, Gougue CP, Moifo LL, Mangala N, Tchendjou P*, et al.* The effect of the Universal Test and Treat policy uptake on CD4 count testing and incidence of opportunistic infections among people living with HIV infection in Cameroon: a retrospective analysis of routine data. Dialogues Health. 2023; 2(100120):1-8.

41. Centers for Disease Control and Prevention. Monitoring selected national HIV prevention and care objectives by using HIV surveillance data—United States and 6 dependent areas, 2019. HIV Surveillance Supplemental Report. Atlanta, Georgia, USA. 2021; Document number: 2. [updated: May 2021. cited: 2023/11/30.] Available from: https://www.cdc.gov/hiv/library/reports/hiv-surveillance/vol-26-no-2/content/technical-notes.html#print

42. Boyd MAA, van Bockel D, Munier CML, Kelleher AD. Navigating the complexity of chronic HIV-1 associated immune dysregulation. Curr. Opin. Immunol. 2022; 76(102186):1-6.

43. Chandran A, Kanagasabapathy B, Ramesh C, Patil TR, Manikandan G, Palaniswamy A*, et al.* HIV-associated thrombocytopenia: Profiling hematological changes in the HIV infected and AIDS patients. Ann Clin Lab Res. 2020; 8(3):1-7.

44. Vidya Vijayan KK, Karthigeyan KP, Tripathi SP, Hanna LE. Pathophysiology of CD4+ T-Cell Depletion in HIV-1 and HIV-2 Infections. Front. Immunol. 2017; 8(580):1-8.

45. Kervevan J, Chakrabarti LA. Role of CD4+ T Cells in the Control of Viral Infections: Recent Advances and Open Questions. Int. J. Mol. Sci. 2021; 22(2):523.

46. Akase IE, Musa BOP, Obiako RO, Ahmad Elfulatiy A, Mohammed AA. Immune Dysfunction in HIV: A Possible Role for Pro- and Anti-Inflammatory Cytokines in HIV Staging. J Immunol Res. 2017; 2017(4128398):1-5.

47. Perdomo-Celis F, Taborda NA, Rugeles MT. CD8(+) T-Cell Response to HIV Infection in the Era of Antiretroviral Therapy. Front. Immunol. 2019; 10(1896):1-19.

48. Nasi A, Chiodi F. Mechanisms regulating expansion of CD8+ T cells during HIV-1 infection. J Intern Med. 2018; 283(3):257-67.

49. Cao W, Mehraj V, Kaufmann DE, Li T, Routy JP. Elevation and persistence of CD8 T-cells in HIV infection: the Achilles heel in the ART era. J. Int. AIDS Soc. 2016; 19(1):20697.

50. Zhang C, Hu W, Jin J, Zhou M, Song J, Deng J*, et al.* The role of CD8 T cells in controlling HIV beyond the antigen-specific face. HIV Med. 2020; 21(11):692-700.

51. Sachdeva M, Fischl MA, Pahwa R, Sachdeva N, Pahwa S. Immune exhaustion occurs concomitantly with immune activation and decrease in regulatory T cells in viremic chronically HIV-1-infected patients. J. Acquir. Immune Defic. Syndr. 2010; 54(5):447-54.

52. Wankah PN, Tagny CT, Mbanya DNS. Profile of blood cell abnormalities among antiretroviral therapy naïve HIV patients attending the Yaounde University Teaching Hospital, Cameroon. BMC Hematol. 2014; 14(1):15.

53. Fan L, Li C, Zhao H. Prevalence and Risk Factors of Cytopenia in HIV-Infected Patients before and after the Initiation of HAART. Biomed Res Int. 2020; 2020(3132589):1-10.

54. Malapati B, Nadeem SM, Shah MM. Analysis of blood parameters in HIV-positive patients. International Journal of Clinical Biochemistry and Research. 2020; 7(3):388-94.

55. Martina NA, Chinemerem EJ. Haematological Changes in HIV Subjects Receiving Antiretroviral Treatment in Enugu State University of Science and Technology Teaching Hospital, Enugu, Nigeria. Afr. J. Health Sci. 2022; 35(4):417-25.

56. Katemba C, Muzoora C, Muwanguzi E, Mwambi B, Atuhairwe C, Taremwa IM. Hematological abnormalities in HIV-antiretroviral therapy naive clients as seen at an immune suppression syndrome clinic at Mbarara Regional Referral Hospital, southwestern Uganda. J. Blood Med. 2018; 9:105-10.

57. Ciccacci F, Lucaroni F, Latagliata R, Morciano L, Mondlane E, Balama M*, et al.* Hematologic alterations and early mortality in a cohort of HIV positive African patients. PLoS One. 2020; 15(11):e0242068.

58. Usman A, Balogun O, Shuaib BI, Musa BOP, Yusuf AA, Ajayi EIO. Prevalence of Cytopenia and its Correlation with Immunosuppression in Naïve HIV-1 Infected Patients Initiating First-Line Antiretroviral Therapy: A Pilot Study. Infect Chemother. 2023; 55(4):479-89.

59. Swafo RM, Moraba MM, Mashinya F. Haematological disorders in HIV-infected people in a semi-rural area in the Capricorn district of Limpopo province, South Africa. AJPHES. 2021; 27(1):59-71.

60. Takuva S, Maskew M, Brennan AT, Sanne I, MacPhail AP, Fox MP. Anemia among HIV-Infected Patients Initiating Antiretroviral Therapy in South Africa: Improvement in Hemoglobin regardless of Degree of Immunosuppression and the Initiating ART Regimen. J. Trop. Med. 2013; 2013(162950):1-6.

61. Suja S, Saravanan T, Karthikeyan S. Profile of hematological abnormalities and its correlation with absolute CD4 count and human immunodeficiency virus viral load in human immunodeficiency virus-infected patients in a tertiary care hospital. Indian J Sex Transm Dis AIDS. 2020; 41(2):156-61.

62. He Y, Hou J, Huo W, Li L, Liu X, Mao Z*, et al.* Prevalence and morphological subtype distributions of anaemia in a Chinese rural population: the Henan Rural Cohort study. Public Health Nutr. 2023; 26(6):1254-63.

63. Obeagu EI, Obeagu GU, Ukibe NR, Oyebadejo SA. Anemia, iron, and HIV: decoding the interconnected pathways: A review. Medicine. 2024; 103(2):e36937.

64. Aslinia F, Mazza JJ, Yale SH. Megaloblastic anemia and other causes of macrocytosis. Clin. Med. Res. 2006; 4(3):236-41.

65. Enawgaw B, Alem M, Addis Z, Melku M. Determination of hematological and immunological parameters among HIV positive patients taking highly active antiretroviral treatment and treatment naïve in the antiretroviral therapy clinic of Gondar University Hospital, Gondar, Northwest Ethiopia: a comparative cross-sectional study. BMC Hematol. 2014; 14(1):8.

66. O'Mahony D, Mabunda SA, Mntonintshi M, Iruedo J, Kaswa R, Blanco-Blanco E*, et al.* Causes of Moderate and Severe Anaemia in a High-HIV and TB-Prevalent Adult Population in the Eastern Cape Province, South Africa. Int. J. Environ. Res. Public Health. 2023; 20(4):1-17.

67. Nemeth E, Ganz T. Anemia of inflammation. Hematol. Oncol. Clin. North Am. 2014; 28(4):671-81, vi.

68. Panjeta M, Tahirović I, Sofić E, Ćorić J, Dervišević A. Interpretation of Erythropoietin and Haemoglobin Levels in Patients with Various Stages of Chronic Kidney Disease. J Med Biochem. 2017; 36(2):145-52.

69. Panjeta M, Tahirovic I, Karamehic J, Sofic E, Ridic O, Coric J. The Relation of Erythropoietin Towards Hemoglobin and Hematocrit in Varying Degrees of Renal Insufficiency. Mater Sociomed. 2015; 27(3):144-8.

70. Gupta R, Musallam KM, Taher AT, Rivella S. Ineffective Erythropoiesis: Anemia and Iron Overload. Hematol. Oncol. Clin. North Am. 2018; 32(2):213-21.

71. Zivot A, Lipton JM, Narla A, Blanc L. Erythropoiesis: insights into pathophysiology and treatments in 2017. Mol. Med. 2018; 24(11):1-15.

72. Yu JC, Shliakhtsitsava K, Wang YM, Paul M, Farnaes L, Wong V*, et al.* Hematologic Manifestations of Nutritional Deficiencies: Early Recognition is Essential to Prevent Serious Complications. J. Pediatr. Hematol. Oncol. 2019; 41(3):e182-e5.

73. Bhoopalan SV, Huang LJ, Weiss MJ. Erythropoietin regulation of red blood cell production: from bench to bedside and back. F1000Res. 2020; 9(F1000 Faculty Rev-1153):3-15.

74. Raadsen M, Du Toit J, Langerak T, van Bussel B, van Gorp E, Goeijenbier M. Thrombocytopenia in Virus Infections. J Clin Med 2021; 10(4):877.

75. Madzime M, Rossouw TM, Theron AJ, Anderson R, Steel HC. Interactions of HIV and Antiretroviral Therapy With Neutrophils and Platelets. Front. Immunol. 2021; 12(634386):1-20.

76. Nka AD, Sosso SM, Fokam J, Bouba Y, Teto G, Simo Rachel R*, et al.* Thrombocytopenia according to antiretroviral drug combinations, viremia and CD4 lymphocytes among HIV-infected patients in Cameroon: a snapshot from the City of Yaoundé. BMC Res. Notes. 2019; 12(632):1-5.

77. Gibellini D, Clo A, Morini S, Miserocchi A, Ponti C, Re MC. Effects of human immunodeficiency virus on the erythrocyte and megakaryocyte lineages. World J Virol. 2013; 2(2):91-101.

78. Lv X, Li P, Yue P, Tang P, Zhou F. Risk factors and prognosis of thrombocytopenia in people living with HIV/AIDS. Ther Adv Reprod Health. 2023; 14(7316456,):1-13.

79. Awamura T, Nakasone ES, Gangcuangco LM, Subia NT, Bali A-J, Chow DC*, et al.* Platelet and HIV Interactions and Their Contribution to Non-AIDS Comorbidities. Biomolecules. 2023; 13(11):1608.

80. Real F, Capron C, Sennepin A, Arrigucci R, Zhu A, Sannier G*, et al.* Platelets from HIV-infected individuals on antiretroviral drug therapy with poor CD4+ T cell recovery can harbor replication-competent HIV despite viral suppression. Sci. Transl. Med. 2020; 12(535):eaat6263.

81. Princy JJD, Singh KB, Biplab N, Reema N, Boini R, Gowde A. Hematological Profile of HIV-Infected Patients on First-Line Highly Active Antiretroviral Therapy and Its Correlation With CD4 Count. Int J Recent Surg Med Sci. 2021; 7(02):54-63.

82. Tilahun M, Gedefie A, Ebrahim E, Seid A, Ali A, Shibabaw A*, et al.* Immuno-Haematological Abnormalities of HIV-Infected Patients Before and After Initiation of Highly Active Antiretroviral Therapy in the Antiretroviral Therapy Clinics of Six Health Facilities at Dessie Town, Northeast Ethiopia. J. Blood Med. 2022; 13:243-53.

83. Mozalyova OL, Samarina AV, Rassokhin VV. Anemia and thrombocytopenia in HIV-positive pregnant women. Journal of obstetrics and women's diseases. 2022; 71(2):69-78.

84. James AH. Iron Deficiency Anemia in Pregnancy. Obstet. Gynecol. 2021; 138(4):663-74.

85. Al-Khaffaf A, Frattini F, Gaiardoni R, Mimiola E, Sissa C, Franchini M. Diagnosis of anemia in pregnancy. J Lab Precis Med. 2020; 5(9):1-6.

86. Subtil SFC, Mendes JMB, Areia A, Moura J. Update on Thrombocytopenia in Pregnancy. Rev. Bras. Ginecol. Obstet. 2020; 42(12):834-40.

87. Reese JA, Peck JD, Deschamps DR, McIntosh JJ, Knudtson EJ, Terrell DR*, et al.* Platelet Counts during Pregnancy. N. Engl. J. Med. 2018; 379(1):32-43.

88. Park YH. Diagnosis and management of thrombocytopenia in pregnancy. Blood Res. 2022; 57(S1):S79-S85.

89. Habas E, Sr., Rayani A, Alfitori G, Eldin Ahmed G, Elzouki AY. Gestational Thrombocytopenia: A Review on Recent Updates. Cureus. 2022; 14(3):e23204.

90. Ciobanu AM, Colibaba S, Cimpoca B, Peltecu G, Panaitescu AM. Thrombocytopenia in Pregnancy. Maedica (Bucur). 2016; 11(1):55-60.

Chapter 6: General discussion and concluding remarks

6.1. Discussion

Haematopoietic stem and progenitor cells (HSPCs) reside in the bone marrow (BM) and are at the top of the haematopoietic system hierarchy[1]. Haematopoietic stem and progenitor cells are a vital cell population as they are responsible for generating and sustaining both blood and immune cells[2, 3]. Human immunodeficiency virus (HIV) infection generates a chronic pro-inflammatory environment that disrupts haematopoietic homeostasis[4]. Therefore, in alignment with objective one, the frequencies of circulating HSPCs present in the peripheral blood of treatment-naïve, HIV-positive and HIV-negative (control) individuals were determined and compared. Chapter three reported a significant reduction in both the frequency (p=0.0096) and absolute count (p=0.023) of the circulating HSPCs in the treatment-naïve, HIV-positive group. When the phenotype of the $Lin^{-}CD34^{++}$ HSPCs was further explored using a combination of key markers $CD38^{-/+}/CD133^{-/+}/CD117^{-/+}$ no significant differences were reported between the treatment-naïve, HIV-positive group and the HIV-negative (control) group. These findings suggest that HIV infection decreases the proportion of $Lin^{-}CD34^{++}$ HSPCs, exiting quiescence and entering the periphery. The virus interactions with HSPCs, however, did not affect the phenotypic characteristics of circulating $Lin^{-}CD34^{++}$ HSPCs.

Objectives two and three set out to assess the ability of the circulating HSPCs to form colony forming units (CFUs). That is, to determine if the circulating HSPCs are functional (objective two) and to phenotypically characterise the differentiated colonies (pooled) such as to identify if HIV exposure induced any differences in the phenotypic distributions of the differentiated cells (objective three). Chapter three demonstrated a non-significant decrease in the number of colonies produced by HSPCs from the treatment-naïve, HIV-positive group when compared to the HIV-negative (control) group. Furthermore, the circulating $Lin^{-}CD34^{++}$ HSPCs of the treatment-naïve, HIV-positive group maintained a differentiation pattern into the various blood cell types, similar to the HIV-negative (control) group. The resultant colonies did not present with any differences between the phenotypic distributions nor the viability assessment. These results suggest that during HIV infection, haematopoiesis at the HSPC level continues at a lower rate, or alternatively, that circulating HSPCs home back to the BM at a faster rate with very little to no impact on their resultant colony forming abilities.

During untreated HIV infection, there is an increased demand for immune cells as they are rapidly activated and depleted. It is also well known that HIV affects the immune system. As such, the basic immune profiles [T-cells, B-cells, natural killer (NK)-cells, monocytes, and neutrophils] of the treatment-naïve, HIV-positive and HIV-negative (control) patients were determined and compared as per objective number four. The data obtained in chapter four, reported that the frequency ($p<0.0001$) and absolute count ($p=0.0009$) of $CD4^+$ T cells were significantly lower when compared to the HIV-negative (control) group. The frequency of $CD8^+$ T cells was significantly higher ($p<0.0001$) in the treatment-naïve, HIV-positive individuals compared to the HIV-negative (control) individuals. The depletion of the $CD4^+$ T cells and the expansion of the $CD8^+$ T cells are established hallmarks of untreated HIV infection[5]. A significant decrease was also observed in the frequency ($p=0.032$) and absolute counts ($p=0.0066$) of $CD56^{++}$ cytokine producing NK cells, as well as the frequency ($p=0.010$) and absolute counts ($p=0.0027$) of the $CD3^+CD56^+CD4^+$ NKT cells.

All sub-populations noted above are classified as lymphocytes, with $CD4^+$ and $CD8^+$ T cells functioning as effector cells of the adaptive immune system[6]. Whilst the cytokine producing NK and $CD4^+$ NKT sub-populations are considered as innate lymphocytes, that bridge immune responses between the innate and adaptive immune systems[7-9]. The aforementioned NK sub-populations maintain a wide variety of functions, but primarily function in an immunoregulatory capacity, secreting a wide variety of chemokines and cytokines[10, 11].

As such, the HIV-mediated depletion and dysregulation of these innate and adaptive lymphocyte sub-populations promotes an immunodeficient environment, allowing for viral replication to continue unchecked at an accelerated rate, potentially resulting in a decrease in the effectiveness of the adaptive immune response. This may lead to increased susceptibility to opportunistic infections in the treatment-naïve HIV-positive cohort.

It must be noted that the above-mentioned cells all derive from common lymphoid progenitors (CLPs). However, cells derived from common myeloid progenitors (CMPs) are also negatively impacted by HIV infection. During the differentiation cascade, HIV interactions with CMPs results in impaired myelopoiesis[12-14].

As the virus continues to evade the immune system, pro-inflammatory cytokines are continuously secreted, resulting in chronic inflammation that supports myeloid derived suppressor cells (MDSC) expansion[15, 16]. Myeloid derived suppressor cells exert their potent immunosuppressive abilities on immune cells, but primarily on T cells, inhibiting T cell proliferation and activity[17]. As such, in accordance with objective five, MDSC frequencies were determined and compared between the treatment-naïve, HIV-positive group and the HIV-negative (control) group. It is important to note that the majority of studies on MDSCs are cancer studies with the number of studies on HIV and MDSCs steadily increasing. However, there is a limited number of studies pertaining to MDSCs and a treatment-naïve, HIV-positive cohort.

Chapter four demonstrated non-significantly lower median values in the treatment-naïve, HIV-positive group regarding the PMN-MDSC sub-populations when compared to the HIV-negative (control) group. However, there were significantly lower median values within both the M-MDSCs ($p=0.030$) and i-MDSCs ($p=0.047$) in the treatment-naïve, HIV-positive group when compared to the HIV-negative (control) group. Our observation was contradictory to what was expected (hypothesised). This may be due to the small study population or alternatively that the MDSCs in circulation are very slowly accumulating. Under steady state conditions, MDSCs are rare in blood, but expand when inflammatory and danger signals that arise due to HIV infection stimulate emergency haematopoiesis[18, 19]. Emergency haematopoiesis is triggered to ensure that both myeloid and lymphoid cells are replenished according to the increased demand[19]. During HIV-mediated immunosuppression MDSCs increase their expression of programmed death ligand (PD-L1), which binds to HSPCs and triggers haematopoietic cell death resulting in ineffective haematopoiesis in the BM[20].

The relationship between the CD4 count and MDSC sub-populations of the treatment-naïve, HIV-positive group, was investigated. Myeloid derived suppressor cells augment T cell dysfunction through inhibiting T cell activation, proliferation, homing capabilities and/or effector functions of $CD4^+$ T cells[13, 21]. No correlation between MDSCs sub-populations and $CD4^+$ T cells could be concluded in this study. This was likely due to the small sample numbers for the MDSC analysis, a limitation of this study. Taken together the basic immune profile data demonstrates that untreated HIV infection results in immune dysregulation. According to literature, immune dysregulation is associated with MDSC suppressive activity and

haematological abnormalities[22, 23]. Although larger study participant numbers, especially in the treatment-naïve , HIV-positive group, are required to make definitive conclusions, it is possible that the presence of MDSCs intensifies the HIV-mediated depletion of T lymphocytes. It does so by inhibiting T cell activity but also extends its suppressive functions to CMPs leading to the onset of haematological abnormalities.

Human immunodeficiency virus infection also results in ineffective haematopoiesis[24]. This therefore supports the importance of assessing the haematological profile in the treatment-naïve, HIV-positive group, as HIV infection often results in cytopenias[25]. The haematological profile of the treatment-naïve, HIV-positive cohort was reported on and compared with an HIV-negative (control) group in alignment with objective five. Chapter five highlights the HIV-mediated reduction of mature blood cells that results in haematological abnormalities (cytopenias). It is important to note that, for this part of the analysis, HIV-negative (control) individuals with cytopenias were included as a part of the HIV-negative (control) group, during the analyses. In addition to this, the individuals enrolled into this study were all pregnant. Given that pregnancy itself is associated with the manifestation of cytopenias, it was a confounding factor in the study.

There are four types of major peripheral blood cytopenias that are associated with HIV infection. These include leukopenia, anaemia, thrombocytopenia, and neutropenia which are classified as abnormally low white cell count (WCC), red blood cell count (RBC)/haemoglobin (HGB), platelets (PLTs) and absolute neutrophil counts respectively[26]. Therefore, to determine the presence of any of the aforementioned cytopenias a complete blood count was performed. The haematological indices of the treatment-naïve, HIV-positive group were compared to the HIV-negative (control) group. The WCC ($p=0.045$) RBC ($p=0.045$ and HGB ($p=0.0031$) mean values of the HIV-positive group were significantly lower than the HIV-negative (control) group. The PLT count was non-significantly higher in the treatment-naïve, HIV-positive group when compared to the HIV-negative (control) group whilst the absolute neutrophil count was significantly lower ($p=0.015$) in the treatment-naïve HIV-positive group compared to the HIV-negative (control) group.

Among the treatment-naïve HIV-positive group, anaemia was the most prevalent (69.23%), followed by thrombocytopenia (7.69%), with the remaining (23.08%) having no cytopenia.

Among the HIV-negative (control) group, the majority presented with no cytopenias (47.17%), followed by anaemia (30.19%), thrombocytopenia (18.87%) and bicytopenia (anaemia and thrombocytopenia; 3.77%) Notably, there was a higher prevalence of cytopenia, particularly anaemia observed in the treatment-naïve , HIV-positive group in comparison to the HIV-negative (control) group. Cytopenias in the HIV-negative (control) group can be attributed to physiological changes and/or nutrient deficiencies that occur during pregnancy. Peripheral blood cytopenias during HIV infection are more commonly associated with low CD4 counts[27]. The findings of chapter five demonstrated this as among the treatment-naïve, HIV-positive group, individuals with CD4 counts <200 cells/µL all presented with a cytopenia [the majority being anaemia (80%) and a single case of thrombocytopenia (20%)].

6.2. Concluding remarks

The overall aim of the project was to determine the relationship of immature circulating haematopoietic cells, namely HSPCs and MDSCs, with the haematological profile of treatment-naïve, HIV-positive participants.

This study depicts the relationship of the immature haematopoietic cells with haematopoiesis during untreated HIV infection by demonstrating the abnormal haematopoiesis characterised by the reduced circulating $Lin^{-}CD34^{++}$ HSPCs and immune dysfunction triggered by HIV. The latter was in addition to the haematological abnormalities (anaemia and thrombocytopenia) observed in the study, likely due to impaired myelopoiesis. The study population is too small to draw concise conclusions regarding the relationship with MDSCs. However, it is probable that if HIV infection continues untreated, MDSC frequencies will progressively accumulate due to HIV-mediated chronic inflammation.

In conclusion, the study depicts the HIV-mediated imbalance of steady state haematopoiesis. Altogether the data suggests that HIV disrupts haematopoietic homeostasis in the BM that resulted in lower WCC, RBC, HGB and neutrophil mean values as well as changes (decrease) in the circulating HSPC frequency. Although not monitored in this study, haematopoiesis is likely to return to steady state conditions when treatment is initiated.

6.3. Limitations of this study

1. A small treatment-naïve HIV-positive study population due to a single recruitment site and the COVID-19 pandemic restrictions. This resulted in a disproportionate number of treatment-naïve HIV positive individuals recruited in comparison with the number of HIV negative (control) individuals recruited.

2. The individuals enrolled into this study were pregnant, therefore, pregnancy was a confounding factor in the analysis of haematological abnormalities among the treatment-naïve, HIV-positive group.

3. The absence of viral load data. This information, together with the CD4 count would allow for a deeper assessment into disease status.

6.4. Future work

1. To investigate the expansion of MDSC activity, in a larger treatment-naïve, HIV-positive study population to observe the extent of its immunosuppressive activity on haematopoiesis.

2. To determine the cytokine levels present in plasma during acute untreated HIV infection. HIV generates a pro-inflammatory environment that is sustained throughout infection. It would therefore be important to investigate the levels of the various cytokines associated with haematopoiesis regulation.

3. To further explore the mechanisms involved in HIV-mediated impairment of haematopoiesis. This would allow for the generation of better, more specific treatment regimens and further provide significant insight into the pathogenicity of HIV-1C which is most prevalent in Sub-Saharan Africa.

4. To investigate the C-reactive protein levels present in plasma during untreated HIV infection. This will determine the associations between C-reactive protein levels, pro-inflammatory cytokine levels and presence of cytopenias. Furthermore, it will highlight the presence of any underlying inflammatory/infectious conditions present in the HIV-

negative (control) group that are contributing to occurrence the peripheral blood cytopenias observed.

6.5. References

1. Gao X, Xu C, Asada N, Frenette PS. The hematopoietic stem cell niche: from embryo to adult. Development. 2018; 145(2):dev139691.

2. Morales-Mantilla DE, Kain B, Le D, Flores AR, Paust S, King KY. Hematopoietic stem and progenitor cells improve survival from sepsis by boosting immunomodulatory cells. Elife. 2022; 11(e74561):1-20.

3. Wildes TJ, Flores CT, Mitchell DA. Concise Review: Modulating Cancer Immunity with Hematopoietic Stem and Progenitor Cells. Stem Cells. 2018; 37(2):166-75.

4. Hormaechea-Agulla D, Le DT, King KY. Common Sources of Inflammation and Their Impact on Hematopoietic Stem Cell Biology. Curr. Stem Cell Rep. 2020; 6(3):96-107.

5. Pastor L, Urrea V, Carrillo J, Parker E, Fuente-Soro L, Jairoce C, et al. Dynamics of CD4 and CD8 T-Cell Subsets and Inflammatory Biomarkers during Early and Chronic HIV Infection in Mozambican Adults. Front. Immunol. 2018; 8(1925):1-13.

6. Charles A, Janeway J, Travers P, Walport M, Shlomchik M. Immunobiology: the immune system in health and disease [Internet]. New York:Garland Publishing Inc; 2001 [cited: 2023/03/10]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK10757/

7. Heller NM, Berga-Bolanos R, Naler L, Sen JM. Natural killer T (NKT) cells in mice and men [Internet]. In: Soboloff J, Kappes DJ, editors. Signaling Mechanisms Regulating T Cell Diversity and Function. Boca Raton, Florida: CRC Press/Taylor & Francis; 2017. p. 119-46. [cited: 2024/02/12]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK532332/

8. Mujal AM, Delconte RB, Sun JC. Natural Killer Cells: From Innate to Adaptive Features. Annu. Rev. Immunol. 2021; 39:417-47.

9. Courtney AN, Tian G, Metelitsa LS. Natural killer T cells and other innate-like T lymphocytes as emerging platforms for allogeneic cancer cell therapy. Blood. 2023; 141(8):869-76.

10. Li D, Xu X-N. NKT cells in HIV-1 infection. Cell Res. 2008; 18(8):817-22.

11. Perera Molligoda Arachchige AS. Human NK cells: From development to effector functions. Innate Immun. 2021; 27(3):212-29.

12. Nixon CC, Vatakis DN, Reichelderfer SN, Dixit D, Kim SG, Uittenbogaart CH, et al. HIV-1 infection of hematopoietic progenitor cells in vivo in humanized mice. Blood. 2013; 122(13):2195-204.

13. Singh B, Singh DK, Ganatra SR, Escobedo RA, Khader S, Schlesinger LS, et al. Myeloid-Derived Suppressor Cells Mediate T Cell Dysfunction in Nonhuman Primate TB Granulomas. mBio. 2021; 12(6):e03189-21.

14. Bhardwaj V, Ansell SM. Modulation of T-cell function by myeloid-derived suppressor cells in hematological malignancies. Front Cell Dev Biol. 2023; 11(1129343):1-19.

15. Green WR, O'Connor MA. HIV vaccines: Unmasking myeloid derived suppressor cells. eBioMedicine. 2020; 61(103063):1-2.

16. Yaseen MM, Abuharfeil NM, Darmani H. Myeloid-derived suppressor cells and the pathogenesis of human immunodeficiency virus infection. Open Biol. 2021; 11(11):210216.

17. Ren R, Xiong C, Ma R, Wang Y, Yue T, Yu J, et al. The recent progress of myeloid-derived suppressor cell and its targeted therapies in cancers. MedComm (2020). 2023; 4(4):e323.

18. Schrijver IT, Karakike E, Théroude C, Baumgartner P, Harari A, Giamarellos-Bourboulis EJ, et al. High levels of monocytic myeloid-derived suppressor cells are associated with favorable outcome in patients with pneumonia and sepsis with multi-organ failure. Intensive Care Med. Exp. 2022; 10(5):1-14.

19. Ademe M. Paradoxes in the phenotype, frequency and roles of myeloid-derived suppressor cells during HIV infection. HIV AIDS (Auckl.). 2020; 12:151-6.

20. Yu S, Ren X, Li L. Myeloid-derived suppressor cells in hematologic malignancies: two sides of the same coin. Exp Hematol Oncol. 2022; 11(43):1-15.

21. Peñaloza HF, Alvarez D, Muñoz-Durango N, Schultz BM, González PA, Kalergis AM, et al. The role of myeloid-derived suppressor cells in chronic infectious diseases and the current methodology available for their study. J. Leukoc. Biol. 2019; 105(5):857-72.

22. Xu D, Li C, Xu Y, Huang M, Cui D, Xie J. Myeloid-derived suppressor cell: A crucial player in autoimmune diseases. Front. Immunol. 2022; 13(1021612):1-17.

23. Bizymi N, Bjelica S, Kittang AO, Mojsilovic S, Velegraki M, Pontikoglou C, et al. Myeloid-Derived Suppressor Cells in Hematologic Diseases: Promising Biomarkers and Treatment Targets. Hemasphere. 2019; 3(1):e168.

24. Balogun TM, Alao AO, Olaosebikan OF, Aremu AJ, Adegbite OO. The Pattern of Haematological Changes in the Baseline Blood Cell Counts and the $CD4^+$ T Lymphocyte Levels among Antiretroviral Therapy Naïve Adult HIV Positive Patients in a Nigerian Hospital. Open J. Blood Dis. 2020; 10(03):77-88.

25. Gebreweld A, Fiseha T, Girma N, Haileslasie H, Gebretsadik D. Prevalence of cytopenia and its associated factors among HIV infected adults on highly active antiretroviral therapy at Mehal Meda Hospital, North Shewa Zone, Ethiopia. PLoS One. 2020; 15(9):e0239215.

26. Durandt C, Potgieter JC, Mellet J, Herd C, Khoosal R, Nel JG, et al. HIV and haematopoiesis. S. Afr. Med. J. 2019; 109(8b):40-5.

27. Adesina OA, Fasola F, Adekanbi O, Ogunbosi B, Akinyemi J, Kuti MA, et al. Burden of Cytopaenias among Hiv Positive Pregnant Women at the University College Hospital, Ibadan. Ann Ib Postgrad Med. 2018; 16(2):99-108.

Appendix I: Normal cell surface expression of CD markers used in this study

CD Markers	Cells expressing
CD3	- Thymocytes - Mature peripheral T cells (cytoplasmic expression at early T cell differentiation) - NK cells (cytoplasmic in 56%)
CD4	- T helper cells - Thymocytes (80 - 90%) - Granulocytes - Monocytes/Macrophages - Langerhans cells - Dendritic cells
CD8	- Thymocytes (70 – 80%) - Mature peripheral T cells (25 – 35%, mostly cytotoxic T cells) - NK cells (30%) - Dendritic cells
CD10	- Pre-B cells - Pre-T cells - Some T cells - Follicular centre (germinal centre) cells - Granulocytes
CD11b	- Granulocytes - Macrophages - Follicular dendritic cells - Promyelocytes - NK cells - Some B/T cells
CD14	- Monocytes/Macrophages (90%) - Langerhans cells - Dendritic cells - B cells

	- Granulocytes-weak (30%)
CD15	- Myeloid cells - Eosinophils - Activated B and T cells - Variable monocytes and basophils
CD16	- NK cells - Granulocytes - Monocytes/Macrophages - T cells (reactive) - Immature thymocytes - Placental trophoblast
CD19	- Pre-B cells - B cells - Follicular dendritic cells - Haematogones in bone marrow by flow cytometry
CD20	- Most B cells (considered a pan B cell antigen) - Follicular dendritic cells - Haematogones
CD33	- Progenitor and other myeloid cells (decreasing expression with maturation) - Basophils - Granulocytes (low level expression) - Mast cells - Monocytes (majority) - NK cells - Plasmacytoid dendritic cells - T cells (some) - Epidermal Langerhans cells (variable)
CD34	- Hematopoietic progenitor cells/ hematogones (less mature) - Dendritic interstitial cells - Mast cells

	- Megakaryocytes - Osteoblasts - Umbilical cord blood
CD38	- Plasma cells - Hematopoietic progenitor cells - NK cells - B and T cells - Monocytes - Basophils - Neurons - Double positive thymocytes - Erythrocytes
CD41a	- Platelets - Megakaryocytes - Haematopoietic progenitor cells (erythrocytes, myeloid, megakaryocytes, mast cells)
CD45	- Haematopoietic cells (including basophils, granulocytes, lymphocytes, macrophages/histiocytes, mast cells, monocytes and plasma cells; **NOT** mature red blood cells and their immediate progenitors platelets or megakaryocytes) - Dendritic cells - Fibrocytes - Medullary thymocyte
CD45RA	- B cells - NK cells - Naïve/resting T cells (including those not exposed to antigens) - Medullary thymocytes - Plasmacytoid dendritic cells
CD49f	- Epithelial cells - Memory T cells

	- Monocytes - Platelets - Megakaryocytes - Thymocytes
CD56	- NK cells (80-90%) - Large granular lymphocytes - Activated T cells - Osteoblasts - Skeletal muscle
CD61	- Platelets - Megakaryocytes - Myeloid progenitor cells - Endothelial cells
CD64	- Antigen presenting cells inclusive of macrophages/monocytes - Activated granulocytes - Dendritic cells - Early myeloid cells
CD71	- Erythroid precursors
CD90	- Immature haematopoietic stem cells
CD117	- Haematopoietic progenitor cells - Mast cells
CD133	- CD34 bright hematopoietic stem and progenitor cells (umbilical cord blood-derived $CD133^+$ cells can differentiate into endothelial cells and induce new blood vessel growth)
CXCR4 (CD184)	- All mature blood cells - Blood progenitor cells
CCR5 (CD195)	- T cells - Macrophages
CD235a	- Erythroid cells
Lin	- T cells

(composition of CD3, CD14, CD16, CD19, CD20, CD56)	- B cells - NK cells - Monocytes - Neutrophils - Dendritic cells - Basophils
HLA-DR	- Basophils (immature) - Osteoblasts - Antigen resenting cells (B cells, dendritic cells, macrophages, and monocytes) - Precursor T cells and $CD4^+$ T cells

NK cells: Natural Killer cells.

Appendix II: Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance:

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027
- IORG #: IORG0001762 OMB No. 0990 0279 Approved for use through June 30, 2025 and Expires 07/23/2020.

Faculty of Health Sciences **Research Ethics Committee**

10 October 2023

Approval Certificate
Annual Renewal

Dear Ms TV Mashingaidze,

Ethics Reference No.: 738/2020 – Line 4
Title: The haematological profile of a treatment naïve HIV positive cohort: A pilot study

The **Annual Renewal** as supported by documents received between 2023-09-19 and 2023-10-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-10-09 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-10-10.
- Please remember to use your protocol number (738/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 1 69, Level 1, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel: (27) (0)12 356 3084
Email: deap-ekb.behalf@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Litsebenzi tša Maphelo

Appendix III: Informed consent documents

PATIENT INFORMATION LEAFLET (HIV-POSITIVE STUDY PARTICIPANTS)

STUDY TITLE

IMMATURE CIRCULATING HAEMATOPOIETIC CELLS, HAEMATOPOIESIS-ASSOCIATED CYTOKINE PLASMA LEVELS, AND SEVERITY OF HAEMATOLOGICAL ABNORMALITIES IN A TREATMENT-NAÏVE , HIV-POSITIVE COHORT

Dear Patient/Participant: _____ Date:_____

INTRODUCTION

You are invited to be part of a research study managed by clinicians and researchers from Sefako Makgatho Health Sciences University and University of Pretoria. This information leaflet is to help you decide if you would like to participate in the study. It is important that you fully understand what is involved before you agree to donate 3 tubes (equivalent to 3 teaspoons of blood) of your blood for the study. Do not hesitate to ask the researcher, the nurse, or your doctor if something is not clear to you or you have any questions. You should not agree to take part unless you are completely happy about all the information provided.

THE PURPOSE OF THE RESEARCH

Blood consists out of many different cell types that are suspended in a liquid known as plasma. Blood cells can be grouped into three broad categories, namely immune cells (also known as white blood cells), red blood cells and platelets. Each group of cells have an important function in the body. Immune cells help the body to fight infections (viruses, bacteria, etc), red blood cells supply body tissues with oxygen, and platelets play an important role during blood clotting. The body produces (makes) all of these cells (white blood cells, red blood cells and platelets) in the bone marrow from immature cells, known as haematopoietic stem cells (HSCs). HSCs have the ability to become any blood cell type the body needs.

HIV is a huge problem in South Africa and many people (approximately 7.5 million South Africans) are living with HIV. HIV mainly enters and kills immune cells, known as CD4-positive T cells, causing persons living with HIV to get sick easily if not treated. Current treatments are effective but are not able to kill all the HIV in the body. The reason for this is that HIV is able to “hide” in certain cells in the body without killing these cells.

Researchers identified some, but not all of the cells, which can “hide” HIV. When HIV “hides” in cells, the virus causes the cells not to function (work) properly. One of the functions that HIV affects is the way in which the cells that host (“house”) HIV communicate (talk) with other cells in the body. Cells in the body communicate (talks) with each other by releasing (bio)chemicals, known as cytokines, in the blood stream. These biochemical molecules serve as signals to inform other cells in the body what are expected from them.

Some researchers believe that HIV can possibly “hide” in bone marrow cells. There are many cell types, including HSCs, present in the bone marrow. Many of the cells in the bone marrow send (bio)chemical messages to HSCs when they need to produce new blood cells. It is

possible that when HIV “hides” in these cells, the virus changes the signals (messages) that these cells send to the HSCs. If HSCs do not to receive the correct messages, they are not able to form the blood cells the body need. In this research study, we want to investigate (research) how HIV interferes with the (bio)chemical messages that the body send to the HSCs and how this disruption affects blood cell formation.

Many of the experiments that researchers perform require the isolation of genetic material, also known as DNA and RNA, from cells. Genetic material contains information about the virus and/or cell that only can be revealed if researchers perform specialized tests on the RNA or DNA. These tests are often necessary in order to completely understand the characteristics of the virus and cells. The information the RNA and DNA provides allows researchers to better understand how HIV affects the cells in the body. Tests using RNA and DNA are also the most sensitive tests available to detect if cells are infected with HIV.

WHAT IS EXPECTED FROM YOU

We need 3 tubes of blood for our research. The volume (amount) of blood we would like to collect is equivalent (similar) to 3 teaspoons of blood. We also need some basic information from your file, such as your age, gender, ethnicity, medicine you are currently taking and if you have any other diseases and/or infections. If you agree to be part of the study, you will give us permission to draw 3 tubes of blood and also collect the information we need from your file. The blood will be drawn by a qualified nurse or doctor at the clinic.

As soon as the blood is collected, the blood tubes will be labelled with a unique participant code to protect your identity. The researchers may also need to test your blood for Hepatitis B and C. There may be a delay of approximately 1 month before the Hepatitis B and C results will be known. Dr Chrisna Durandt will forward the unique participant codes of all Hepatitis B and/or C results that came back as positive to Prof Moodley. Dr Durandt or Prof Moodley will then liaise with either Dr D Rankoe or Dr Mashamba (if you are pregnant) which will then contact you and discuss the results with you. Please be assured that your identity will be protected at all time.

RIGHTS OF THE PARTICIPANT

Your participation in this study is entirely voluntary and you may withdraw your participation at any time. Access to medical care and the quality of the medical care you will receive, won’t be affected if you decide not to participate. You will not receive any money if you participate. It will also not cost you anything to participate in the study.

CONFIDENTIALITY

The blood tubes will be labelled with a unique code. The information from your file and the results of the blood tests will be linked this unique sample code to protect your identity. Research reports and articles in scientific journals will not include any information that may identify you.

In some isolated cases it might however be important for the doctors or researchers involved in the study to share medical information to medical personnel or the appropriate Research Ethics committees. In such a case, you by signing this document, give permission to the researcher to release the necessary information to regulatory health authorities or the

Research Ethics committee. If necessary, these medical professionals will discuss the results with your doctor and everyone will act in your best interest.

ETHICAL APPROVAL

Researchers often do not use all the plasma, cells or genetic material (RNA and DNA) isolated from the blood you donated for the research study. When researchers and post-graduate students decide to use some of these stored (frozen) genetic material (RNA and DNA) or cells, they first need to get permission from the Ethics Committee at the Faculty of Health Sciences, University of Pretoria before they can start their studies.

The research done at the University of Pretoria is structured according to international guidelines (Declaration of Helsinki),

You are also welcome to contact the Faculty of Health Sciences Ethics Committee at the University of Pretoria if you have any concerns or questions. Their contact details are:

The Research Ethics Office:
Tel: 012 - 354 1330 or 012 - 354 1677
Fax: 012 - 354 1367

INFORMATION AND CONTACT PERSON

If at any time you would like to find out more information or have any questions regarding the study, please do not hesitate to contact the researchers.

Dr. C. Durandt:	012 -319 2101 / 084 484 5561
Dr. D Rankoe	082 924 4602

If you are pregnant and have questions please contact:

Dr. T Mashamba:	012-521 4461 / 082 825 0215

SEFAKO MAKGATHO HEALTH SCIENCES UNIVERSITY ENGLISH CONSENT FORM

Statement concerning participation in a Research Project.

Name of Study

IMMATURE CIRCULATING HAEMATOPOIETIC CELLS, HAEMATOPOIESIS-ASSOCIATED CYTOKINE PLASMA LEVELS, AND SEVERITY OF HAEMATOLOGICAL ABNORMALITIES IN A TREATMENT-NAÏVE , HIV-POSITIVE COHORT

I have read the information on the aims and objectives of the proposed study and was provided the opportunity to ask questions and given adequate time to rethink the issue. The aim and objectives of the study are sufficiently clear to me. I have not been pressurized to participate in any way.

I understand that participation in this study is completely voluntary and that I may withdraw from it at any time and without supplying reasons. This will have no influence on the regular treatment that holds for my condition neither will it influence the care that I receive from my regular doctor.

I know that this study has been approved by both the Sefako Makgatho Health Sciences University / Dr George Mukhari Hospital and Faculty of Health Sciences, University of Pretoria Research Ethics Committees. I am fully aware that the results of this study will be used for scientific purposes and may be published. I agree to this, provided my privacy is guaranteed.

I also understand that certain laboratory tests may require the isolation of genetic material, also known as DNA and RNA, and give herewith permission that the researchers may extract RNA/DNA from cells isolated from the blood I have donated.

YES	NO

I hereby give the researchers permission to perform routine HIV, hepatitis B and hepatitis C tests on the blood. It is important that we confirm your HIV status before we use your blood in the research study.

YES	NO

Study Participant Consent

I hereby give consent to participate in this study.

....

Name of patient/volunteer Signature of patient.

....

Place Date Witness

Statement by the Researcher

I provided written information regarding this study.

I agree to answer any future questions concerning the study as best as I am able.

I will adhere to the approved protocol.

.....	.....	.....	.....
Name of Researcher	Signature	Date	Place

INFORMATION LEAFLET – HIV-NEGATIVE CONTROLS

STUDY TITLE

IMMATURE CIRCULATING HAEMATOPOIETIC CELLS, HAEMATOPOIESIS-ASSOCIATED CYTOKINE PLASMA LEVELS, AND SEVERITY OF HAEMATOLOGICAL ABNORMALITIES IN A TREATMENT-NAÏVE , HIV-POSITIVE COHORT

Dear Patient/Participant: _____ Date:_____

INTRODUCTION

You are invited to be part of a research study managed by clinicians and researchers from Sefako Makgatho Health Sciences University (SMU) and University of Pretoria (UP). This information leaflet is to help you decide if you would like to participate in the study. It is important that you fully understand what is involved before you agree to donate three tubes (equivalent to 3 teaspoons of blood) of your blood for the study. Do not hesitate to ask the researcher, the nurse, or your doctor if something is not clear to you or you have any questions. You should not agree to take part unless you are completely happy about all the information provided.

THE PURPOSE OF THE RESEARCH

Blood consists out of many different cell types that are suspended in a liquid known as plasma. Blood cells can be grouped into three broad categories, namely immune cells (also known as white blood cells), red blood cells and platelets. Each group of cells have an important function in the body. Immune cells help the body to fight infections (viruses, bacteria, etc), red blood cells supply body tissues with oxygen, and platelets play an important role during blood clotting. The body produces (makes) all of these cells (white blood cells, red blood cells and platelets) in the bone marrow from immature cells, known as haematopoietic stem cells (HSCs). HSCs have the ability to become any blood cell type the body needs.

Human immunodeficiency virus (HIV) infection is a huge problem in South Africa and many people (approximately 7.5 million South Africans) are living with HIV. HIV mainly enters and kills immune cells, known as CD4-positive T cells, causing persons living with HIV to get sick easily if not treated. Current treatments are effective, but are not able to kill all the HIV in the body. The reason for this is that HIV is able to “hide” in certain cells in the body without killing these cells.

Researchers identified some, but not all of the cells, which can “hide” HIV. When HIV “hides” in cells, the virus causes the cells not to function (work) properly. One of the functions that HIV affects is the way in which the cells that host (“house”) HIV communicate (talk) with other cells in the body. Cells in the body communicate (talks) with each other by releasing (bio)chemicals, known as cytokines, in the blood stream. These biochemical molecules serve as signals to inform other cells in the body what are expected from them.

Some researchers believe that HIV can possibly “hide” in bone marrow cells. There are many cell types, including HSCs, present in the bone marrow. Many of the cells in the bone marrow send (bio)chemical messages to HSCs when they need to produce new blood cells. It is

possible that when HIV “hides” in these cells, the virus changes the signals (messages) that these cells send to the HSCs. If HSCs do not to receive the correct messages, they are not able to form the blood cells the body need. In this research study, we want to investigate (research) how HIV interferes with the (bio)chemical messages that the body send to the HSCs and how this disruption affects blood cell formation.

Many of the experiments that researchers perform require the isolation of genetic material, also known as DNA and RNA, from cells. Genetic material contains information about the virus and/or cell that only can be revealed if researchers perform specialized tests on the RNA or DNA. These tests are often necessary in order to completely understand the characteristics of the virus and cells. The information the RNA and DNA provides allows researchers to better understand how HIV affects the cells in the body. Tests using RNA and DNA are also the most sensitive tests available to detect if cells are infected with HIV.

To measure the effect of HIV, we need to compare the results we got using blood from individuals living with HIV with results we got from blood donated by healthy (HIV-negative) persons. You indicated that you are HIV-negative and therefore, your participation will help us to know what the normal levels of the biochemicals (cytokines) and the different cell types are in the blood of HIV-negative individuals. Knowing this will help us to understand how HIV affects the different kinds of cytokines (biochemical molecules) and cells in the blood.

WHAT IS EXPECTED FROM YOU

We need three tubes of blood for our research. The volume (amount) of blood we would like to collect is equivalent (similar) to 3 teaspoons of blood. The blood will be drawn by a qualified nurse or doctor. We also need some necessary information from you, such as your age, gender, ethnicity, and medicine you are currently using.

It is essential that the researchers only use HIV-negative blood to measure the normal levels of the biochemicals (cytokines) and cell types. The researchers will, therefore, need to confirm that your blood is HIV-negative. The researchers will also test your blood for Hepatitis B and C. You will receive pre-test HIV counselling from an HIV counsellor, before the blood is collected. As soon as the blood is collected, the blood tubes will be labelled with a unique participant code to protect your identity. Only Prof Vanessa Moodley (Haematologist, co-investigator, Sefako Makgatho Health Sciences University) and Dr Chrisna Durandt will have access to the electronic document that links the unique participant code with your identity. The document will be password protected and the information will be treated with the utmost confidentiality. If your HIV test results came back as positive, the HIV counsellor will further counsel you and guide you on how to proceed. The results of the HIV test should be available almost immediately, but there will be a delay of approximately 1 month before the Hepatitis B and C results will be known. Dr Chrisna Durandt will forward the unique participant codes of all Hepatitis B and/or C results that came back as positive to Prof Moodley. Dr Durandt or Prof Moodley will then liaise with either Dr K Kangawaza or Dr Mashamba (if you are pregnant) which will then contact you and discuss the results with you. Hepatitis B and C infections can be treated successfully, and either Dr Kangawaza or Dr Mashamba will refer you to a clinic or specialist that will assist you in receiving the correct treatment. Please be assured that your identity will be protected at all times.

You may withdraw your participation in the study at <u>any stage</u>. It is important to understand that your decision to withdraw from the study will not be used against you in any way or at any stage. <u>You are fully entitled to withdraw your participation any stage, even directly after pre-test counselling, without disclosing any reason for your decision.</u>

RIGHTS OF THE PARTICIPANT

Your participation in this study is entirely voluntary. You will not receive any money if you participate. It will also not cost you anything to participate in the study.

CONFIDENTIALITY

The blood tubes will be labelled with a unique code. The information you provided, and the results of the blood tests will be linked to this unique sample code to protect your identity. Research reports and articles in scientific journals will not include any information that may identify you.

ETHICAL APPROVAL

Researchers often do not use all the plasma, cells, or genetic material (RNA and DNA) isolated from the blood you donated for the research study. When researchers and post-graduate students decide to use some of these stored (frozen) genetic material (RNA and DNA) or cells, they first need to get permission from the Ethics Committee at the Faculty of Health Sciences, University of Pretoria before they can start their studies.
The research done at the University of Pretoria is structured according to international guidelines (Declaration of Helsinki),
You are also welcome to contact the Faculty of Health Sciences Ethics Committee at the University of Pretoria if you have any concerns or questions. Their contact details are:

The Research Ethics Office:
Tel: 012 - 354 1330 or 012 - 354 1677
Fax: 012 - 354 1367

INFORMATION AND CONTACT PERSON

If at any time you would like to find out more information or have any questions regarding the study, please do not hesitate to contact:

Dr. C. Durandt:	012 -319 2101 / 084 4845561
Dr. E Kangawaza`	012-521 4584 / 082 863 0259

If you are pregnant and have questions please contact:

Dr. T Mashamba:	012-521 4661 / 082 825 0215

SEFAKO MAKGATHO HEALTH SCIENCES UNIVERSITY ENGLISH CONSENT FORM

Statement concerning participation in a Research Project.

Name of Study

IMMATURE CIRCULATING HAEMATOPOIETIC CELLS, HAEMATOPOIESIS-ASSOCIATED CYTOKINE PLASMA LEVELS, AND SEVERITY OF HAEMATOLOGICAL ABNORMALITIES IN A TREATMENT-NAÏVE , HIV-POSITIVE COHORT

I have read the information on the aims and objectives of the proposed study and was provided the opportunity to ask questions and given adequate time to rethink the issue. The aim and objectives of the study are sufficiently clear to me. I have not been pressurized to participate in any way.

I understand that participation in this study is completely voluntary and that I may withdraw from it at any time and without supplying reasons. This will have no influence on the regular treatment that holds for my condition neither will it influence the care that I receive from my regular doctor.

I know that this study has been approved by both the Sefako Makgatho Health Sciences University / Dr George Mukhari Hospital and Faculty of Health Sciences, University of Pretoria Research Ethics Committees. I am fully aware that the results of this study will be used for scientific purposes and may be published. I agree to this, provided my privacy is guaranteed.

I also understand that certain laboratory tests may require the isolation of genetic material, also known as DNA and RNA, and give herewith permission that the researchers may extract RNA/DNA from cells isolated from the blood I have donated.

YES	NO

I hereby give the researchers permission to perform routine HIV, hepatitis B and hepatitis C tests on study. the blood. It is important that we confirm your HIV status before we use your blood in the research

YES	NO

Study Participant Consent

I hereby give consent to participate in this study.

.....	.....
Name of patient/volunteer	Signature of patient/volunteer

.....	.....	.....
Place	Date	Witness

HIV counsellor statement

The study participant received pre-test HIV counselling

YES	NO

Please provide a reason if NO was indicated above.

_____
_____
_____
_____

.....	.....	.....	.....
Name of HIV counsellor	Signature	Date	Place

_____

Researcher statement

I provided written information regarding this study.

I agree to answer any future questions concerning the study as best as I am able.

I will adhere to the approved protocol.

.....	.....	.....	.....
Name of Researcher	Signature	Date	Place

Appendix IV: Standard Operating Procedure (SOP)

Standard Operating Procedure (SOP)

Study Sample: _____ Performed by: _____ Date: _____

	A: While waiting for the sample to arrive 1. Remove the following from the fridges/freezers to reach room temperature. Do this as soon as you were notified that there is a sample on the way.	
	a. Sterile PBS	
	b. Methocult medium	
	c. Histopaque	
	d. RPMI media	
	e. Foetal Bovine Serum (FBS)	
Already @ RT	f. VersaLyse™	
	g. Basic Immune Phenotype Duraclone tube	
	h. Dimethyl sulfoxide (DMSO)	

	2. Prepare the flow cabinet	
	a. Switch on cabinet. Mare sure the fan is switched on.	
	b. Spray surfaces with 70% ethanol and wipe dry lightly.	
	c. Spray tissue paper well with 70% ethanol and wipe the pipette shafts (end of pipettes) with the paper towel.	
	d. Spray the following well with 70% ethanol before placing it in the flow cabinet.	
	i. Waste bucket lined with plastic bag. Add bleach to bucket. **DO NOT** overfill bucket. Leave enough space to add scrunched up paper towel to absorb liquid when you discard.	

	ii. Plastic container to discard empty tubes, used plastic Pasteur pipettes, used serological pipettes, etc.	
	iii. Two tube racks for smaller and larger tubes. **DO NOT** spray racks containing tubes. Don't spray tubes. Wipe tubes with paper towel sprayed with 70% ethanol.	
	iv. Serological pipettes (5 mL and 10 mL).	
	v. Sterile plastic Pasteur pipettes.	
	3. Add monoclonal antibodies to the following tubes. • Please make sure that monoclonal antibodies are added to the bottom of the tubes. • Work in a flow cabinet. • Use a clean pipette for each new antibody and when adding antibodies to tubes already containing antibodies. • Cap the tubes after adding the antibodies and place the tubes in the dark, room temperature. • Work systematically moving antibodies to the opposite side or a row up (if using a tube rack) to ensure that you do not get confused. • Make sure that monoclonal antibody vials are tightly closed after use. It is important to prevent evaporation. • **Do not** add viability dyes to the tubes at this stage.	
Used in B-5	a. **MNC Count tube** i. Add 10 µL CD45 FITC to the tube.	
Used in B-9	b. **MDSC tube** Please refer to the ''Addition of Monoclonal Antibodies" page for the list of monoclonal antibodies and the appropriate volume of each antibody that should be added to the tubes labelled "MDSC".	
Used in B-8	c. **HSPC Isotypic Control tube** Please refer to the ''Addition of Monoclonal Antibodies" page for the list of monoclonal antibodies and the appropriate volume of each antibody that should be added to the tube labelled "HSPC".	
Used in B-8	d. **HSPC tube**	

	Please refer to the ''Addition of Monoclonal Antibodies" page for the list of monoclonal antibodies and the appropriate volume of each antibody that should be added to the tubes labelled "HSPC".	
Used in B-8	e. **CFU tube** Please refer to the ''Addition of Monoclonal Antibodies" page for the list of monoclonal antibodies and the appropriate volume of each antibody that should be added to the tubes labelled "CFU"	
	B. On arrival of blood	
	1. Pool the blood in the two tubes into a 15 mL tube. Measure and record the volume of blood received. Volume of blood:_____	
Plasma Collection (B-6)	**2.** Add 1 mL of blood to four microcentrifuge tubes (labelled in 2a), i.e. 4 microcentrifuge tubes containing 1 mL blood each.	
Basic Immunophenotype (B-7)	**3.** Add 100 µL of blood to the bottom of the DuraClone Basic Immunophenotype tube. a. Cap the tube and vortex for 10 – 15 seconds. b. Start the timer (15 minutes).	
	4. Isolation of mononuclear cells	
	a. Volume of remaining blood:_____ (Volume recorded in B-1 minus 4 mL minus 100 µL)	
	b. Add an equal volume (B-4a) of Histopaque to the bottom of a 15 mL tube. Split the blood equally between two 15 mL tubes if too much for one 15 mL tube. Adjust the volume of Histopaque accordingly.	
	c. Layer the blood carefully on top of the Histopaque, using a sterile plastic Pasteur pipette to do this.	
Continue Plasma Collection (B-6) Continue with Basic Immunophenotype (B-7)	d. Centrifuge for 30 minutes at 300 g (Program 4). No break!	
	e. Carefully remove the plasma/histopaque top layer with a sterile plastic Pasteur pipette and discard. Do not disturb the mononuclear cell (MNC) layer.	

	f. Carefully aspirate the MNC layer, using a sterile plastic Pasteur pipette and transfer to a new (labelled) 15 mL tube. Make sure that all the MNCs are aspirated. Do not disturb the neutrophil layer on top of the red blood cells. Do not aspirate any of the neutrophils and red blood cells.	
	g. Fill the tube with sterile PBS– total volume in the tube 14 - 15 mL.	
	h. Centrifuge for 10 minutes at 1700 rpm (Program 2).	
	i. Carefully aspirate the supernatant and discard. Do not disturb the cell pellet.	
	j. Resuspend the MNCs in residual PBS. Residual PBS refers to the left over PBS in the tube that what was not aspirated.	
	k. Add 2 mL of VersaLyse™ to the MNC cell suspension and mix. Make sure the cell pellet is completely resuspended in the VersaLyse™.	
	l. Incubate for 15 minutes at room temperature.	
	m. Fill the tube with sterile PBS– total volume in the tube 14 - 15 mL.	
	n. Centrifuge for 10 minutes at 300 g (Program 2).	
	o. Carefully aspirate the supernatant. Do not disturb the cell pellet.	
	p. Resuspend the MNCs in the residual PBS. Residual PBS refers to left over PBS in the tube that was not aspirated.	
	q. Add 1mL sterile PBS to the MNC cell population and mix well. Make sure the cell pellet is complete resuspended.	
	5. Counting MNCs	
	a. Add 50 µL MNC cell suspension (B-4q) to a flow cytometry tube prepared in A-3a. Make sure the MNC cell population is well mixed before transferring the 50 µL to the flow cytometry tube.	
IMPORTANT NOTE	b. Add 10 µL 7AAD to the cell suspension in the flow cytometry tube. Only add 7AAD if the flow cytometer is available and the count will be performed immediately after staining.	

	DO NOT add 7AAD, if you need to wait for the flow cytometer to become available or are unable to do the count as soon as staining is complete. **REMEMBER** to add 7AAD before analysis on the flow cytometer. Remember to incubate cells with 7AAD for at least 10 minutes, but not longer than 15 minutes, before counting on the flow cytometer.	
	c. Tap the tubes a few times to mix the monoclonal antibody and 7AAD with the cell suspension. You may also vortex for 10 seconds.	
	d. Incubate for 15 minutes at room temperature, out of direct light.	
	e. Add 3 mL PBS to the tube.	
	f. Centrifuge for 10 minutes at 300 g (Program 2).	
	g. Carefully aspirate the supernatant. Do not disturb the cell pellet.	
	h. Resuspend the MNCs in the residual PBS. Residual PBS refers to left over PBS in the tube that was not aspirated.	
	i. Add 200 µL of PBS to the tube. Make sure that the cell pellet is completely suspended.	
	j. Add 50 µL Flow Count beads to the tube. Calibration factor: _____	
	k. Mix by tapping the flow tube hard without spilling the content	
	l. Analyse on the flow cytometer. CD45+ Cell Count: _____ cells/µL	
	m. Save the flow cytometry raw data file as a pdf file for future reference.	
	6. Plasma Collection	
	a. Make sure the microcentrifuge tubes containing 1 mL blood are tightly closed.	
	b. Place the 4 microcentrifuge tubes into the microcentrifuge.	
	c. Centrifuge at 12,000 g for 10 minutes.	
	d. Remove the tube carefully from the microcentrifuge. Do not disturb the plasma / cells interphase.	

	e. Carefully aspirate the plasma and transfer to a new 4 mL microcentrifuge tube. Do not insert your pipette to deep. Aspirate from the top.	
	f. Centrifuge microcentrifuge containing plasma at 12,000 g for 10 minutes to remove any residual cells.	
	g. Remove the tube carefully from the microcentrifuge.	
	h. Aliquot plasma into microcentrifuge tubes as indicated below: Do not insert your pipette to deep. Aspirate from the top. • 6 aliquots of 100 µL • Aliquot the rest of the plasma in volumes of 500 µL. Please indicate the volumes aliquoted on the microcentrifuge tubes.	
	i. Make sure the microcentrifuge tubes are tightly closed.	
	j. Immediately store plasma aliquots in -80 ^{0}C freezer. Stored in Box_____ Number of aliquots stored: 100 µL aliquots: _____ 500 µL aliquots: _____ Volume of aliquots_____	

	7. Basic Immune Profile (continue from B-3)	
	a. Add 2mL VersaLyse™ to the blood.	
	b. Vortex for 10 seconds.	
	c. Cap the tube and incubate for 15 minutes at room temperature (out of direct light).	
	d. Centrifuge at 300 g for 10 minutes (Program 2).	
	e. Carefully aspirate the supernatant. Do not disturb the cell pellet.	
	f. Resuspend the cell pellet in the remaining (residual) PBS/VersaLyse™. Make cell that the cell pellet is completed resuspended.	
	g. Add 3 mL PBS to the tube.	
	h. Centrifuge at 300 g for 10 minutes (Program 2).	
	i. Carefully aspirate the supernatant. Do not disturb the cell pellet.	
	j. Resuspend the cell pellet in the remaining, residual PBS.	
	k. Add 250 µL PBS to the tube.	
IMPORTANT NOTE	l. Add 10 µL 7-AAD to the cell suspension. Only add 7AAD if the flow cytometer is available and the count will be performed immediately after staining. **DO NOT** add 7AAD, if you need to wait for the flow cytometer to become available or are unable to do the count as soon as staining is complete. **REMEMBER** to add 7AAD before analysis on the flow cytometer. Remember to incubate cells with 7AAD for at least 10 minutes, but not longer than 15 minutes, before counting on the flow cytometer.	
	m. Incubate for 15 minutes at room temperature (out of direct light).	
	n. Add 100 µL Flow Count to the tube.	
	o. Acquire on the CytoFLEX flow cytometer.	
	8. HSPC phenotype staining	

	• **This will be acquired on the BD FACSAria™ Fusion cell sorter) (first choice).** • **Please acquire on the Beckman Coulter CytoFLEX if there is a problem with the BD FACSAria™ Fusion (second choice).** • **HSPCs will be sorted for CFU while phenotyping.**	
	a. If **HIV-POSITIVE,** then label 5 sterile tubes accordingly: • 1 X HSPC isotype • 1 X HSPC phenotype • 3 X CFU tubes b. If **HIV NEGATIVE,** then label 3 sterile tubes accordingly: • 1 X HSPC isotype • 1 X HSPC phenotype • 1 X CFU tubes	
NB: If there is not enough for ALL panels, then exclude the MDSC panel	c. Add 1 x 10^6 MNCs to the HSPC isotype, the HSPC phenotype tubes, and the CFU tubes respectively (A-3c – 3e). Use the following formula to calculate the volume that should be added of the MNC cell suspension prepared in B-4q $= 1000\ 000 \div MNC\ cell\ count$ = Volume that should be added to the tube (µL) MNC count documented in B-5l (cells/µL)	
	d. Cap the tubes and vortex for 15 seconds.	
	e. Incubate for 15 minutes at room temperature, out of direct light.	
	f. Add 3 mL PBS to the tubes.	
	g. Centrifuge at 300 g for 10 minutes (Program 2)	
	h. Carefully aspirate the supernatant. Do not disturb the cell pellet.	
	i. Resuspend the cell pellet in the remaining, residual PBS.	
	j. Add 250 µL PBS to the tubes.	
	k. Prepare Zombie violet viability dye.	
	i-a. Remove Zombie violet aliquot from the -20^{0}C freezer.	
	i-b. Thaw in your hand and pipette 1 µL in a PCR tube.	

	i-c. Add 1 µL DMSO to the Zombie violet in the PCR tube (1:1 dilution).	
	i-d. Adjust your pipette to 2 µL and pipette up and down a few times to mix DMSO with Zombie violet dye.	
	i.e. Centrifuge PCR tube (containing Zombie Violet) on a microcentrifuge).	
NB: If there is no Zombie Violet then use 1 µL DAPI. Do not add DMSO to the DAPI.	l. Add 1 µL Zombie violet viability dye to the cell suspension (B-8i) Only add Zombie violet viability due to cell suspension if the flow cytometer is available and the count will be performed immediately after staining. **DO NOT** add Zombie violet viability dye, if you need to wait for the flow cytometer to become available or you are unable to do the count as soon as staining is complete. **REMEMBER** to add Zombie violet viability dye before analysis on the flow cytometer. Remember to incubate cells with Zombie violet viability dye for at least 10 minutes, but not longer than 15 minutes, before counting on the flow cytometer.	
	m. Vortex tube for 10 seconds.	
NB: Only for HIV-positive samples.	n. Pool ALL 3 CFU tubes into a new sterile tube in preparation of CFU sorting.	
	o. Incubate for 15 minutes at room temperature, out of direct light.	
	p. In a 48 well plate, pipette 500 µL of Methocult in 4 wells. **Ensure no bubbles are present** and place into incubator.	
	q. Analyse on BD FACSAria™ Fusion cell sorter.	
Steps 8o -8r will not be performed on all samples but will only be performed on 15 randomly selected samples		
	r. Sort HSPCs into 48 well plate while acquiring the samples – 50 HSPCs per wells; 5 wells.	
	s. In surrounding wells, pipette 500 µL of PBS and place into incubator. Analyse for colony forming units after 14 days.	
	t. Spray outside of cello tape role with 70% ethanol and wipe dry.	
	u. Tape 4 sides of lid securely on plate (4 pieces of tape – from top of lid to bottom of plate).	

	9. MDSC phenotype staining • **This will be acquired on CytoFLEX flow cytometer.**	
	a. Add 1 x 10^6 MNCs to the HSPC phenotype tube (A-3b). Use the following formula to calculate the volume that should be added of the MNS cell suspension prepared in B-4q $= 1000\ 000 \div MNC\ cell\ count$ = Volume that should be added to the tube (µL) MNC count documented in B-5l (cells/µL)	
	b. Cap the tube and vortex for 15 seconds.	
	c. Incubate for 15 minutes at room temperature, out of direct light.	
	d. Add 3 mL PBS to the tube.	
	e. Centrifuge at 300 g for 10 minutes (Program 2)	
	f. Carefully aspirate the supernatant. Do not disturb the cell pellet.	
	g. Resuspend the cell pellet in the remaining, residual PBS.	
	h. Add 250 µL PBS to the tube.	
	i. Add 10 µL 7AAD and incubate for 15 minutes.	
	j. Analyse sample on CytoFLEX flow cytometer.	
	10. Cryopreservation of MNCs	
	a. Calculate remainder of cells which will be stored in Mr/Mrs Frosty = 950 µL - Remainder of volume of cells post-HSPC & MDSC panel= _____ µL = ____ µL x $CD45^+$ cell count obtained in 5l =	
	b. Add sterile PBS to the cells to a total volume of 2 mL.	
	c. Centrifuge at 300 g for 10 minutes (Program 2).	
	d. Discard the supernatant and resuspend pellet in 1 mL freezing medium. If necessary, adjust volume until the concentration is in the region of 5 x 10^6 MNCs/mL.	
	e. Label a 1.5 mL tube with i) Study sample number ii) Date dd/mm/yyyy	

<table>
<tr><td></td><td>iii) Total number of cells</td><td></td></tr>
<tr><td></td><td>f. Transfer resuspended cell pellet (v) to the labelled 1.5 mL tube – 1 mL per cryovial.</td><td></td></tr>
<tr><td></td><td>g. Freezing Medium:
<table>
<tr><td>Number of Cryovials:</td><td></td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="2">Add the following to a 15 mL tube:</td></tr>
<tr><td>RPMI (volume/µL)</td><td>= # of cryovials x 800 µL
= # of cryovials x 800 µL
=</td></tr>
<tr><td>Plasma from same sample</td><td>= # of cryovials x 100 µL
=</td></tr>
<tr><td>DMSO</td><td>= # of cryovials x 100 µL
=</td></tr>
<tr><td>Total Volume</td><td>1000 µL/vial</td></tr>
</table></td><td></td></tr>
<tr><td></td><td>h. Immediately transfer cryovials to Mr/Mrs frosty.</td><td></td></tr>
<tr><td></td><td>i. Take Mr/Mrs frosty to the -80 °C freezer immediately.</td><td></td></tr>
<tr><td></td><td>j. Indicate on board in cell culture which Mr/Mrs frosty was used and contained the MNCs.</td><td></td></tr>
<tr><td></td><td>k. After 24 hours, transfer cryovials to storage boxes in the -80^{0}C freezer.
Stored in Box_____</td><td></td></tr>
</table>

Appendix V: Supplementary figures

HIV-positive, test group

Spearman r values

	WBC	RBC	HGB	HCT	MCV	PLT	Neuts count	%Neuts	Lymph count	%Lymph	Lin$^-$CD34^{++}count	%Lin$^-$CD34^{++}
WBC	1	-0.39065	-0.39065	-0.46703	-0.08791	0.093535	0.825174825	0.398601	0.083916084	-0.4335664	0.247252747	0.093924085
RBC	-0.39065	1	0.549587	0.519945	-0.63274	-0.21763	-0.378284293	-0.00701	-0.10507897	0.08756581	0.176066191	0.569852762
HGB	-0.39065	0.549587	1	0.971115	0.140303	-0.33609	-0.535902748	-0.44483	0.126094764	0.42381851	0.239339979	0.561553935
HCT	-0.46703	0.519945	0.971115	1	0.203297	-0.36864	-0.517482517	-0.42657	0.146853147	0.3986014	0.241758242	0.546969674
MCV	-0.08791	-0.63274	0.140303	0.203297	1	-0.02751	-0.020979021	-0.38462	0.167832168	0.27272727	-0.175824176	-0.281772256
PLT	0.093535	-0.21763	-0.33609	-0.36864	-0.02751	1	0.287215852	0.241682	-0.353765867	-0.2241685	0.321871006	-0.348550718
Neuts count	0.825175	-0.37828	-0.5359	-0.51748	-0.02098	0.287216	1	0.776224	-0.363636364	-0.7972028	0.391608392	0.098593994
%Neuts	0.398601	-0.00701	-0.44483	-0.42657	-0.38462	0.241682	0.776223776	1	-0.741258741	-0.965035	0.412587413	0.253527413
Lymph count	0.083916	-0.10508	0.126095	0.146853	0.167832	-0.35377	-0.363636364	-0.74126	1	0.72727273	-0.27972028	-0.19014556
%Lymph	-0.43357	0.087566	0.423819	0.398601	0.272727	-0.22417	-0.797202797	-0.96503	0.727272727	1	-0.447552448	-0.246484985
Lin$^-$CD34^{++}count	0.247253	0.176066	0.23934	0.241758	-0.17582	0.321871	0.391608392	0.412587	-0.27972028	-0.4475524	1	0.635368813
%Lin$^-$CD34^{++}	0.093924	0.569853	0.561554	0.54697	-0.28177	-0.34855	0.098593994	0.253527	-0.19014556	-0.246485	0.635368813	1

p-value

	WBC	RBC	HGB	HCT	MCV	PLT	Neuts count	%Neuts	Lymph count	%Lymph	Lin$^-$CD34^{++}count	%Lin$^-$CD34^{++}
WBC		0.186425	0.186433	0.110329	0.778404	0.760528	0.001591991	0.200954	0.800385105	0.16160877	0.415071123	0.763239043
RBC	0.186425		0.054019	0.071127	0.022952	0.47116	0.224156696	0.986451	0.744492595	0.78755507	0.562208959	0.044899126
HGB	0.186433	0.054019		4.09E-07	0.645951	0.259183	0.075583973	0.148451	0.694690957	0.17032033	0.428131057	0.048655555
HCT	0.110329	0.071127	4.09E-07		0.505345	0.213998	0.088795194	0.168896	0.650893634	0.20095438	0.425726196	0.056493293
MCV	0.778404	0.022952	0.645951	0.505345		0.931062	0.955960076	0.218343	0.603894096	0.39116306	0.565947159	0.350603139
PLT	0.760528	0.47116	0.259183	0.213998	0.931062		0.362269955	0.446328	0.257843949	0.480303	0.281528342	0.241614341
Neuts count	0.001592	0.224157	0.075584	0.088795	0.95596	0.36227		0.004331	0.246411152	0.00291209	0.209711467	0.764451334
%Neuts	0.200954	0.986451	0.148451	0.168896	0.218343	0.446328	0.004331317		0.007797999	3.4739E-06	0.184465542	0.426631118
Lymph count	0.800385	0.744493	0.694691	0.650894	0.603894	0.257844	0.246411152	0.007798		0.00963085	0.378913695	0.55526197
%Lymph	0.161609	0.787555	0.17032	0.200954	0.391163	0.480303	0.002912095	3.47E-06	0.009630853		0.14735927	0.440530153
Lin$^-$CD34^{++}count	0.415071	0.562209	0.428131	0.425726	0.565947	0.281528	0.209711467	0.184466	0.378913695	0.14735927		0.022655468
%Lin$^-$CD34^{++}	0.763239	0.044899	0.048656	0.056493	0.350603	0.241614	0.764451334	0.426631	0.55526197	0.44053015	0.022655468	

Figure 5.S1 *Spearman r and p-values obtained from correlation analysis for the treatment-naïve, HIV-positive group.*

HIV-negative, control group

Spearman r values

	WBC	RBC	HGB	HCT	MCV	PLT	Neuts count	%Neuts	Lymph count	%Lymph	Lin$^-$CD34^{++}count	%Lin$^-$CD34^{++}
WBC	1	0.043676	0.034035	0.063842	-0.08555	0.204306	0.952099388	0.428919	0.459536241	-0.48067	0.300661687	0.255597427
RBC	0.043676	1	0.511479	0.638173	-0.37817	-0.0957	0.085747841	0.085703	-0.006535669	-0.08615	-0.113454892	-0.265599671
HGB	0.034035	0.511479	1	0.914729	0.468695	-0.16976	0.035691413	0.007566	0.0887654	-0.01748	0.03043844	-0.092573987
HCT	0.063842	0.638173	0.914729	1	0.383498	-0.19432	0.060383484	0.041059	0.090561524	-0.04907	-0.022973292	-0.184977058
MCV	-0.08555	-0.37817	0.468695	0.383498	1	-0.09334	-0.135311701	-0.12957	0.061035773	0.089752	0.071216619	-0.012521103
PLT	0.204306	-0.0957	-0.16976	-0.19432	-0.09334	1	0.121763262	-0.0961	0.188938723	0.094887	0.076844263	0.136926935
Neuts count	0.952099	0.085748	0.035691	0.060383	-0.13531	0.121763	1	0.655965	0.224274531	-0.68902	0.264904858	0.334345052
%Neuts	0.428919	0.085703	0.007566	0.041059	-0.12957	-0.0961	0.655965163	1	-0.429962444	-0.91575	0.032806914	0.295611137
Lymph count	0.459536	-0.00654	0.088765	0.090562	0.061036	0.188939	0.224274531	-0.42996	1	0.451197	0.252631121	-0.063795574
%Lymph	-0.48067	-0.08615	-0.01748	-0.04907	0.089752	0.094887	-0.689023609	-0.91575	0.45119688	1	0.005746996	-0.281395195
Lin$^-$CD34^{++}count	0.300662	-0.11345	0.030438	-0.02297	0.071217	0.076844	0.264904858	0.032807	0.252631121	0.005747	1	0.720145558
%Lin$^-$CD34^{++}	0.255597	-0.2656	-0.09257	-0.18498	-0.01252	0.136927	0.334345052	0.295611	-0.063795574	-0.2814	0.720145558	1

p-value

	WBC	RBC	HGB	HCT	MCV	PLT	Neuts count	%Neuts	Lymph count	%Lymph	Lin$^-$CD34^{++}count	%Lin$^-$CD34^{++}
WBC		0.756154	0.808825	0.649714	0.542465	0.142258	2.19694E-27	0.00151	0.00061014	0.00031	0.030327773	0.067423003
RBC	0.756154		9.07E-05	2.74E-07	0.005239	0.495432	0.545564301	0.545774	0.963322868	0.543658	0.423225745	0.057034791
HGB	0.808825	9.07E-05		1.03E-21	0.000401	0.224275	0.801661098	0.957548	0.531463541	0.902098	0.830384403	0.513926045
HCT	0.649714	2.74E-07	1.03E-21		0.004588	0.163242	0.670665381	0.772577	0.523156372	0.729768	0.871576028	0.189241765
MCV	0.542465	0.005239	0.000401	0.004588		0.506204	0.33885231	0.359946	0.667314135	0.526891	0.615875571	0.929797614
PLT	0.142258	0.495432	0.224275	0.163242	0.506204		0.389839249	0.497945	0.179772827	0.503418	0.588188379	0.333063971
Neuts count	2.2E-27	0.545564	0.801661	0.670665	0.338852	0.389839		1.3E-07	0.109956624	1.63E-08	0.060297163	0.016486413
%Neuts	0.00151	0.545774	0.957548	0.772577	0.359946	0.497945	1.30104E-07		0.001466455	1.93E-21	0.819225368	0.035198269
Lymph count	0.00061	0.963323	0.531464	0.523156	0.667314	0.179773	0.109956624	0.001466		0.000788	0.073686278	0.65650014
%Lymph	0.00031	0.543658	0.902098	0.729768	0.526891	0.503418	1.63049E-08	1.93E-21	0.000787586		0.96807352	0.045460692
Lin$^-$CD34^{++}count	0.030328	0.423226	0.830384	0.871576	0.615876	0.588188	0.060297163	0.819225	0.073686278	0.968074		1.76951E-09
%Lin$^-$CD34^{++}	0.067423	0.057035	0.513926	0.189242	0.929798	0.333064	0.016486413	0.035198	0.65650014	0.045461	1.76951E-09	

Figure 5.S2 *Spearman r and p-values obtained from correlation analysis for the HIV-negative (control) group.*