Immune Activation in HIV-Positive Patients on Combined Anti-Retroviral Treatment (cART) as a High Risk Group for the Development of Cardiovascular Diseases

by

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Declaration

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Abstract

Although roll-out of combined anti-retroviral treatment (cART) has blunted HIV-AIDS onset, studies show increased development of cardio-metabolic complications in HIV-infected individuals. For this study we hypothesized that HIV-induced low-grade inflammation perturbs immune cell function/activation, thereby contributing to an increased risk for cardiovascular diseases (CVD) onset. Here we aimed to identify changes in monocyte and T cell subsets and determine its relationship to: (a) classical markers of HIV progression (CD4 count, viral load); (b) immune activation status; (c) endothelial dysfunction; and (d) traditional lipid profile and subclasses.

Eighty participants were recruited from the Worcester Community Day Center (Worcester, Western Cape, South Africa): n=13 HIV-negative, n=67 HIV-positive. Recruits were divided as HIV-naïve and HIV-treated (on cART), and also groups based on CD4 count (control group, HIV-positive with CD4count > 500 cells/μL, CD4 count from 200–500 cells/μL and CD4 count < 200 cells/μL). Clinical histories and a validated lifestyle questionnaire were completed. Fasted blood was collected and used to assess monocyte subpopulation phenotype (non-classical, intermediate, classical) by flow cytometry together with tissue factor (a marker for thrombus formation) and CD38 (a marker for immune activation) expression on monocyte and T cell subsets (CD4, CD8). Classical regulatory T cells ($CD4^+CD25^{++}FOXP3^+$) with activation markers (glycoprotein A repetitions predominant [GARP] and special AT-rich sequence binding protein 1 [SATB-1]) were also evaluated together with an assessment of endothelial function (flow-mediated dilatation). C-reactive protein levels together with the traditional lipid profile were also evaluated. In addition, an assessment of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) subclasses was also completed.

Our data revealed a robust increase in inflammation/immune activation and coagulation markers on $CD8^+$ and $CD4^+$ T cell populations, respectively ($CD8^+CD142^+$ [P = 0.01] and $CD4^+CD142^+$ [P = 0.0003]). Increased co-expression of inflammation and coagulation markers were also observed on both $CD8^+$ and $CD4^+$ T cells ($CD8^+142^+CD38^+$ [P = 0.0001] and $CD4^+142^+38^+$ [< 0.0001]). In addition, we found an expansion of both non-classical (P = 0.0001) and intermediate monocytes (P = 0.05) that were highly correlated with immune activation, coagulation and HIV disease progression markers. There was also an expansion of $CD4^+FOXP3^+$ regulatory T cells (P = 0.0005), together with higher levels of GARP (P = 0.001) and SATB-1 (P = 0.04) (especially in patients with relatively low CD4 counts). The lipid profile data revealed interesting changes, i.e. a significant decrease during early HIV-infection but with substantial increase after cART initiation. In addition, we also found significant changes in HDL and LDL subclasses.

The most novel findings of this study are: a) the identification of a unique coagulation marker (CD142) expressed on CD8 and CD4 T cells and its relatively early expression in HIV-infected individuals (treatment naïve). CD142 is also co-expressed with immune activation and strongly correlates with disease progression markers; b) changes in lipid subclasses that significantly correlate to HIV immunological markers despite a decrease in terms of the traditional lipid profile expected. Such subclass changes may also be a driver for

CVD onset, although further research is needed to pursue this question; and c) upregulation of both anti-inflammatory GARP and pro-inflammatory SATB-1 in regulatory T cells in HIV-treated individuals (with immune dysregulation) altering regulatory T cell function and also potentially contributing to CVD onset. Thus we propose that clinicians could be aware of immune activation and coagulation with HIV infection (even at relatively early stages of disease progression) as monitoring and control of these factors could result in improved healthcare and the long-term well-being for such patients.

Opsomming

Hoewel die uitrol van gekombineerde anti-retrovirale behandeling (kARK) die aanvang van MIV-VIGS laat daal het, toon studies die verhoogde ontwikkeling van kardio-metaboliese komplikasies in MIV-geïnfekteerde individue. In hierdie projek word daar vermoed dat MIV-geïnduseerde laegraadse inflammasie immuunsel funksie / aktivering ontwrig en sodoende bydra tot 'n verhoogde risiko vir kardiovaskulêre siektes (KVS).

Die studie het die volgende doelwitte: identifiseer veranderinge in monosiet en regulerende T-sel subtipes en hul verhouding tot: (a) klassieke merkers van MIV vordering (CD4-telling, virale lading); (b) immuun aktivering status en; (c) endoteel wanfunksie.

Tagtig deelnemers is gewerf (Worcester CDC [Wes-Kaap]): n=13 MIV-negatief, n=67 MIV-positief (gegroepeer as MIV-naïewe, MIV-behandeling op kARK), asook groepe op grond van CD4-telling (kontrole groep, MIV-positief met CD4-telling > 500 selle/µL, CD4-telling 200-500 selle/µL en CD4-telling < 200 selle/µL) Kliniese geskiedenis is aangeteken en 'n gevestigde leefstyl vraelys is voltooi.

Bloed monsters, geneem tydens ‘n tydeperk van vas, is ingesamel en gebruik om monosiet subpopulasie (nie-klassieke, intermediêre, klassieke) fenotipe deur vloeisitometrie te evalueer tesame met weefsel faktor (merker: trombus ontwikkeling) en CD38 (merker: immuun aktivering) uitdrukking op monosiet subtipes en CD8 T-selle.

Klassieke regulerende T-selle (CD4+CD25++Foxp3+) met aktivering merkers (GARP en SATB-1) is ook beoordeel tesame met endoteel funksie toets (vloei-bemiddelde ontsluiting). CRP met tradisionele lipiedprofiel is ook geëvalueer en verdere evaluering van HDL met LDL subklasse is uitgevoer.

Die data het 'n beduidende toename in inflammasie / immuun aktivering (P <0.0001) en stolling merkers CD8+CD142+ (P = 0,01) getoon. 'n Hoë mede-uitdrukking van inflammasie en stolling merkers CD8+42+CD38+ is ook waargeneem (P = 0,0001).

Die resultate het opregulering van beide nie-klassieke monosiete (P = 0,0001) en intermediêre monosiete (P = 0,05) getoon, en hierdie subtipes het hoogs gekorreleer met immuun aktivering en stolling en MIV siekte progressie merker. Daar was ook 'n uitbreiding van CD4+Foxp3+ T-selle (P = 0,0005), tesame met hoër vlakke van bykomende T-reg merkers GARP (P = 0,001) en SATB-1 (P = 0,04) (veral in pasiënte met relatiewe lae CD4 tellings). 'n Beduidende afname in lipiedprofiel vroeg na MIV-infeksie, met ‘n aansienlike toename na kARK inisiasie, saam met 'n beduidende verandering in HDL en LDL subklasse, is ook waargeneem.

Die mees beduidende nuwe bevindinge van hierdie studie is: a) die identifisering van 'n unieke koagulasie merker (CD142) wat op CD8 T-selle uitgedruk word en sy relatiewe vroeë uitdrukking in MIV-geïnfekteerde individue (naïewe). Verder het ons gevind dat dit ‘n mede-uitdrukking met immuun aktivering deel en sterk ooreenstem met die siekte merkers; b) veranderinge in lipied subklasse wat beduidend korreleer met MIV immunologiese merkers ten spyte van 'n afname in terme van die verwagte tradisionele lipiedprofiel. Sulke

subklas veranderinge kan dalk ook die aanvang van KVS bevorder, alhoewel verdere navorsing benodig word om hierdie interessante idee aan te spreek; en c) opregulering van beide anti-inflammatoriese GARP en pro-inflammatoriese SATB-1 in Treg selle in MIV-behandelde individue (met immuun wanregulering) kan die balaans van Treg sel funksie ontwrig en ook potensiëel bydra tot die aanvang van KVS.

Ons stel voor dat dokters moet bewus wees van immuun aktivering en stolling met MIV-infeksie (selfs by relatiewe vroeë stadiums van die siekte) aangesien dit sal lei tot verbeterde gesondheidsorg en welsyn vir sulke pasiënte.

Acknowledgements

My first word of thanks is to my supervisor, Prof Faadiel Essop, whose unwavering encouragement and support enabled me to successfully complete this study. His academic experience, patience and vision indeed helped me to continue, even in difficult times and under trying circumstances. I am also very grateful for his outstanding editing and linguistic assistance.

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Studying in a foreign country is not always easy and it has been especially challenging for myself (as a Libyan citizen). In addition to the language barrier, I found it difficult to be so far away my extended family and friends during these tumultuous times in my home country. However, the enduring prayers, love and support of my parents (even though far away) provided me with the energy and focus to complete this study. I am forever grateful to them for their constant encouragement. As a mother and wife, the time taken up by this study has resulted in matters at home not always being easy to manage! My heartfelt thanks go to my husband (Emad) and my three daughters, Sarah, Tasneem and Ream for their unwavering love, support and patience throughout this study. Thank you for all the sacrifices that you made to support me - I am proud and grateful to call you my family!

Last but not least, thanks and praise to Allah. This study would not have been possible without His presence and guidance to keep me focused and motivated. To Him be all honour and glory; *Alhamdulillah*.

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List of Abbreviations

Abbreviation	Description
AB	Antibodies
AGM	African Green Monkey
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
APC	Antigen Presenting Cell
APC-H7	Allophycoyanin-Hilite 7
ART	Antiretroviral Therapy
BD	Becton Dickson
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CD	Cluster Differential
CMI	Cell-mediated Immunity
CTL	Cytotoxic T Lymphocyte
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FDP	Fibrin Degradation Product
FITC	Fluorescein Isothiocyanate
FIV	Feline Immunodeficiency Virus
FMO	Fluorescence Minus One
FOXP3	Forkhead Box Protein P3
FSC	Forward Scatter
GARP	Glycoprotein A Repetitions Predominant
GIT	Gastrointestinal Tract
Gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HDL	High-density Lipoprotein
HIV	Human Immunodeficiency Virus
ICAM-1	Intracellular Adhesion Molecule-1
IDL	Intermediate-density Lipoprotein
IFN- γ	Interferon-gamma
IL	Interleukin
LDL	Low-density Lipoprotein

LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
MHC	Major Histocompatibility Complex
NHLS	National Health Laboratory Service
OX-LDL	Oxidized Low-density Lipoprotein
PAMP	Pathogen-associated Molecular Pattern
PBS	Phosphate-buffered Saline
PE	Phycoerythrin
PI	Protease Inhibitor
PRR	Pattern Recognition Receptor
RNA	Ribonucleic Acid
SATB1	Special AT-rich Sequence Binding Protein 1
SBP	Systolic Blood Pressure
SCD	Sudden Cardiac Death
SD	Standard Deviation
SEM	Standard Error of the Mean
SIV	Simian Immunodeficiency Virus
SM	Sootey Mangabey
SSC	Side Scatter
Tat	Trans-activator of Transcription
TC	Total Cholesterol
TCR	T-cell Receptor
TF	Tissue Factor
TG	Triglycerides
TGF-β	Transforming Growth Factor Beta
TH1 Cells	Type 1 T Helper Cells
TH2 Cells	Type 2 T Helper Cells
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
Treg Cells	Regulatory T Cells
UNAIDS	Joint United Nations Programme on HIV/AIDS
VCAM	Vascular Cell Adhesion Molecule
VL	Viral Load
VLDL	Very Low-density Lipoprotein

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

Previous studies demonstrated a robust association between human immunodeficiency virus (HIV) infection and the onset of cardiovascular diseases (CVD), especially coronary heart disease (Triant et al., 2012; Hansson et al., 2001). Despite this concern, the underlying mechanisms driving HIV-mediated CVD onset require additional investigations. However, immune activation and inflammation (the focus of this study) emerge as potential contributors to this process and require further evaluation (Baker et al., 2011; Hsue et al., 2012; Beltran et al., 2015). How could this be a contributing factor in the era of combination antiretroviral therapy (cART)? Although most HIV-infected individuals on cART achieve viral suppression, this may not necessarily result in complete immunological recovery in such individuals (Prabhakar et al., 2011; Pinzone et al., 2012). For example, significant CD4 T cell depletion from gut mucosa (primarily chemokine receptor 5 [CCR5+] memory T cells with activated phenotype) during the early stages of infection results in damage to the gut mucosal epithelium and subsequent microbial translocation. Moreover, ongoing microbial translocation is linked to systemic immune activation and permanent changes to host immune cells (Brenchley et al., 2006; Cassol et al., 2010). Microbial translocation can in turn induce persistent macrophage activation that is unrelated to the HIV viral load (VL) (Wallet et al., 2010). In addition, viral co-infections (e.g. cytomegalovirus [CMV]) can contribute to the phenomenon of persistent immune activation. Moreover, increased T cell death caused by activation-induced cell death (AICD) increases turnover (thymic output) to replace dying cells as an attempt to maintain T cell homeostasis (Février et al., 2011). Another aspect of ongoing immune activation is the development of T cells with an “exhausted” phenotype.

Such perturbations elicit effects on both adaptive and innate immune cells that subsequently lead to profound changes in immune signals, and production of cytokines and chemokines. Together such processes and related immune dysfunction can contribute to the onset of CVD, e.g. atherosclerosis. In light of this, the current study hypothesised that the onset of atherosclerosis in HIV-infected individuals occurs due to a) increased pro-inflammatory stimuli impacting on immune cell function, and b) failure of regulatory systems to control immune activation, or a combination of both. Here the aim was to describe changes in monocyte and T cell subsets in a local HIV-positive population and then ascertain how this relate to a) classical markers of HIV disease progression (CD4 count and VL), b) immune activation status and c) risk markers and functional assessments for CVD (Figure 1.1).

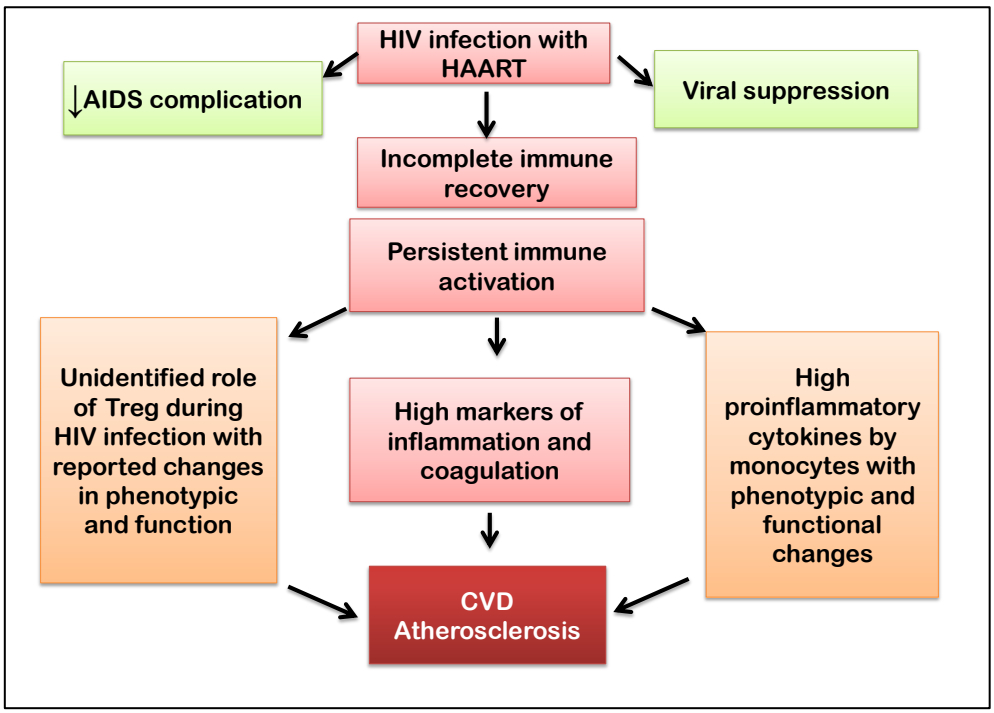

Figure 1-1: The role of immune activation in triggering atherosclerosis. HAART: Highly Active Antiretroviral Therapy; AIDS: Acquired Immune Deficiency Syndrome; Treg: Regulatory T Cells

The remainder of this thesis will cover the following: **Chapter 2** consists of a review of current literature that focuses on the link between HIV and CVD onset – with emphasis on the role of immune activation. This chapter will culminate in the motivation, hypothesis, aims and objectives of the current study. **Chapter 3** covers a description of the materials and methods used to perform the research work, while data generated are covered in **Chapter 4.** This is followed by a discussion and conclusion – covered in **Chapter 5 (**Figure 1.2).

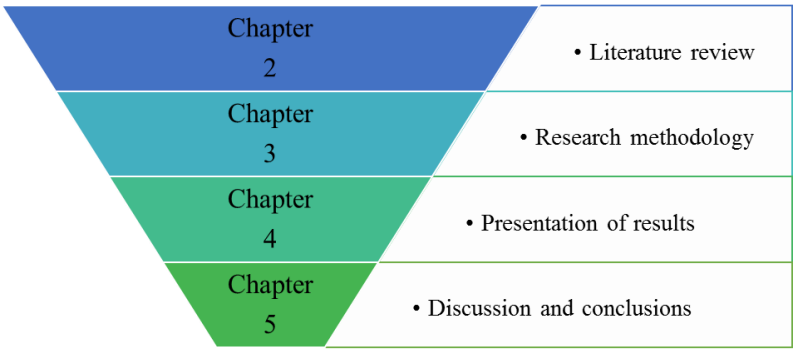

Figure 1-2 : Research layout and thesis roadmap.

CHAPTER 2 LITERATURE REVIEW

2.1 HIV-POSITIVE PATIENTS AND THE LINK TO CVD

The advent of HIV-mediated CVD has been shown in many studies, particularly an increased prevalence of coronary artery disease (Triant et al., 2012; Hansson et al., 2001). In support, the rate of myocardial infarction is twice as high for treated HIV-infected individuals versus non-infected counterparts (Triant et al., 2012). Moreover, with successful combination antiretroviral treatment (cART) roll-out, HIV is increasingly managed as a chronic disease, meaning that treated individuals display increasing lifespans leading to an aging population. As a result complications such as pulmonary hypertension and sudden cardiac death (SCD) are becoming increasingly prevalent in HIV-positive individuals. For example, Tseng et al. (2012) demonstrated that SCD accounted for 86% of all cardiac-related deaths in this group of patients.

Thus although cART prolongs the lives of HIV-infected individuals, the risk of developing non-acquired immune deficiency syndrome (AIDS) co-morbidities such as CVD remains high (Serrano-Villar et al., 2016). CVD have therefore surfaced as a major cause of morbidity and mortality in HIV-infected individuals (Tseng et al., 2012) and contribute to ~20% of deaths in this cohort (Zaaqoq et al., 2014). Increasing efforts are therefore being made to understand the underlying mechanisms driving this process. Studies completed thus far indicate that the onset of CVD in HIV-positive individuals is a complex process that involves multiple parameters that may include traditional risk factors, drug toxicity and persistent immune activation (Baker et al., 2011). Thus while the traditional risk factors and drug toxicity play a role in this process, immune activation emerges as a significant role player (Dixon et al., 2011; Kaplan et al., 2011) (refer Figure 2.1). As the focus of this thesis is on the relatively understudied phenomenon of immune activation as a driver of CVD onset in HIV-positive patients, the nature of this link will be the major focus of this literature review.

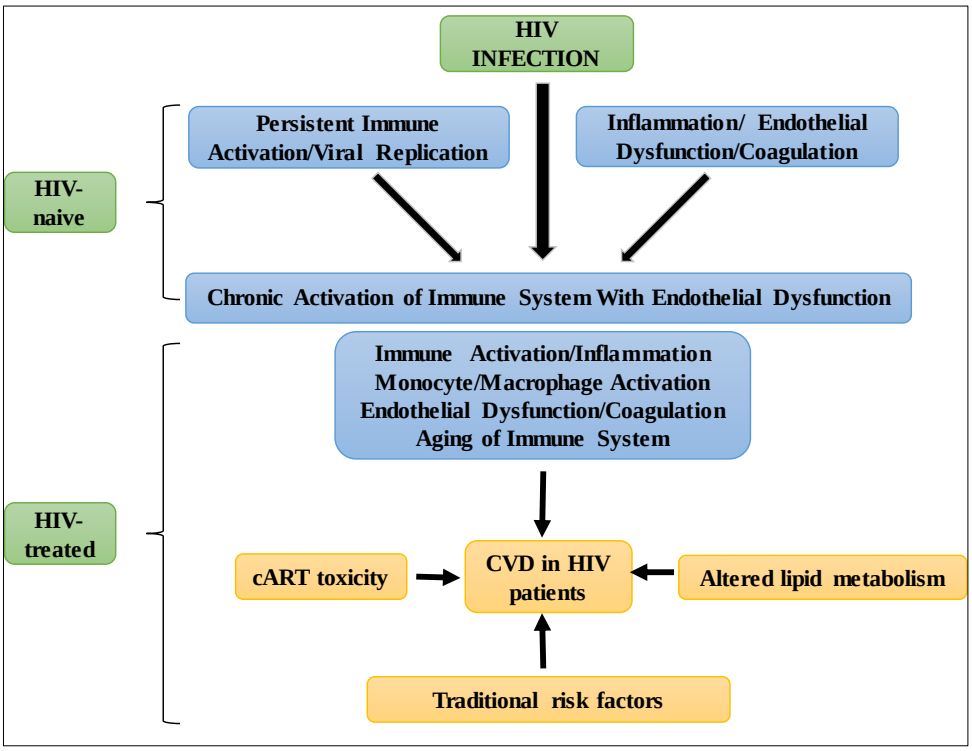

Figure 2-1: CVD in HIV-infected individuals. (a) For HIV-naïve patients (not on treatment) the role of HIV infection itself together with immune activation and coagulation are linked to increased CVD onset; (b) For HIV-treated individuals (long-term cART) the interaction between cART toxicity, traditional risk factors, altered lipid metabolism as well as immune activation and coagulation is shown. Together these factors all contribute to CVD onset in HIV-treated individuals.

2.2 THE HUMAN IMMUNODEFICIENCY VIRUS

2.2.1 The Global HIV/Aids Pandemic

Since the inception of the HIV/AIDS pandemic ~30 years ago, it has become one of the biggest global health problems. For example, since 1981 a total of ~78 million persons were infected with HIV, while ~35 million died from HIV/AIDS-related causes (World Health Organization, 2015) (Figure 2.2).

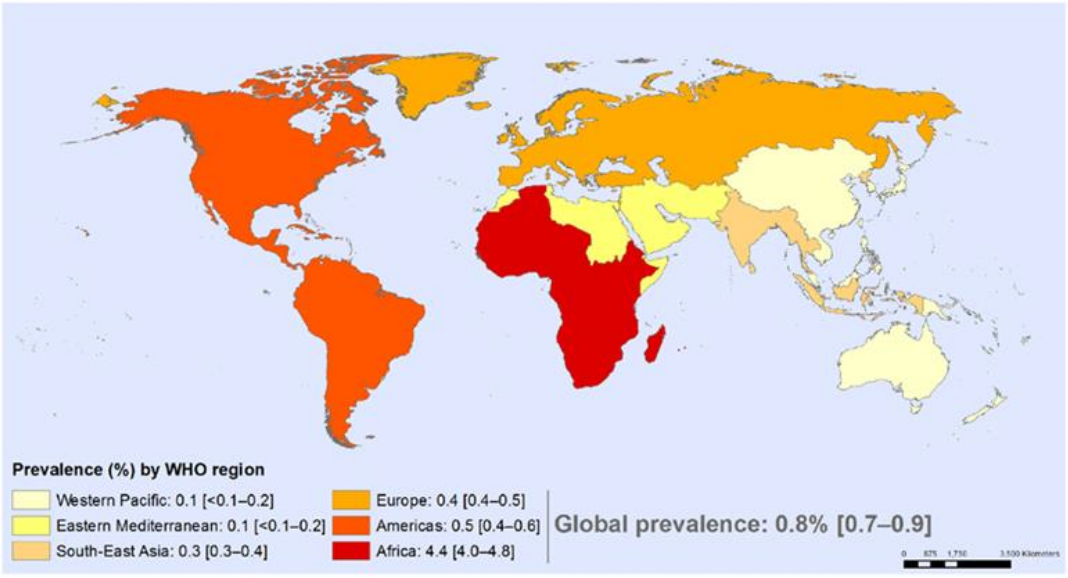

Figure 2-2: Global HIV prevalence in adults (15 – 49 years) 2015 by WHO region (World Health Organization, 2015)

Current statistics reveal that there are ~37 million individuals globally living with HIV/AIDS (World Health Organization, 2015), with ~3 million under the age of 15 years. More than two thirds of all persons living with HIV are located in sub-Saharan Africa, equating to ~26 million people. Here South Africa is saddled with the highest numbers with ~7 million individuals identified as HIV-positive (Statistic SA/UNAIDS, 2015) and ~180, 000 South Africans dying of AIDS-related causes during 2015 (UNAIDS, Report on the Global AIDS Epidemic, Geneva: UNAIDS, 2015). Although cART roll-out is relatively successful (reaching ~17 million individuals) new infections continue to fuel this burden of disease. For example, during 2015 there were ~2 million newly-infected individuals, with ~240, 000 under the age of 15 years (UNAIDS, Report on the Global AIDS Epidemic, Geneva: UNAIDS, 2015). Since 2000 there has been significant progress in terms of the universal goal to lower and ultimately controlling HIV infection, e.g. a 35% decrease in new infections since this time together with a 24% decline in AIDS-related diseases for individuals receiving cART (UNAIDS, 2015). However, as HIV-AIDS has evolved into a more chronically managed clinical condition there has been a parallel rise in non-AIDS related complications such CVD (Triant et al., 2012; Hansson et al., 2001).

2.2.2 HIV Structure

The HIV belongs to genus lentivirus within the family Retroviridae. Retroviruses have a single-stranded ribonucleic acid (RNA) genome, with HIV possessing two copies. The life-cycle involves the process of reverse transcription, whereby RNA is converted to double-stranded copy DNA (cDNA) before integration into the host genome (Figure 2.3). Before replication can take place, viruses need to enter host cells and here HIV virions target $CD4^+$ T cells (i.e. $CD4^+$ T cell tropic) as this cell surface marker represents the viral receptor (Hu and Hughes. 2012).

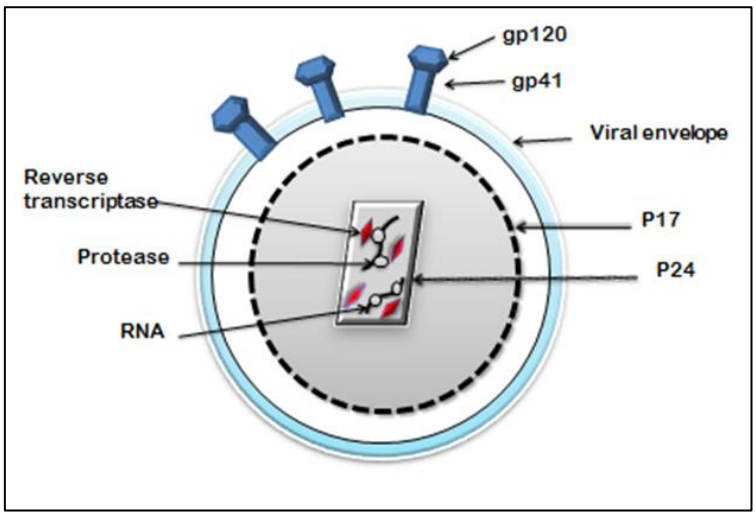

Figure 2-3: The structure of an HIV-1 virion. The diagram illustrates the lipid membrane, including gp120 and gp41, as well as the p17 matrix protein and the p24 core antigen.

The HIV particle is spherical with a diameter measuring ~120 nm and the intact virus structure (virion) consists of:

- Viral envelope: the outer membrane coat of the virus that is composed of two phospholipid layers obtained from the host cell membrane when newly-formed virions bud from the cell. The envelope contains a virally-encoded glycoprotein (gp120) that is needed to attach to the host cell. In addition, gp41 is crucial for the fusion process between the virion and host cell.
- Viral matrix: here the viral protein p17 surrounds the capsid and maintains the integrity of the virion particle; it is situated between the envelope and the core.
- Viral core: it contains a viral capsid protein p24 that surrounds two single strands of HIV RNA and the enzymes needed for HIV replication (reverse transcriptase, protease and integrase).

A The Genomic Structure of HIV

The HIV genome contains nine genes that are located between two long terminal repeat regions (LTR); of these there are three (group antigen [*gag*], polymerase [*pol*] and envelope [*env*]) that encode structural proteins for new viral particles (Figure 2.4). The classical structural scheme of a retroviral genome usually is 5' LTR-*gag*-*pol*-*env*-3' LTR. The nine genes comprise *gag* that in turn includes *p17*, *p24* and *p72*; *pol* that includes reverse transcriptase *p66*, integrase *p32* and protease *p11*, and *env* that includes *gp120* and *gp41*. Additionally, *tat* and *rev* genes are regulatory proteins for gene regulation while *vif*, *vpu*, *vpr* and *nef* code for accessory proteins (Frankel and Young et al., 1998).

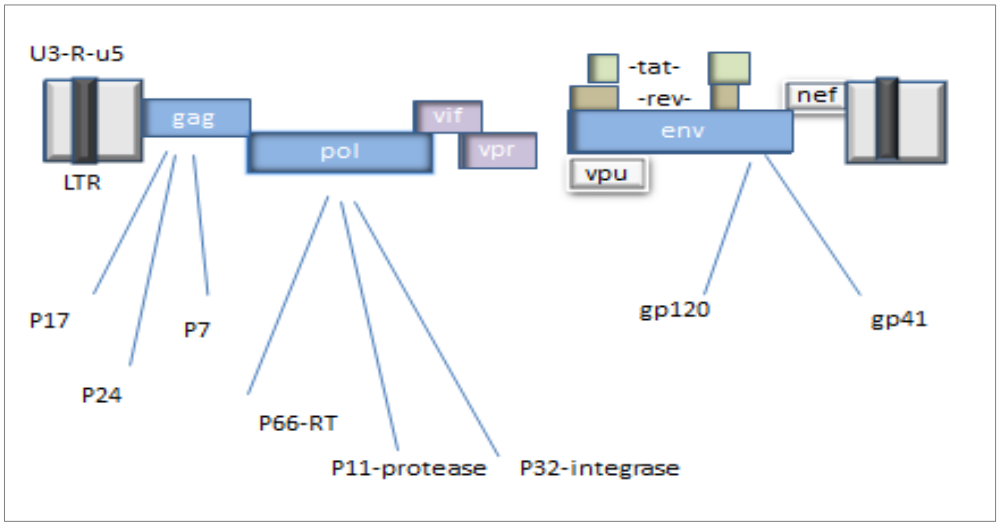

Figure 2-4: The genomic structure of an HIV-1 virion. (Frankel and Young et al., 1998).

2.2.3 The HIV Life Cycle

The life cycle starts when an HIV virion infects CD4-expressing cells, usually T helper cells or $CD4^+$ monocytes and macrophages. Here gp120 binds to the CD4 receptor, with co-receptors CCR5 or C-X-C chemokine receptor type 4(CXCR4) also required in this process. After such binding the viral envelope fuses with the host membrane and the viral core enters the cytoplasm. This leads to the release of virion enzymes and viral genetic material (Fanales-Belasio et al., 2010). The reverse transcriptase enzyme subsequently converts the two RNA strands to cDNA (Figure 2.5). Thereafter, the enzyme integrase starts to facilitate the entry of viral DNA (provirus) into the host nucleus that is then transcribed into mRNA by the host cell's enzymatic machinery. Such mRNA transcripts encode viral proteins and forms the basis for new progeny genomic RNA. The protease enzyme mediates the conversion of newly-translated polypeptides into functional proteins and newly assembled virions subsequently move to the cell surface (Goto et al., 1998). During the budding process, the virus may also incorporate different host proteins from the host membrane into its lipoprotein layer. This may include proteins such as human leucocyte antigen (HLA class I and II) or adhesion proteins such as intracellular adhesion molecule-1 (ICAM-1) that may facilitate adhesion to other target cells (AL-Jabri et al., 2003).

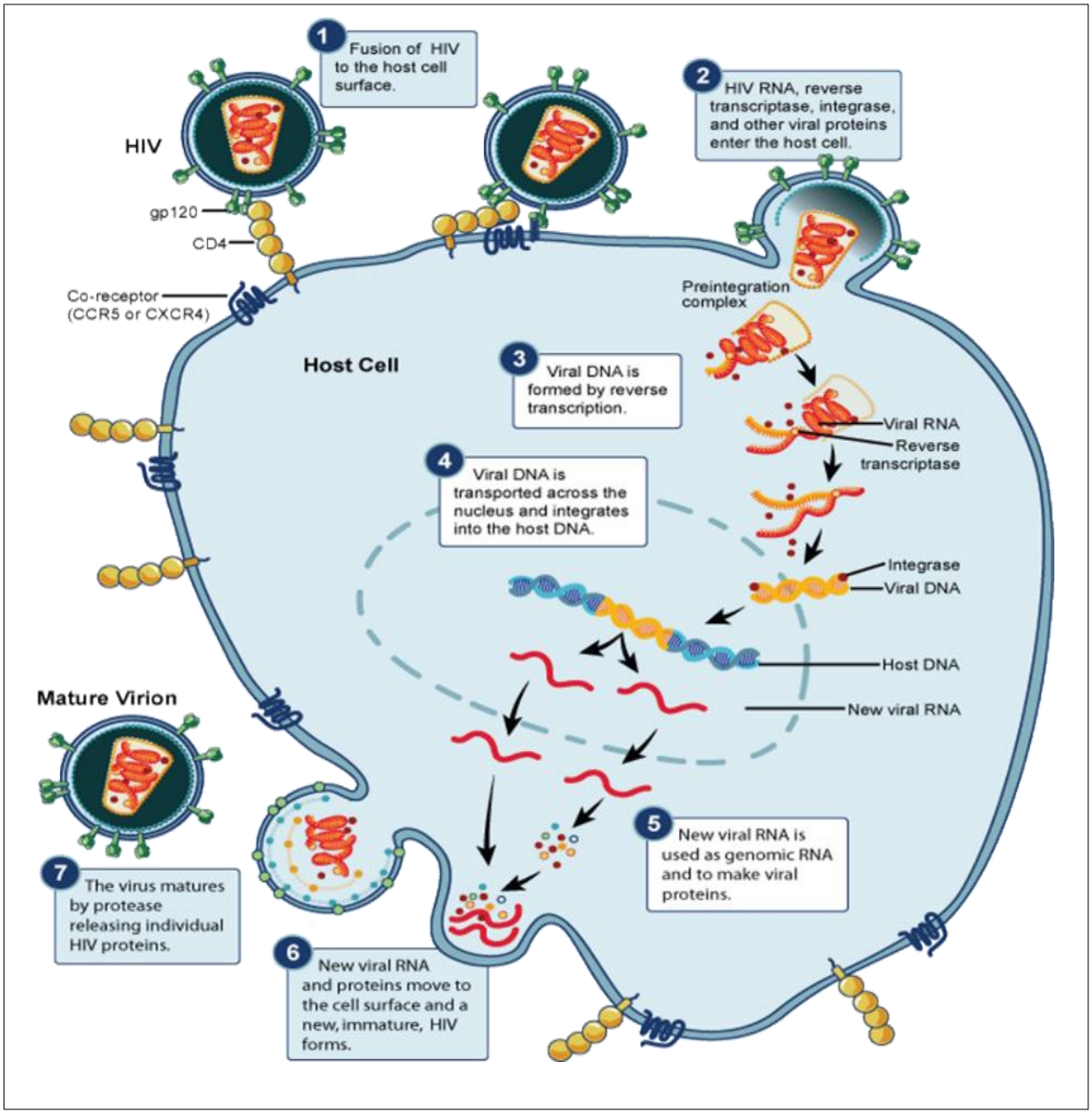

Figure 2-5: The HIV life cycle. Step #1: binding; Step #2: fusion; Step #3: reverse transcription; Step #4: integration; Step #5: replication; Step #6: assembly; and Step #7: budding (Goto et al., 1998); Figure copied from NIAID Source https://www.niaid.nih.gov.

2.3 HIV/CVD: RELATIONSHIP WITH THE IMMUNE SYSTEM AND INFLAMMATORY RESPONSES

HIV infection induces an immune response and this may result in a chronic stimulation due to the inability of the immune system to successfully deal with this stressor (i.e. the infecting virus). As HIV infection is linked to an immune response, the next sections will briefly review the nature of inflammation and then contextualize the role of HIV within this setting.

2.3.1 Immune Cells

Immune cells are produced in the bone marrow, the generative tissue for all lymphoid and myeloid cells. Bone marrow provides a suitable environment for formation and differentiation of haematopoietic immune cells (Figure 2.6).

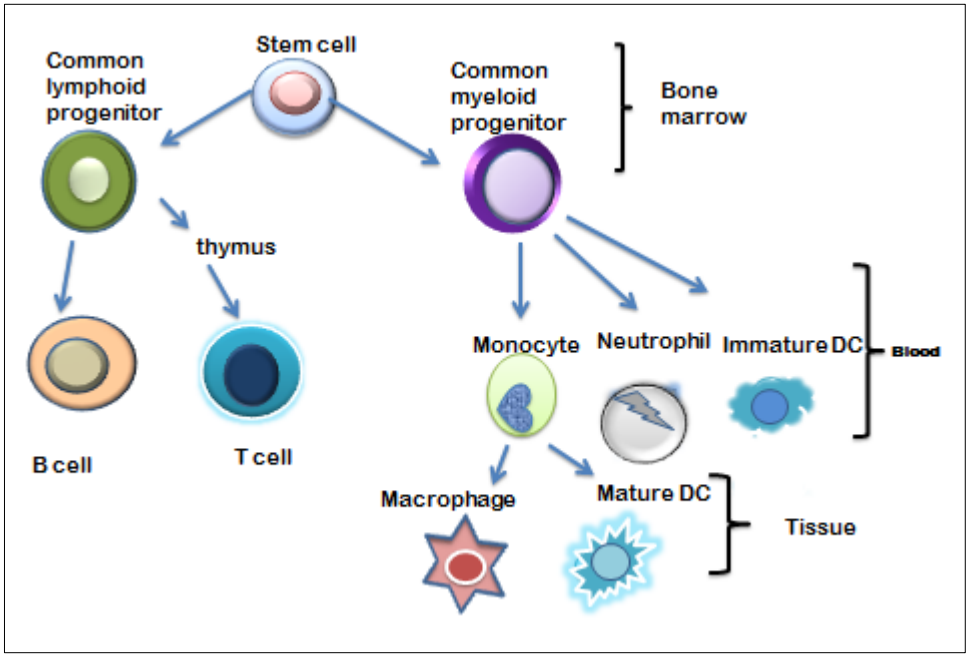

Figure 2-6: Immune cells in the body. This includes the innate immune system (that arises from a common myeloid progenitor) and an adaptive immune system (that arises from a common lymphoid progenitor).

2.3.2 Inflammation

The Roman medical writer Cornelius Celsus write about the four basic signs of inflammation (redness, swelling, heat, and pain) around 2000 years ago (Medzhitov et al., 2010). Some 200 years later the Greek physician Galen put forward the notion that inflammation is a crucial response to injury. More recently, Rudolf Virchow added loss of function as a fifth cardinal sign, thereby indicating that inflammation constitutes a pathological condition (Medzhitov et al., 2010).

The inflammatory process is initiated by infection or injury when specialized pattern recognition receptors (PRRs) such as toll like receptors (TLRs) recognize pathogen or damage-associated molecular patterns (PAMPs or DAMPs) (Land et al., 2015). TLRs (and other PRRs) stimulate the production of various mediators that include cytokines, chemokines and other bioactive substances. TLRs also activate adaptive immune cells that can initiate changes at various target tissues as a response to pathogens or injuries (Land et al., 2015).

A Immune activation and inflammation

Inflammation is a broad term that explains the natural process arising from infection and/or injury and which involves the interaction between soluble factors and immune cells. When inflammatory cells recognize pathogens there is an initiation of pro-inflammatory cytokine and chemokine secretion (Tedgui and Mallat. 2006). Inflammation is usually a transitory phenomenon with its signs apparent over a two to three-week period. Once pathogens are cleared then activation levels of the immune system reverts back to normal.

During acute inflammation, numerous types of immune cells and plasma proteins are activated at the infection site. Such actions are initiated by a family of PRRs expressed on macrophage/monocyte cells and also expressed on natural killer (NK) cells and dendritic cells (DCs). PRRs expressed on phagocytes possess the ability to recognise pathogens. These cells are usually stimulated early during inflammation when macrophages and NK cells start producing pro-inflammatory cytokines (interleukin-1 [IL-1], IL-6, and tumor necrosis factor-alpha [TNF-α]) and chemokine receptors chemotactic ligand-2 (CCL2) and chemokine (C-X-C motif) ligand 8 (CXCL8) (Duque and Descoteaux. 2014). Acquired immunity then follows – all these reactions can take place concurrently. Antigen-specific T cells can also amplify such an inflammatory response by cytokine production (Mogensen et al., 2009). CD4 T helper cells manage the overall immune response, while CD8 T killer cells or cytotoxic T cells have the primary task of killing pathogens, virus-infected cells and malignant cells (Mogensen et al., 2009). Together such inflammatory mediators act on target tissues like blood vessels to cause vasodilatation, leakage of plasma, the migration of neutrophils together with the migration of tissue macrophages and mast cells to the infected site. Immune cells such as neutrophils, activated macrophages and NK cells can also kill and destroy infectious agents (Medzhitov et al., 2010). They also have the ability to terminate and eliminate the trigger once the damaged tissue is repaired - this is a highly-regulated resolution of inflammation. Here a regulatory process develops that switches mechanisms from a pro- to anti-inflammatory nature. Mediators possess anti-inflammatory and pro-resolution properties (termed protectins and resolvins, respectively) that possess the ability to stimulate mucosal antimicrobial defense and clearance. This switch in turn recruits monocytes/macrophages (M2) (instead of neutrophils) and plays a role as an anti-inflammatory regulator that expresses IL-10 which is involved in tissue repair (Serhan et al., 2008); if the resolution phase is not achieved, chronic inflammation arises.

2.3.3 The Role of HIV in Inflammation

Under normal conditions the immune system is self-regulated, i.e. will turn itself off when pathogens are cleared. However, during HIV infection such inflammation can become chronic due to the inability of the immune system to clear the virus during the acute stage of infection (Paiardini and Müller-Trutwin. 2013). What prevents the immune system from completely eradicating the HIV in this instance? There are several reasons that can help explain this phenomenon. In the first instance, HIV infects some CD4 T helper cells and also other cells that express CD4 (e.g. monocytes, macrophages) and destroys a large number of CD4 T cells, especially memory cells (Février et al., 2011). The loss of a key immune regulatory cell type will

subsequently impact on other cells that make up the immune system. The invading virus can also establish latent reservoirs in memory cells so that it can be reactivated at a later stage to once again replicate. Secondly, there is the suggestion that the shift from Type 1 T helper (TH1) to Type 2 helper (TH2) cells, and also regulatory T cells (Treg cells) may contribute in this instance. However, this shift may be due to the loss of TH1 cells. Another option is that the immune system tries to rectify the pro-inflammatory milieu by up-regulating "exhaustion" markers – such as programmed cell death protein markers (PD-1) and T cells immunoglobulin mucin domain-3 (Tim-3) – that in turn can render T cells non-functional. Finally, there may also be increased insensitivity of HIV to neutralized antibodies and cellular immunity as a result of its high mutation rate that allows it to escape immune surveillance and destructive systems (Miedema et al., 2013).

Such factors may all contribute to continuous stimulation of the immune system and thus chronic inflammation can manifest in a systemic manner that now affects the entire body. This in turn can lead to the phenomenon of persistent immune activation that will occur even with the roll-out of cART. Such immune activation can be detected by CD38 and human leukocyte antigen-DR (HLA-DR) expression on CD8 T cells where elevations of such markers indicate persistent immune activation in HIV-infected individuals (Deeks et al., 2004). In support, research shows that with cART the co-expression of CD38 and HLA-DR decreases slightly but not to baseline levels measured in non-infected individuals (Al-Harthi et al., 2004). Together it is clear that HIV pathogenesis is driven by chronic inflammation that is strongly associated with infection in concert with robust increases of inflammatory markers such as IL-6, IL-10, TNF-α, interferon-gamma (IFN-γ) and IFN-α (Stacy et al., 2009).

2.3.4 HIV infection and CVD Link

The HIV impacts on host immune responses in multiple ways including modulation of cytokine production and chemokine signaling networks and also as a driver of inflammation. The virus stimulates various cell types of the immune system to produce a range of inflammatory cytokines. The importance of HIV and viral components in driving immune activation/inflammation is clearly demonstrated by relatively lower inflammation and immune activation that accompany cART-mediated attenuation of HIV replication (Pinzone et al., 2012; Hunt et al., 2007). Moreover, a comparison between an HIV control group with a VL of less than 20 copies/ mL (i.e. below standard detection limit), and a low level viremia group (VL 20–200 copies/ mL) showed that low level viremia is associated with higher levels of inflammation than controls (Reus et al., 2013).

The risk of developing CVD in treatment-naïve patients is based on the potential impact of HIV infection as a driver of atherosclerosis. This can occur through mechanisms related to persistent immune activation and inflammation, accompanied by an increase in coagulation markers and lipid disorders (Baker et al., 2013). In support, the SMART (Strategies for Management of Antiretroviral Therapy) Study demonstrated the role of HIV replication and relatively high immune activation and an increased risk for CVD onset after cART was interrupted (Kuller et al., 2008). Huse et al. (2012) also established a robust association between elevated markers of immune activation and coronary atherosclerosis, supporting the important role of immune

activation in the onset and progression of atherosclerosis. Thus it emerges that continuous low-level viremia from HIV infection leads to persistent immune activation and chronic inflammation in HIV-infected persons. In addition, co-infection and microbial translocation across compromised epithelial gastrointestinal barriers (as a result of HIV infection) can fuel immune system activation and persistent chronic inflammation (Brenchley and Douek. 2008). In parallel with such an inflammatory response, HIV-infected individuals (cART-naïve and cART-treated) can thus develop atherosclerosis (Sandler and Douek. 2012)

Long-term cART can also impact on heart function and the vasculature, thereby contributing to endothelial dysfunction, lipid disorders and an increased risk of cardiac complications (Cerrato et al., 2015). For example, the Data Collection on Adverse events of Anti-HIV Drugs (D:A:D) Study showed that HIV protease inhibitors (PI) are linked to an increased risk for myocardial infarction. This is a consequence of PI-mediated effects on lipid metabolism, endothelial function and contributing to insulin resistance. cART is also associated with increased oxidative stress and mononuclear cell adhesion to the endothelium of vascular walls causing increased lipid accumulation and endothelial permeability (Dube et al., 2008; Reyskens et al., 2013).

Thus HIV infection can elicit direct effects on endothelial function (as discussed) while the production of relatively high levels of inflammatory cytokines enhance generalized inflammation and the formation of atheroma by macrophage cells.

2.3.5 Adaptive Immune Activation and CVD Link

Activation of adaptive immunity during HIV infection occurs relatively early during acute infection and upon initial exposure to the virus (McMichael et al. 2010). Here incoming HIV particles are taken up by DCs (professional antigen presenting cells [APCs]); that recognize PAMPs through their TLRs. DCs then break up the pathogens into protein fragments that are termed epitopes. Then the class II major histocompatibility complex (MHC-II) presents such epitopes to CD4 T cells, while class I MHC presents it to CD8 T cells. The T cell receptors (TCRs) on both CD4 and CD8 cells recognize the corresponding pathogen epitopes and eventually become activated (Gasper et al., 2014). The activated T cells then divide and proliferate to form an epitope-specific clonally expanded population. Activated T cells then start to secrete proteins (chemokines and cytokines), with respective subsets (e.g. effector versus Treg cells) each displaying a specific function.

Persistent T cell activation during HIV infection leads to the exhaustion of T cells - manifesting as attenuated proliferative capacity (Hazenberg et al., 2000). This in turn results in the loss of cytokine production capacity, the expression of exhaustion phenotype markers (PD-1, Tim-3) and lowered expression of CD28 co-stimulatory molecules. The increased number of $CD8^{+}CD28^{-}$ T cells will lead to sub-clinical CVD that are associated with morbidity and mortality (Shahbaz et al., 2015; Kaplan et al., 2011). As T cells lose its ability to regenerate, senescence of the immune system develops. This is reflected by expression of premature aging markers, attenuated naïve CD4 and CD8 T cell numbers (due to decreased thymic output)

and a gradual depletion of total CD4 count that leads to a skewed CD4:CD8 ratio. Of note, lower CD4 counts can be indicative of an increased risk for atherosclerosis (Kaplan et al., 2011 Lichtenstein et al., 2010). Here data revealed a greater frequency of carotid plaques in individuals with CD4 counts of less than 200 cells/µL. There is also strong evidence that both CD4 depletion and loss of function are associated with atherosclerosis (Lichtenstein et al., 2010; Zaaqoq et al, 2015).

The TH cell-mediated response also contributes to the development of atherosclerosis. In parallel, Treg cells inhibit the proliferation of effector T cells and the development of atherosclerosis. With CVD an imbalance exists between pathogenic TH and Treg cells that are associated with lesion development (Foks et al., 2015). Several *in vitro* and *in vivo* studies show that natural Treg cells can inhibit effector T cell proliferation, demonstrating its pathogenic role in atherosclerosis (Pastrana et al., 2013; Taleb et al., 2008). Induced Treg cells are generated in peripheral blood in the presence of transforming growth factor-beta (TGF-β) and IL-2. Treg cells mediate their suppressor function through the production of IL-10 and TGF-β. The disruption of TGF-β signaling as an anti-inflammatory cytokine in T cells accelerates atherosclerosis which in turn inhibits the proliferation and differentiation of T cells toward pathogenic TH cells (Mallat et al., 2009). There are further studies which show a protective role for IL-10 in atherosclerosis – this is achieved by inhibiting the production of inflammatory mediators, matrix metalloproteinases (MMPs), tissue factor (TF) production and also by exerting anti-apoptotic effects (Mallat et al., 2002).

Thus Treg cells play an important role in the prevention of atherosclerosis and also to control inflammation during HIV infection. However, many studies show that Tregs play a role in inducing chronic infection by preventing the protective immune response of T and B cells, thus enabling chronic viremia and immune activation and inflammation (Chevalier et al., 2013). The elucidation of the crucial role of Tregs is therefore central to a better understanding of the underlying mechanisms driving the onset of atherosclerosis.

A Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTLs) play a major role in controlling virus levels during a symptomatic period; CD8 T cells recognize the infected cell through an MHC-I dependent process and can also lyse such cells by perforin and granzymes secretion (Zhang et al., 2003). One of the characteristics of untreated HIV is the expansion of CD8 T cells. Such cells are mature and senescent and the reason for a decreased CD4/CD8 ratio. The latter can predict morbidity and the eventual outcome(s) of HIV infection (Lu W et al., 2015; Serrano-Villar et al., 2014). CD8 T cells are detected in relatively low numbers during the early stages of the atherosclerotic process but become more dominant in plaque lesions during the latter stages (Ait-Oufella et al., 2014). $CD8^+$ T cells may also possess a more direct pro-atherogenic function. For example, a relatively high number of activated circulating cells are directly linked to atherosclerotic plaques in treated HIV-positive individuals. Increased CD8 T cells with HIV infection also express the fractalkine receptor (CX3CR1) and are enriched for protease-activated receptor-1 (PAR-1) (Mudd and Lederman. 2014; Hurley et al., 2012).

2.3.6 Innate Immune Activation and CVD Link

The innate immune activation and response occur relatively early during infection and results in a significant, non-specific early response during the acute phase of infection. The mononuclear phagocyte system, which originates from myeloid progenitors in bone marrow and comprises monocytes, macrophages and DCs forms the foundation of innate immunity. There is strong evidence that innate immune activation plays an important role in HIV pathogenesis and that its components may contribute to the onset of atherosclerosis. Oxidized low-density lipoprotein (OX-LDL) activates adhesion molecules (selectin, immunoglobulin superfamily, integrin, and mucin-glycoprotein) which in turn recruit monocytes and macrophages (Chen et al., 2015). The early recruitment of monocytes/macrophages to atherosclerotic plaques is initiated as a defensive or adaptive response. Furthermore, OX-LDL activates target cells to produce monocyte chemo attractant protein (MCP-1) and vascular endothelial growth factor (VEGF) that can both contribute to plaque development. After activating adhesion molecules and monocyte recruitment to endothelial cells, OX-LDL also increases monocyte-platelet aggregation on endothelial cells that further contributes to plaque formation (Chen et al., 2015).

Blood monocytes and tissue macrophages can initiate the thrombotic pathway by TF (initiating factor of the extrinsic coagulation pathway) expression on their cell surfaces (Funderburg et al., 2010). In addition, changes in monocyte subsets (numbers and relative proportion of total monocytes) during different stages of HIV infection also play a crucial role in HIV pathogenesis (Zawada et al., 2012). Monocytes can be classified according to the relative expression of CD14 and CD16. Three different subpopulations have been identified, each with a distinct function. The non-classical ($CD14^{+}CD16^{++}$) and intermediate monocyte ($CD14^{++}CD16^{+}$) subsets are increased in HIV-infected patients and these subsets express relatively high levels of CCR5, CX3CR1 and CCR2 that are all associated with an increased risk for atherosclerosis, while classical monocytes are involved in phagocytosis and antimicrobial role (Yang et al., 2014).

The differentiation of macrophages into M1 and M2 subtypes also plays an important role in the development of atherosclerosis. Here the pro-inflammatory M1 macrophages reflect the TH1 response and lead to the activation of endothelial and smooth muscle cells, while M2 macrophage cells possess a pro-atherogenic role. The latter occurs via the expression of IL-4 and CD36 on macrophages that promote the uptake of OX-LDL and enhances foam cell formation (Moore and Tabas. 2011).

Persistent activation of monocytes/macrophages in HIV infected-individuals is triggered by lipopolysaccharide (LPS);, while activated macrophages play an essential role in promoting systemic and chronic inflammation during HIV infection (Shalhoub et al., 2011; Mogensen et al., 2010). Monocytes are key pro-inflammatory cells, producing powerful cytokines such as IL-6 and TNF-α. During the course of a normal immune response, immune cells possess the ability to eliminate the trigger (i.e. clear the pathogen) and terminate the response with the simultaneous restoration of damaged tissue – a process termed resolution of inflammation. At this point, there is a firmly established regulatory process at play, which changes mechanisms from a pro- to anti-inflammatory nature. This in turn employs monocytes/macrophages instead

of neutrophils as anti-inflammatory cells for tissue repair (Serhan et al., 2008). If the resolution phase is not achieved, chronic inflammation ensues and aberrant repair can occur that leads to fibrosis and the development of end-organ disease (Wynn et al., 2008). Fibrosis is a key feature of many chronic unresolved inflammatory diseases that include lymph node fibrosis with HIV infection and vascular fibrosis associated with atherosclerotic plaque formation. Recent work has also shown a correlation between the monocyte/macrophage marker, sCD163 and non-calcified coronary plaque formation lesions in both the general and the HIV-infected population, representing additional inflammatory activation driven by HIV (Burdo et al., 2011).

As the immune system cannot completely eradicate HIV, activation of both adaptive and innate immunity can contribute to the pathogenesis of complications such as CVD, renal disease and cancer. This eventually results in CD4 depletion and the development of immunodeficiency in HIV-positive persons even though administered with cART.

2.3.7 Immune activation and HIV pathogenesis

There is currently strong evidence that immune activation is the major driver of HIV pathogenesis (Paiardini and Müller-Trutwin. 2013; Pandrea et al., 2008). Whether the development of immune deficiency complications like AIDS or non-immune deficiency complications such as CVD occurs (Hunt et al., 2007), the link between immune activation and HIV pathogenesis is quite clear from studies of different monkey species infected with simian immunodeficiency virus (SIV). Here the natural host or non-pathogenic species (e.g. African Green Monkey) did not develop immune activation and AIDS despite a relatively high viral load. By contrast, pathogenic species (e.g. Rhesus macaque) developed AIDS with a relatively high level of immune activation (Pandrea et al., 2008) (Table 2.1).

Table 2-1: HIV pathogenesis: a comparison between non-pathogenic and pathogenic species (Pandrea et al., 2008: Miedema et al., 2013).

Non-pathogenic species (e.g. African Green Monkey)	**Pathogenic species** (e.g. Rhesus macaque)
Stable viral replication	High viral replication
Normal level of immune activation and apoptosis	High level of immune activation and apoptosis
Restoration of CD4 T cells in peripheral blood and intestine	No restoration of CD4 T cells
Lower levels of CCR5 expressing CD4 T cells, with lack of disease progression	Higher levels of CCR5 expressing CD4 T cells, with disease progression
Preservation of immune cell subset function	Abnormal immune cell subsets function
Lack of microbial translocation, with absent of aberrant immune activation	High levels of microbial translocation, with aberrant immune cell activation
Establishment of early anti-inflammatory milieu	No early anti-inflammatory role

These comparative analyses show that major differences are reflected by how host immune cells respond to HIV (Christiaansen et al., 2015). For example, with non-pathogenic SIV infection immune activation is

dampened by increased IL-10 production, decreased T cell activation and attenuated apoptosis (Klatt et al., 2012). In addition, monocytes become unresponsive to LPS stimulation and no longer secrete TNF-α, a powerful pro-inflammatory cytokine that impacts on the initiation of the immune response. Furthermore, there is an absence of microbial translocation in SIV natural hosts with a relatively low level of immune activation (Klatt et al., 2013; Brenchley et al., 2013). Thus, it is apparent that the nature of the early response of innate immune cells plays a key role in terms of the degree of HIV-mediated pathogenesis (Mogensen et al., 2010)

2.3.8 Inflammation and Atherosclerosis

Atherosclerosis is a chronic inflammatory disease that is characterized by plaque formation that includes a number of co-factors such as OX-LDL, activated T cells and macrophages, cell death, smooth muscle and fibrosis (Tedgui et al., 2006). Persistent chronic inflammation and immune activation lead to increased utilization of antioxidants, resulting in oxidative stress that can be detected in several tissues of HIV-infected individuals (Wanjiku et al., 2013). Oxidative stress induces formation of OX-LDL with downstream effects such as the activation of monocytes and T lymphocytes, thereby increasing its ability to infiltrate vascular walls (Chen et al., 2015; Singh et al., 2002).

Atherosclerosis is initiated by the infiltration of OX-LDL through arterial walls leading to increased expression of vascular cell adhesion and intracellular adhesion molecules, an early sign of an aberrant immune cell response (Hansson et al., 2001; Syed et al., 2013). Activated monocytes can also migrate from blood to the intima and sub-intima, where it can take up OX-LDL by scavenger receptors and resulting in monocytes forming early fatty plaques streaks (foam cells) (Woollard and Geissmann et al., 2010). Zidar et al. (2015) demonstrated that increased OX-LDL uptake by activated monocytes in HIV-infected patients is associated with higher levels of reactive oxygen species (ROS). DCs encounter atherosclerosis-related antigens with vascular changes and then migrate to the secondary lymphoid organ to present antigens and to promote antigen-specific pathogenic or Treg cell activation (Mallat et al., 2009). Activated T cells also produce mediators such as IFN-γ that can contribute to increased inflammation and atherosclerosis (Andersson et al., 2010) (Figure 2.7).

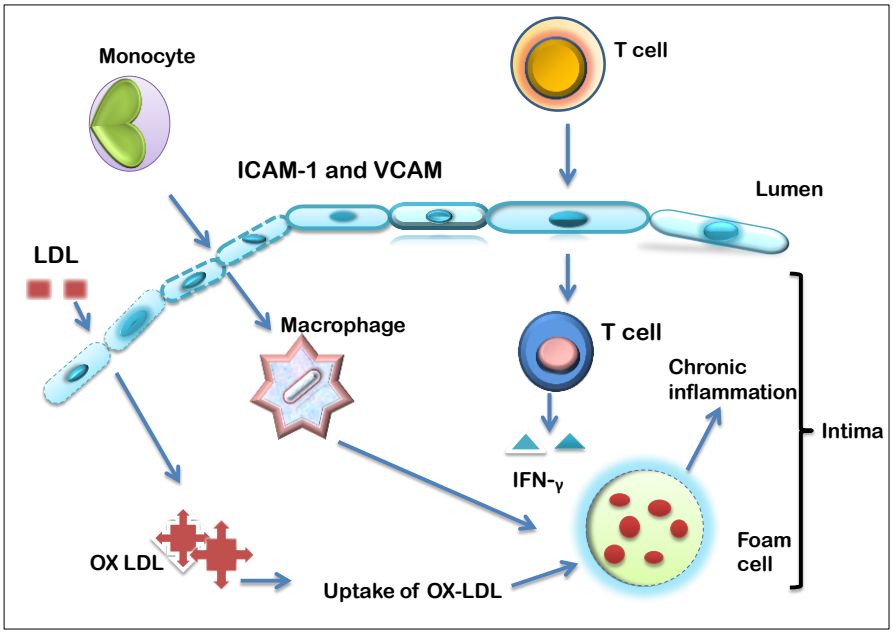

Figure 2-7: The involvement of immune cells in the initiation of atherosclerosis. Infiltration of OX-LDL through the arterial wall activates both adhesion molecules and the recruitment of immune cells to the site of injury with downstream effects.

Thus the variety of immune cells plays an important role to initiate atherosclerosis and continues to contribute to its subsequent progression (Libby and Hansson. 2015). This notion can be exploited within the clinical setting as studies show that the elevation of inflammatory markers such as C-reactive protein (CRP), IL-6 and D-dimer can help predict outcomes of patients burdened with acute coronary syndrome (Baker and Duprez. 2010; Funderburg et al., 2012).

2.3.9 Viral and Immune System Interaction as a Driver of Atherosclerosis

Although both the innate and adaptive immune responses are activated during HIV infection, this response is inadequate to eliminate/clear the invading virus. There are several reasons why this may occur, e.g. the ability of the virus to integrate into the host genome and establish a latent reservoir ensures that it can survive even a well-coordinated immune defense system. Moreover, the picture is complicated as cells that are expected to eliminate the virus can also play a damaging role by facilitating continued immune activation and progressive immune deficiency (Mogensen et al., 2010) (Figure 2.8). Thus in order to gain an improved understanding of mechanisms underlying CVD risk in HIV-affected individuals, there should be a focus on the interaction between HIV and host immune systems.

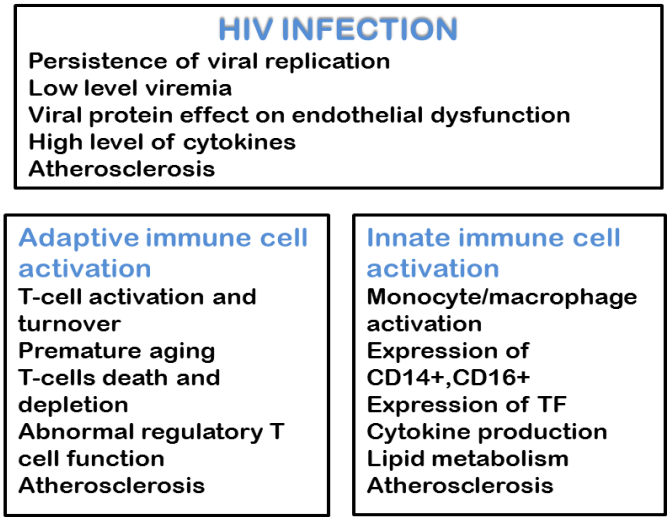

Figure 2-8: The interaction between HIV and immune cells in the context of atherosclerosis.

A The immune system's response to HIV

The viral protein trans-activator of transcription (Tat) directly affects the IFN-γ-mediated intracellular signaling pathway that usually increases antigen presentation capacity by stimulating the expression of MHC-II on antigen-presenting cells (Wu and KewalRamani. 2011). However, viral Tat decreases the antigen presentation capacity of macrophages and DCs by interfering with IFN-γ-mediated signaling and thereby limiting the overall immune response to invading viruses. IFN-γ is the main cytokine produced by TH1 and NK cells and it is main role is to stimulate cytotoxic T lymphocytes to eliminate the invading virus. IFN-γ also activates macrophages to enhance their eliminating ability, while an increased level at the time of infection suppresses the role of TH2 cells.

There is a positive correlation between TNF-α elevation and HIV-1-induced viremia as the former is one of the most powerful pro-inflammatory cytokines that can elicit severe inflammatory-related damage (Norris et al., 2006). For example, TNF-α can induce IL-6 and IL-8 that leads to upregulation of viral replication. Both IL-6 and TNF-α, are also necessary during acute stage reactions, thereby contributing to fever and anorexia (processes characteristic of systemic inflammation). With HIV infection other modulators such as TGF-β and glycoprotein A repetitions predominant (GARP) play a role in the transfer of TH1 to Treg cells, and as the disease progresses this shift leads to an increasingly prominent role for TH2 and Treg cells (Christiaansen et al., 2015; Miller et al.,2014). However, the TH2 response is not enough to control HIV pathogenicity and these results in viral persistence and the development of chronic HIV-induced pathogenesis (Keynan et al., 2008).

The induction of anti-inflammatory cytokines such as IL-10 decreases the inflammatory response by the suppression of both inflammatory cytokine-mediated signalling and impaired DC maturation (due to inhibition of effector T cells) (Blanco et al., 2008). By contrast, IL-10 induction is crucial for supporting viral persistence and preventing viral clearance. For example, upregulation of IL-10 and PD-1 by monocytes

during HIV-1 infection leads to reversible CD4 T cell dysfunction and impaired viral clearance (Said et al., 2010). Moreover, others found that the attenuation of IL-10 signalling induced viral clearance and resulted in the restoration of CD4 and CD8 T cell proliferation. This suggests that IL-10 may play a role in HIV-mediated immune exhaustion (Yi et al., 2010). As HIV infection is associated with increased IL-10 levels, this shifts TH1 to TH2 and Treg cells (Christiaansen et al., 2015) (Figures 2.9 and 2.10). This subsequently leads to decreased activity of crucial immune cells responsible for viral clearance thereby causing persistent viral infection and chronic inflammation that eventually results in increased morbidity and mortality.

Thus, although HIV promotes inflammation and immune activation which augments CTLs and helps with virus clearance, inflammation and immune activation also drives viral replication (Paiardini and Müller-Trutwin. 2013). Conversely, IL-10 dampens inflammation and immune activation but also allows the invading virus to better replicate and perpetuate itself. It is important to note that a choice is required, i.e. either the virus is tolerated and reduced to relatively lower levels, or it should be effectively eliminated when it becomes active (as with an acute infection virus).

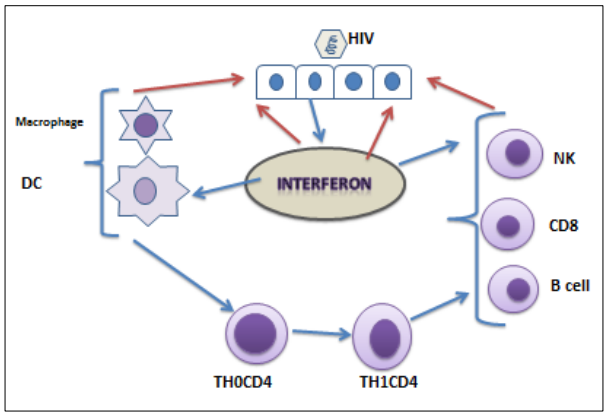

Figure 2-9: Normal antiviral immune response. TH1 activates CD8 to eradicate the virus. This occurs via powerful antibody production to neutralize the virus. In addition, interferon-gamma macrophages, DC and killer cells are all recruited to the site of infection (Christiaansen et al., 2015).

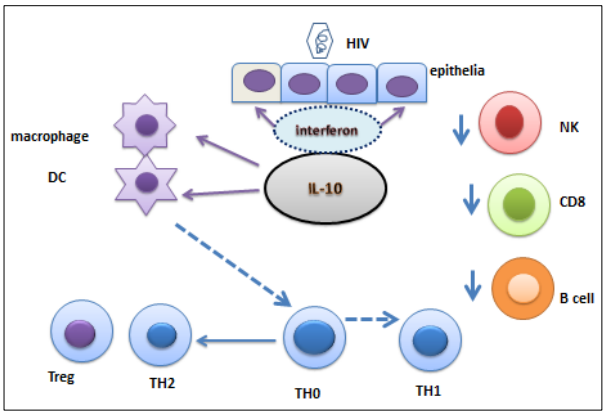

Figure 2-10: HIV's role in skewing immune cells. High IL-10 impairs TH1 and shifts the balance to TH2 and Treg cells. This in turn interferes with CD8 T cells and inhibits antibody production (Christiaansen et al., 2015).

2.3.10 CD4 Depletion

HIV attacks the immune system by targeting CD4 T cells, an integral constituent of the immune system. CD4 T cells coordinate the overall immune response to assist more broadly, e.g. B cells and CTLs. This can be mediated in the form of cytokine- and receptor-ligand-mediated downstream effects (Ait-Oufella et al., 2014). CD4 T cell subsets consist of TH1 cells that assist and support CD8 T cells to kill infected cells and to activate macrophage intracellular killing (Février et al., 2011). TH2 cells support the production of antibodies by B cells and TH17 cells that are a subset involved in responses to extracellular bacteria.

Moreover, Treg cells are $CD4^+$ but not a "helper" subset and can release immune suppressive cytokines to dampen the immune response (Ait-Oufella et al., 2014).

Viral replication is sustained by chronic immune activation that leads to CD4 T cell depletion. Here persistent low-grade inflammation (even during the clinically asymptomatic latent stage and good control of VL) leads to progressive CD4 loss (Deeks et al., 2008). The strongest association between chronic immune activation and HIV/AIDS pathogenesis is provided by SIV infection studies where a relatively high level of viral replication (without immune activation) is not sufficient to produce SIV-mediated pathogenesis and progression to AIDS (Miedema et al., 2013). Such studies not only prove the role of immune activation in AIDS development, but also show that viral replication alone cannot induce disease progression – thus an important point to consider for the onset of CVD in this case. There are a number of causes for CD4 cell depletion that include direct effects of HIV viral replication and infection of CD4-expressing cells (Cummins et al., 2014). Of note, the number of CD4-infected cells is limited during early infection and its production relatively normal at this stage (Hazenberg et al., 2000). CD4 depletion in HIV patients is linked to persistent T cell activation and uninfected cells expressing CD4 can also be targeted for elimination but via activation-induced cell death (AICD) (Février et al., 2011).

2.3.11 Causes of Immune Activation

A Microbial translocation

Microbial translocation is a major contributor to immune activation and disease progression. This was first defined in 2006 when it was recognized that bioactive microbial products were significantly elevated in the plasma of HIV-infected individuals (Brenchley et al., 2006). LPS levels in such individuals correlated directly with the activation of both the adaptive and innate immune systems. During acute HIV infection gastrointestinal tract (GIT) damage occurs as a result of the depletion of gut-associated lymphocytes (Gori et al., 2010; Nazli et al., 2010). Here up to 60% of CD4 T cells within the intestinal mucosa are affected and display viral RNA expression (Brenchyley et al., 2008) Thus increased HIV replication together with CD4 T cell destruction also occurs in the gut mucosa and submucosa (as compared to other lymphoid tissues). Why should this response occur at this unusual location? The gut mucosa is a primary storage site for memory T cells (expressing CCR5 receptors) that possess a semi-activated status and are a preferred target for HIV replication (Veazey et al., 2000).

The normal gut lining transports antigens associated with microbes and nutrients to immune cells located in clusters, i.e. Peyer's patches (Jung et al., 2010). The majority of immune cells are located within such clusters and send an early warning to the immune system by identifying microbes to be eliminated. The main causes of microbial translocation are altered mucosal immunity that occurs due to elimination of a large proportion of CD4 TH cells, particularly TH17 and memory T cells (Février et al., 2011). Subsequently, there is inflammation-associated damage especially to gap junctions within the epithelium. This occurs as maintenance of GIT integrity is dependent on a functional mucosal immune system. However, when TH17

cells (usually responds to bacterial products) are significantly lowered in the GIT this can result in organ damage (Nazli et al., 2010).

B Viremia

The simultaneous decrease in CD4 T cells together with increased plasma viremia are hallmarks of an HIV primary infection. Although there is some normalization after the acute infection stage, this trend can resume with chronic infection (albeit at a more moderate pace). It is clear that HIV itself has a direct impact on immune activation. This effect initially increases during acute infection but decreases after initiating cART. Moreover, inflammatory and immune activation markers decrease within days of initiating therapy but can increase again (together with plasma viremia) as circumstances change, e.g. poor cART adherence (Sandler and Douek. 2012; Pinzone et al., 2012).

The persistence of low-level viremia in HIV patients is a stimulus for immune activation and the most recent cART intensification trials show that the complete eradication of HIV is near impossible (Hatano et al., 2011). Such persistent viremia also acts as an on-going stimulus for the immune system. (Klatt et al., 2013) demonstrated that T cell activation declines with lower levels of viral replication and that activation also further decreased during cART-mediated VL suppression. However, HIV-infected patients can still exhibit persistently abnormal T cell activation due to other factors such as co-infection and GIT leakage.

C Co-infection and immune activation

HIV-positive individuals with chronic, active viral co-infection e.g. CMV, Hepatitis B and C virus (HBV & HCV) possess relatively higher plasma HIV VLs, lower CD4 cell counts and a faster progression to AIDS. Rempel et al. (2013) reported that persons co-infected by HIV/HCV displayed elevated monocyte activation compared to those only infected with HIV. Furthermore, HIV-positive women co-infected with HIV/HCV exhibited increased CD8 T cell activation and a high chance of progressing to AIDS, i.e. their risk of was three times higher versus women with HIV infection only (Kovacs et al. 2010). Co-infection with HIV/CMV also results in relatively higher levels of inflammatory markers and the onset of atherosclerosis (Naeger et al., 2010).

2.3.12 Changes during the Acute and Chronic Stages of Infection

A Acute stage

This phase of HIV infection starts immediately after infection and is characterized by viremia. The latter can be confirmed by laboratory tests that include assessments of HIV-RNA and p24 antigen detection (Tang et al., 2012). This phase usually lasts up to 6 months and ends when anti-HIV antibodies (AB) are detected in plasma. During acute infection, there is a transient loss of circulating CD4 cells (Overbaugh and Morris. 2012). In parallel, there is a significant depletion of CD4 T cells (especially TH17 and effector memory TH cells) from the gastric mucosa due to disruption of lymphoid tissues and changes that occur in mucosal tissues (Février et al., 2011). There is also a dramatic expansion of CD8 T cells early-on during infection that

reflects the induction of antigen-specific CTL (Wherry and Ahmed. 2004). Such an increase is associated with decreased VL, suggesting that CD8T cells play a role in the early suppression of viral replication. Moreover, HIV-positive persons show an inversion of the normal CD4 to CD8 cell ratio, which is indicative of incomplete recovery to pre-infection CD4 T cell numbers. In support, up to half of peripheral CD8 T cells are activated in individuals with long-term HIV infection compared to values typically less than ten per cent in healthy HIV-negative individuals (Sandler and Douek et al. 2012).

B Chronic stage

The chronic HIV-infection stage starts after antibody formation (seroconversion) and is characterized by the progressive dysfunction of the immune system that culminates in its collapse. This is referred to as late stage infection or AIDS. However, HIV infection alone is not sufficient to lead to immune dysfunction. Instead a number of factors are responsible, i.e. HIV together with immune activation and inflammation are strongly linked to immune dysfunction and related morbidity and mortality (Kuller et al., 2008; Kaplan et al., 2011) (Figure 2.11). Chronic infection is characterized by the invading virus being trapped in lymphoid tissues by follicular DCs together with a gradual decline in CD4 T cell counts and low levels of HIV plasma viremia (Mogensen et al., 2010). Although latency may also occur with cART roll-out and thus reduce HIV plasma viremia to lower levels, residual viremia can contribute to immune activation.

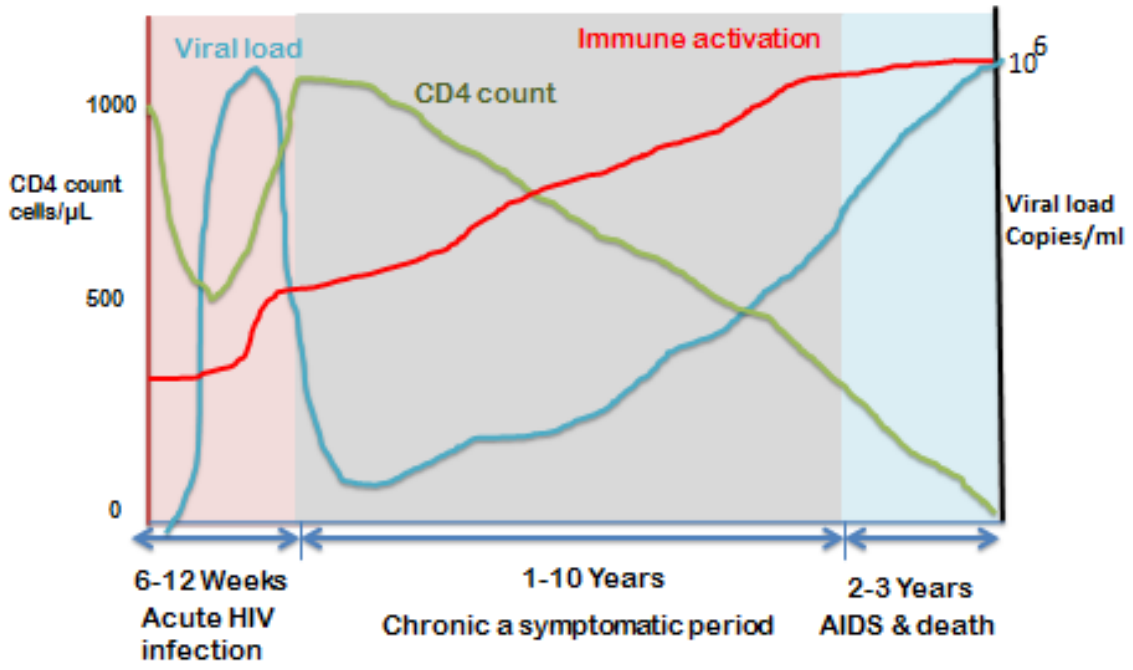

Figure 2-11:The different stages of HIV infection - including length of each respective stage and other parameters such as viral load, immune activation and CD4 T cell counts (Mogensen et al., 2010).

2.3.13 T Cell Senescence

The aging of immune cells and the immune system in general is referred to as immunosenescence. As T cells are the most important immune role player during HIV infection, its senescence is paramount within this context. Persistent immune activation in HIV-infected individuals can lead to T cell activation that can be measured by the expression of CD38 and HLA-DR on CD8 T cells. During a primary infection, there is also an upregulation of telomerase, a crucial enzyme involved in the maintenance of telomere length (Hearps et al., 2012). However, within the context of recurrent immune stimulation such upregulation is attenuated. Thus, memory T cells exhibit short telomere lengths that lead to early or premature aging (Deeks et al., 2011). Senescence occurs when the functional ability of immune cells are impacted by the permanent exhaustion of T cells. The increasing turnover of new T cells then leads to premature aging of all the immune system cells and organs, with an accumulation of aging markers on T cells ($CD28^-$ and $CD57^+$) (Moro-Garcia et al., 2012). Furthermore, peripheral blood monocytes from young HIV-positive individuals show changes in phenotype, function, and telomere length that closely resemble those detected in elderly controls aged approximately 30 years older (Deeks et al., 2011; Hearps et al., 2012). However, such immune-related changes are not fully restored by cART.

2.4 MONOCYTE CELLS

2.4.1 Monocyte Subpopulations

Monocytes are the most dynamic within the mononuclear phagocyte system and display a great deal of plasticity (Figure 2.12). Monocytes develop in the bone marrow (from common myeloid progenitor cells) and form a network of agranulocytic myeloid cells (Bell and Bhandoola et al. 2008). Such cells are responsive to macrophage colony-stimulating factor (M-CSF) that can alter their phenotype and function (Das et al., 2015). Circulating monocytes make up 5–10% of total leukocytes and circulate for up to three days before migrating to specific tissues where they function as precursor cells to macrophages and in some cases as for DCs (Yang et al., 2014). Focusing on morphology, monocyte nuclei can display different forms, i.e. kidney-shaped, oval, or lobulated and can also exhibit varying nucleus-to-cytoplasm ratios. The discovery of monocyte subpopulations has provided a significant advance in the understanding of the crucial role such cells play in terms of chronic inflammation, e.g. as occurs with HIV infection.

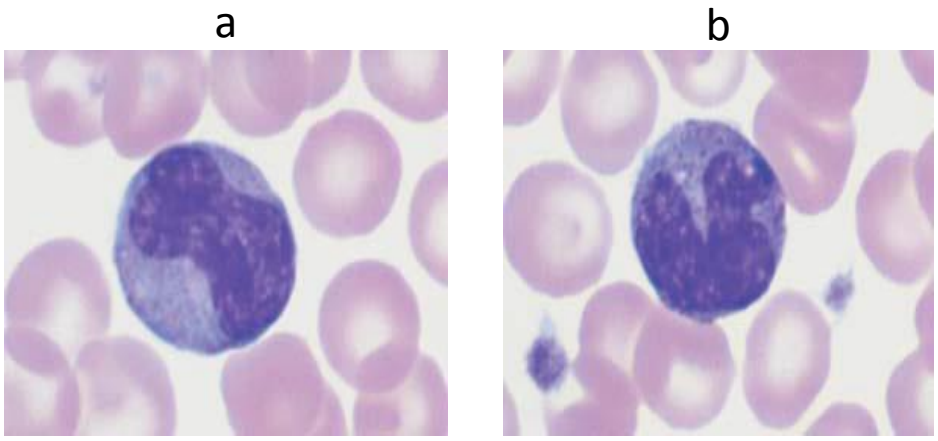

Figure 2-12: Range of appearances of typical monocytes. (a) kidney shape nucleus with gray-blue cytoplasm; (b). Lobed nucleus with gray-blue cytoplasm and fine granulation. (Theml et al. 2004).

Monocytes are key role players of the innate immune system. Both macrophages and DCs are derived from monocytes and are important antigen presenting cells, playing a central role in HIV pathogenesis (Coleman et al., 2009). Monocytes also possess the ability to respond to different environmental stimuli (e.g. cytokines) and are the main responders to microbial LPS (Funderburg et al., 2010). Thus monocytes serve as a crucial link between GIT translocation and immune activation during HIV-1 infection. Monocytes are also responsible for pro-inflammatory pathways that produce cytokines to initiate local and systemic inflammation (Yang et al., 2014; Tedgui and Mallat. 2006). In support, monocytes from HIV-infected persons (with virological suppression) produce high levels of intracellular pro-inflammatory cytokines such as IL-6 and TNF-α compared to uninfected controls (Scully et al., 2016). Of note, the intermediate monocyte subpopulation ($CD14^{++}$ $CD16^{+}$) independently predicts CVD events and this may be explained by relatively high levels of pro-inflammatory cytokines secreted with the expansion of this subset (2012, Rogacev et al).

2.4.2 HIV Infection and Monocyte Subpopulations

Monocytes are usually differentiated according to cell surface expression of phenotypic markers, cytokine production and gene expression profiles (Gren et al., 2015; Wong et al., 2011). They are differentiated into classical monocytes ($CD14^{+}CD16^{-}$) that make up 80–90% of total circulating monocytes and $CD16^{+}$ expressing monocytes ($CD14^{+}CD16^{+}$) that constitute the remaining, circulating monocytes (Yang et al., 2014). The $CD14^{+}CD16^{+}$ monocyte population is further divided into intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$). During HIV infection monocytes show activated phenotypes which increase the expression of the $CD14^{+}CD16^{+}$ population (Funderburg et al., 2012). The $CD14^{+}CD16^{+}$monocyte subset is preferentially susceptible to HIV-1 infection as it expresses relatively high CCR5 levels (Ellery et al., 2007). The identification of such subpopulations during HIV infection is necessary to better understand monocyte function. For example, expansion of both intermediate and non-classical monocyte subsets during HIV infection is proposed to play an important role in HIV-mediated

pathogenesis, especially CVD onset and progression (Funderburg et al., 2012). The significance of $CD16^+$ monocytes in atherosclerosis is highlighted by its pro-inflammatory capacity and increased ability to produce cytokines (IL-6), matrix metalloproteinase (MMPs), expressing CCL2, as well as their potential role to activate endothelial cells.

A Classical monocyte ($CD14^{++}CD16^-$)

The ‘’classical’’ terminology is employed in this instance as their phenotype matches the original description of a monocyte (Ziegler-Heitbrock et al., 2015) and is described as $CD14^{++}$ $CD16^-$ due to bright CD14 staining. Their main function is to facilitate phagocytosis that is also linked to ROS generation (Yang et al., 2014). Classical monocytes also produce cytokines such as TNFα and IL-1β, while expressing a high level of CCR2 as homing markers. In addition, they also secrete IL-10, the anti-inflammatory and immunosuppressive cytokine. These monocytes also play a crucial role during the onset of acute myocardial infarctions and are involved in the early resolution of inflammation (Fang et al., 2015; Yang et al., 2014). By contrast, $CD16^+$ monocytes are involved in the latter stages of inflammation and secrete relatively low levels of IL-10 (Yang et al., 2014).

B Intermediate monocyte ($CD14^{++}CD16^+$)

Despite its naming, the characteristics of intermediate monocytes do not simply fall midway between classical and non-classical monocytes. Instead, it is a specialized subpopulation with the ability to secrete significant amounts of inflammatory cytokines, thus possessing robust pro-inflammatory potential (Zawada et al., 2012). This subpopulation also expresses CCR2 at relatively low levels compared to classical monocytes, while expressing CCR5 as the main HIV receptor (Tacke et al., 2007). During infection intermediate monocytes migrate to the site of infection and invade tissues via CCR2/CCL2 (Yang et al., 2014). This subset also increases during HIV infection and is an independent predictor for CVD events (Rogacev et al., 2012).

C Non-classical monocyte ($CD14^+CD16^{++}$)

The $CD16^+$ monocyte population increases significantly with infections, even if of a localized nature. There is indeed a correlation between CD16 expression, TGF-β and elevated TNF-α serum levels (Ziegler-Heitbrock et al., 2015). $CD14^+CD16^{++}$ monocytes are also considered an inflammatory monocyte subset in humans that is highly correlated with atherosclerosis and CVD. Non-classical monocytes express the chemokine receptor CX3CR1 at relatively high levels and also protect vessel walls against CX3CR1/CCL3 invasion in response to triggering stimuli. Non-classical monocytes with the $Ly6C^-$ phenotype are classified as anti-inflammatory in nature as they and play an important role in patrolling the vascular endothelium (Yang et al., 2014).

Some studies show that the $CD14^+CD16^{++}$ population can generate low pro-inflammatory stimuli (producing TNF-α and IL-6) as well producing relatively high levels of anti-inflammatory cytokines (Thomas et al.,

2015). The $CD16^+$ population is the preferential target for HIV infection as it expresses a relatively high level of CCR5 receptors. Furthermore, there is an association between the $CD16^+$ population and increased TLR-4 levels (Crowe et al., 2010). A recent study demonstrated that both non-classical and intermediate monocytes correlated with viremia, T cell activation and IL-6 (Funderburg et al., 2012). In addition, they showed that non-classical monocytes generally displayed the highest TF expression levels among monocyte subsets, except for intermediate monocytes during HIV infection. Thus, the role of each monocyte subset during HIV-1 infection is a crucial factor to consider especially within the context of inflammation; coagulation and the development of CVD (refer Table 2.2).

Table 2-2: Comparison of the three monocyte cell subpopulations. (Yang et al., 2014; Funderburg et al., 2012 and 2010; Wong et al., 2011; Woollard and Geissmann et al., 2010; Ziegler-Heitbrock et al., 2015 and 2007).

HUMAN MONOCYTE	CLASSICAL	INTERMEDIATE	NON–CLASSICAL
Surface markers	$CD14^{++}CD16^-$	$CD14^{++}CD16^+$	$CD14^+CD16^{++}$
Percentage (Normal range)	80–95%	2–11%	2–8%
Chemokine receptor	$CCR2^{high}$ $CX3CR1^{low}$ $CD62L^+$	$CCR2^{mid}$ $CX3CR1^{high}$ $CCR5^+$	$CCR2^{low}$ $CX3CR1^{high}$
Function	Phagocytosis & immune response	Pro-inflammatory	Patrolling and anti-inflammatory
		Linked to CVD & chronic inflammatory disease & HIV	Inflammatory and HIV infection
Resemble mouse subsets	$Ly6C^+$	$Ly6C^+$	$Ly6C^-$
	High CD62L expression		
Response to LPS	High IL-10 & low TNFα levels	IL-1β and TNF-α	Low IL-10 & high TNF-α levels
Gene signature		Links to T cell activation and antigen presentation	Correlates with T cell activation
Viral load (HIV)	Decrease with HIV-1 infection	Positive correlation	Positive correlation
CD4		Negative correlation	Negative correlation
TF / HIV infection	Low expression	Increased expression	Increased expression
D-dimer/HIV		Positive correlation	Positive correlation

2.4.3 The Role of LPS in Monocyte Activation

LPS is a gram-negative bacterial product that is linked to immune activation with HIV infection (Brenchley et al., 2006). It directly activates monocytes and macrophages and is recognized as a potent activator of especially macrophages. Such activation leads to increased TNFα secretion - the earliest and most potent pro-inflammatory cytokine. LPS stimulates monocytes/macrophages via the TLR4 that is associated with the CD14 molecule (Mogensen et al., 2009; Tedgui and Mallat. 2006). TNF-α displays contrasting roles in non-pathogenic versus pathogenic SIV infection, i.e. for the former immune activation is dampened by rendering monocytes insensitive to LPS and thereby decreasing TNF-α secretion while increasing IL-10 production

(Klatt et al., 2012). IL-10 can in turn inhibit LPS–induced human monocyte TF expression (Lindmark et al., 1998).

2.4.4 Expression of Tissue Factor on the Surface of Monocytes

Tissue factor (a thromboplastin) is a 47-kDa glycoprotein that is expressed on the cell surface of monocytes and platelets. Tissue factor is an important cellular coagulation marker and is responsible for initiating extrinsic clotting pathways (Ferreira et al., 2010). It normally circulates in plasma as an inactive protein until serine protease activates other coagulation pathway factors. Moreover, *in vivo* biological activity of monocyte TF is linked to plasma D-dimer levels.

With hemostasis there is a balance between the formation and dissolution of thrombi. It can be divided into two stages, i.e. the primary one that comprises the platelet and vascular response and a secondary one that includes coagulation factors (Levi et al., 2003). Platelets, vessels and coagulation factors can together stop bleeding and allow for vessel repair by the formation of a stable fibrin platelet plug at the site of injury. In addition, physiological anti-coagulants such as anti-thrombin III (AT-III) and activated protein C oppose thrombosis. Thus clot formation is balanced by plasmin-mediated fibrinolysis that results in the formation of fibrin degradation products (FDPs) such as D-dimer (Levi et al., 2003).

During HIV infection there is a strong correlation between plasma LPS and immune activation, with the former contributing to immunodeficiency that can occur with chronic HIV infection (Brenchley et al., 2006). Moreover, LPS can also increase pro-coagulant TF on circulating blood monocytes, e.g. Funderburg et al. (2012) demonstrated a significant proportion of monocytes expressing TF in HIV-infected blood samples versus matched controls. These data indicate an important role for LPS in terms of monocyte TF induction that in turn can contribute to clotting and ultimately CVD onset in HIV–infected persons (Funderburg et al., 2010; Funderburg and Lederman. 2014). This can be further compounded by monocyte TF expression preventing fibrinolysis and rendering clots resistant to heparin (Semerao et al. 2009).

The pivotal role of inflammation in the development of thrombus formation has been the subject of many studies, supporting a correlation between inflammation and pro-thrombotic factors (Levi et al., 2003). For example, the SMART Study was the first to show a strong association between inflammation and coagulation, and CVD morbidity and mortality in HIV-infected individuals (Kuller et al., 2008). Others also demonstrated increased TF expression with HIV infection and that this correlated with viremia and T cell activation. This was the case especially for the activation of monocyte subsets where - for acute coronary syndrome (ACS) patients - TF expression is robustly linked to non-classical monocyte subsets in HIV positive patients (2012, Funderburg et al) (Figure 2.13).

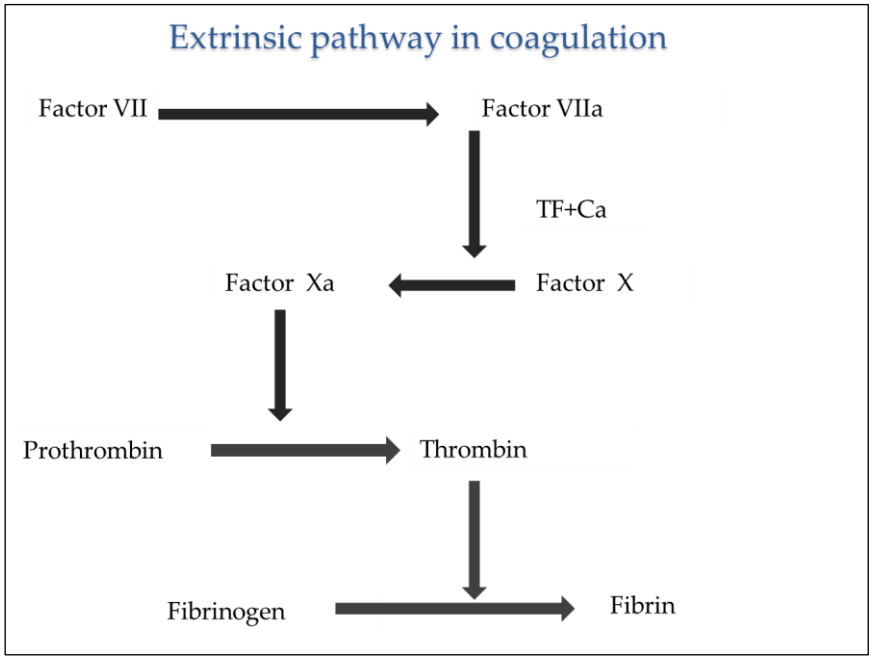

Figure 2-13: The role of TF in the initiation of the extrinsic coagulation pathway. TF binds to factor VIIa and this activates Factor X. This then forms a prothrombinase complex together with activated Factor V to convert prothrombin (Factor II) to thrombin (Factor IIa). Thrombin can then lead to conversion of fibrinogen to fibrin (Levi et al., 2003).

2.4.5 D-dimer

D-dimer is an FDP that is formed when clots are broken down by plasmin actions. It helps to prevent clot formation and is an important step in the hemostasis process (Ruttmann et al., 2006). Such formation of D-dimer can be exploited as a biomarker to predict CVD events and mortality. For example, the SMART Study found that D-dimer (and IL-6) are crucial biomarkers for all-cause and CVD-related mortality in especially HIV-infected persons (Kuller et al., 2008). D-dimer has also been correlated with TF levels, reflecting fibrinolysis and clot formation in HIV–infected persons with immune activation (Funderburg et al., 2010).

2.5 REGULATORY T CELLS

2.5.1 Introduction

Due to their suppressive function, $CD4^+CD25^+FOXP3^+$ Treg cells play an important role in self-tolerance and maintaining immune homeostasis. Here a healthy immune system should maintain a balance between maximizing recognition, the clearance of infectious agents and reducing immune-mediated pathology. Treg cells are a subset of CD4 T lymphocytes and are responsible for this balance and also plays a major role in controlling immune activation and HIV pathogenesis (Chevalier and Weiss. 2013) (Figure 2.14).

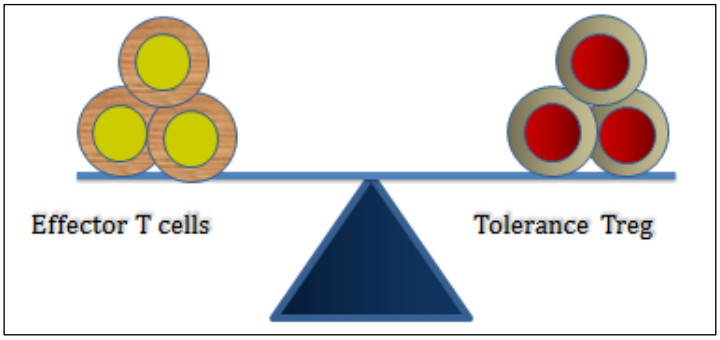

Figure 2-14: The balance between effector T cells and Treg cells.

2.5.2 Regulatory T Cell Phenotype

Tregs normally comprise less than 10% of circulating CD4 T cells and are further characterized by the expression of an interleukin-2 receptor-alpha (IL-2Rα) chain (CD25) (Furtado et al. 2002). Moreover, Sakaguchi et al. (2007) demonstrated that cell tolerance is maintained by active T cells that express the IL-2Rα chain. Treg cells also express relatively low levels of IL-7Rα chains (CD127) (Klein et al., 2010; Seddiki et al., 2006). The latter is a useful marker as it is expressed on the cell surface, thereby eliminating the requirement for intracellular fork head box P3 (FoxP3) protein staining when used in combination with CD4 and CD25. FOXP3 is a master regulator of Treg function and development (Fontenot et al., 2003) and binds numerous gene promoters to ensure increased suppressive activity and inhibition of effector T cells. Here target genes include special AT-rich sequence binding protein (*SATB-1*) and *GARP* (Beyer et al., 2011; Probs-Kepper and Buer. 2010). SATB-1 is a genome organizer that regulates chromatin structure and gene expression. In line with the Treg suppression function its expression should be lowered in regulatory T cells and highly expressed in conventional T cells (Beyer et al., 2011). By contrast, GARP is a transmembrane protein that is selectively expressed by activated regulatory T cells but not by conventional CD4 T cells (Wang et al., 2009). Thus both GARP and FOXP3 are up-regulated in activated Treg cells, with GARP also acting as a receptor for latent TGF-β (Stockis et al., 2009). This determines its role in suppression function. In addition, it may play a role in the shift of TH1 to Treg cells during HIV infection (Miller et al., 2014).

However, no single expression marker (including FOXP3) has thus far been identified as a specific marker for Treg cells as they also express on effector T cells (Wang et al., 2009). This lack of specificity makes the identification of Treg cells quite difficult, especially in cases of immune activation. Although the expression of GARP as a unique marker for activated regulatory T cells is a step in the right direction, the relative lack of its expression in resting inactivated Treg cells is a limitation for Treg cell identification.

2.5.3 Role of Tregs in HIV Infection

Persistence of immune activation is a hallmark of HIV infection (even with long-term cART) and is associated with CD4 depletion and disease progression (Valverde-Villegas et al., 2015). As CD4 T cells perform a central role in the immune system, a decrease in its count and/or function leads to immune

dysregulation and defects in cell mediated–immunity (CMI). A key issue is to understand how the immune system deals with immune activation without suppressing an HIV-specific immune response. Here Treg cells are major role players during immune activation and mediating a response to HIV infection. Although there is robust evidence that Treg cells elicit beneficial effects by limiting immune activation, they can also trigger detrimental outcomes by suppressing the viral immune response (Keynan et al., 2008). Treg cells express CCR5 (in addition to CD4) that are main receptors for HIV to target (Chevalier and Wiess. 2013). After TCR activation, naïve CD45RA Treg cells display increased expression of HIV co-receptors (CCR5, CXCR4) indicating a susceptibility to HIV infection (Oswald-Richter et al., 2004). Previous studies reported increased Treg cell frequency (possibly reflecting overall loss of total CD4 TH cells) with HIV infection together with a decrease in $CD4^+$ Treg cells (Bi X et al., 2009). The higher Treg cell frequency may be helpful during immune activation as this likely reflects increased activity and function.

There is a lack of consensus regarding specific phenotypic markers that define Treg cells during HIV infection. Here Treg cell identification may be complicated as $CD25^+$ Treg and $FOXP3^+$ may be up-regulated in conventional CD4 T cells as a result of immune activation (Chevalier and Wiess. 2013). In this instance GARP can be used as a marker to distinguish $CD25^+$ Treg cells from conventional T cells. This is due to the fact that GARP is more specific to activated Treg cells and is also not expressed in conventional T lymphocytes (Gauthy et al., 2013). Studies have shown that an increased Treg cell frequency during chronic infection is associated with immune activation of Tregs in circulation (Bi X et al., 2009). Others demonstrated that there is an association between immune activation with CD4 depletion and increased production of Treg cells in the thymus of HIV-infected individuals (Bandrea et al., 2010). Furthermore, Epple et al. (2011) showed that mucosal (but not peripheral) $FOXP3^+$ Treg cells are robustly increased during untreated HIV infection and that this normalizes after cART. The number of Treg cells also strongly correlate with both $CD4^+$ and $CD8^+$ T cell activation, suggesting that Treg cells are major contributors to immune activation observed during chronic HIV infection (Eggena et al., 2005).

2.5.4 Regulatory T Cell Function

The suppressive function of Tregs is mediated by IL-10 and TGF-β. Downstream, induced regulatory T (iTreg) cells are stimulated by IL-10 (termed Tr1 cells) and Th3 cells stimulated by TGF-β (Wan and Flavell. 2007). Moreover, Tregs can also mediate suppression by cell-to-cell contact and here IL-35 is needed for suppression of contact of DCs with macrophages (Vignali et al. 2009). Treg-mediated suppression can also be triggered by cytolysis via granzyme A, while Tregs can also target DC maturation and function. CTLA-4 expressed on Tregs can down-regulate co-stimulatory molecules (CD80 and CD86) that usually maintain immaturity and decrease antigen-presenting cells while simultaneously blocking the activation of FOXP3 T cells (Oderup et al., 2006).

2.5.5 Regulatory T Cell Role in Atherosclerosis

The role of Treg cells as a protective mechanism in atherosclerosis is currently an issue of interest, stemming from their role as an anti-inflammatory subset responsible for damping the pathogenic immune response. The involvement of T cells in atherosclerosis is established on the theory that an imbalance between pathogenic T cells (TH1 and TH2) and Tregs leads to the development and progression of atherosclerosis (Mallat et al., 2003). The role of $CD4^{+}CD25^{++}FOXP3^{+}$ Treg cells in maintaining immune homeostasis and inhibiting the pathogenic immune response is facilitated by its suppressive activity. This occurs via the secretion of anti-inflammatory cytokines (TGF-β and IL-10) that inhibit pathogenic T cell function (Mallat et al., 2003). TGF-β plays an important role not only in inducing Tregs from naïve $CD4^{+}$ $CD25^{-}$ cells and generating Th3, but also by mediating protective effects in the context of atherosclerosis (Mallat and Tedgui. 2002). Robertson et al. (2003) demonstrated that TGF-β deficiency leads to an acceleration of atherosclerosis and further showed that it can inhibit the proliferation and differentiation of T cells into pro-atherogenic TH1 and TH2 cells (Mallat and Tedgui. 2002). Greater IL-10 deficiency also increases the infiltration of inflammatory cells into the endothelium, causing activated T cells to increase the secretion of pro-inflammatory cytokines (Zhang and An. 2007). Thus IL-10 plays a crucial role in the prevention of atherosclerosis (Mallat et al., 1999). In addition, Treg cells can limit OX-LDL uptake and foam cell formation through the inhibition of M2 macrophages that usually elicit a pro-atherogenic effect by increasing IL-4 and CD36 expression (Figure 2.15). The latter can promote OX-LDL uptake as well as the formation of foam cells (Tse et al., 2013; Pastrana et al., 2012).

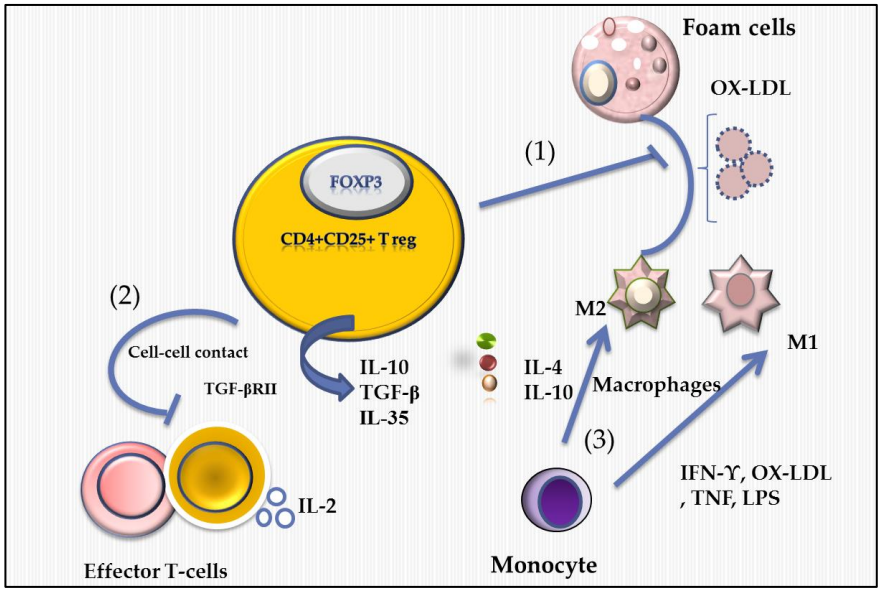

Figure 2-15: The role of Treg cells in atherosclerosis. through anti-inflammatory cytokines (IL-10, TGF-β, IL-35) and cell-to-cell contact. (1) Tregs inhibit OX-LDL uptake and prevent foam cell formation; (2) Tregs also inhibit proliferation to effector T cells (TH1 and TH2); and (3) Monocyte differentiation into anti-inflammatory M1 and proatherogenic M2 (Tse et al., 2013).

2.5.6 Regulatory T cell Types and Development

Although all immune cells develop in the bone marrow, T cell maturation and differentiation occur in the thymus. In addition, natural Treg (nTreg) cells (subset of $CD4^+CD25^+$) develop in the thymus and migrate to peripheral blood where they are responsible for immune homeostasis (Lin et al., 2016) (Figure 2.16) (Table 2.2).

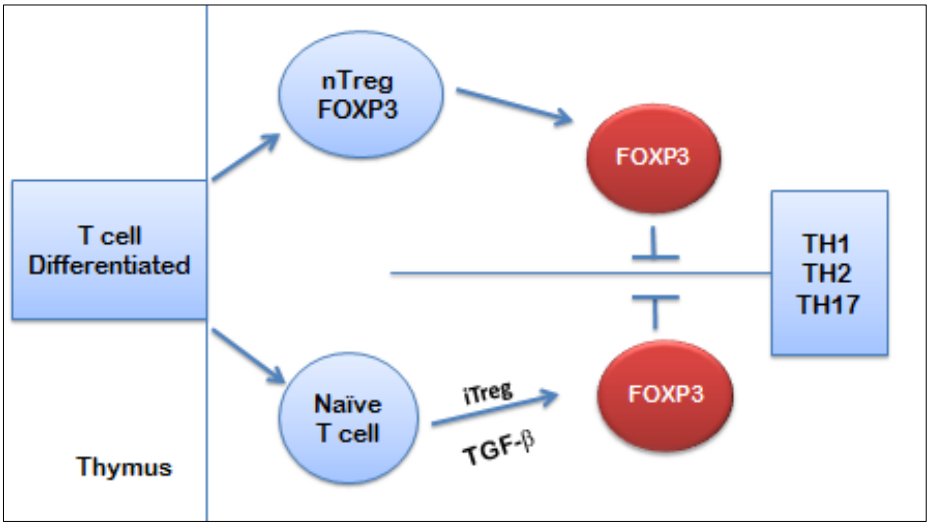

Figure 2-16: The development of natural Treg (nTreg) and adaptive Treg (iTreg) cells.

Another type of Treg cell is the adaptive or induced Treg cell (iTreg) that is a non-regulatory $CD4^+$ T cell that acquires CD25 (IL-2Rα) in circulation (Pastrana et al., 2012). TGF-β has a major role in the differentiation and expression of iTreg from naïve CD4. As anti-inflammatory cytokines, TGF-β and IL-2 enhance the expression of FOXP3 (nuclear transcription factor) and CD25 (α chain of IL-2 receptor) on Tregs (Wan and Flavell. (2007).

Table 2-3: Comparison between natural Treg and adaptive Treg cells (Lin, X. et al., 2013).

NATURAL Treg CELLS	ADAPTIVE (INDUCED) Treg CELLS
Induction in thymus	Induction in periphery
Not based on antigen-stimulation	Based on antigen-stimulation
IL-2 dependent	IL-2 dependent
High CD25 expression	Variable CD25 expression
Cytokine-independent regarding suppressor function (cell-to-cell contact)	Cytokine-dependent in terms of suppressor function (IL-10 and TGF-β)

2.5.7 The Role of GARP in HIV Infection

GARP is found on the surface of activated Treg cells, megakaryocytes and platelets where it acts as a receptor for TGF-β (Stockis et al., 2009). It is mostly expressed on activated natural Treg cells and not on induced Treg (iTreg) cells (in presence of TGF-β) (Wang et al., 2009). GARP expression on $CD4^+CD25^+$ Treg cells indicates the activation of FOXP3 Treg and this may explain its role as a suppressor. The involvement of GARP in Treg cell function is based on induction during TCR stimulation; Treg function is upregulated and controlled by FOXP3. Upon TCR stimulation, human effector $CD4^+CD25^-$ (T helper) can upregulate FOXP3 while ectopic GARP expression in antigen-specific TH cells also occurs (Wang et al., 2009). GARP is induced relatively early after TCR stimulation on $CD4^+CD25^{++}$ Treg cells (Probst-Kepper and Buer, 2010) and its interaction with TGF-β on the cell surface (as a receptor) provides further support for GARP's regulatory role in the immune response. Here TGF-β has a major role in maintaining and inducing FOXP3, which in turn is necessary for suppressive activity during HIV infection (Wang et al., 2009).

An important aspect to consider is how the immune system interacts with immune activation without suppressing the HIV-specific immune response; Treg cells are major contributors to this response. Thus GARP-mediated regulation of Treg cells is important in this instance. GARP is upregulated upon TCR stimulation and together with FOXP3 and TGF-β may help explain the phenomenon of chronic infection and the vicious cycle of immune activation. Here $CD4^+CD25^+$ may be induced by a direct viral infection or by chronic antigenic stimulation. Such activation leads to increased suppressive mechanisms and inhibition of the protective immune response mediated by T and B cells. This leads to chronic viremia and persistent immune activation (Paiardini and Muller. 2013; Keynan et al., 2008).

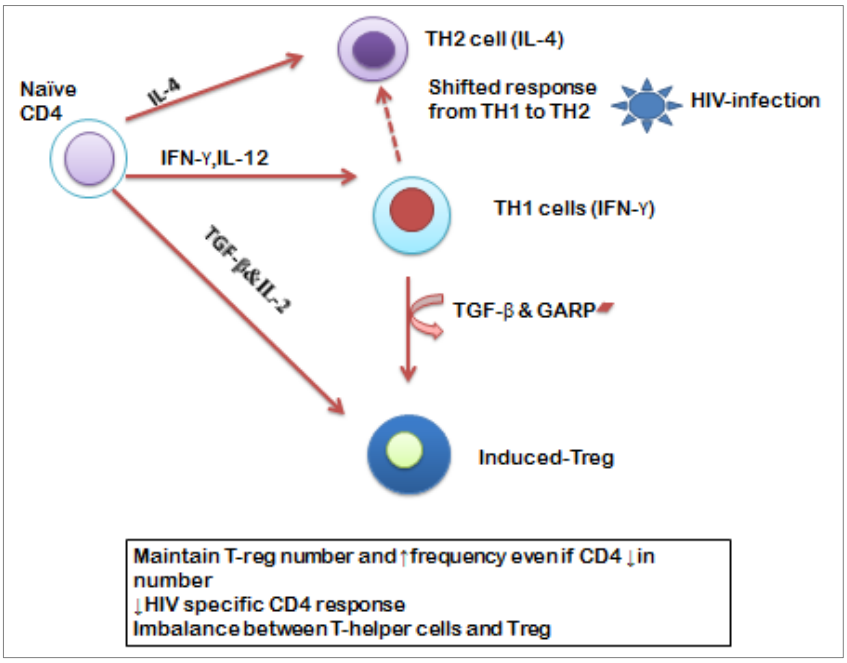

Figure 2-17: FIV infection : role of GARP and TGF- β in the transfer of TH1 to induced-Tregs (Miller et al., 2014).

Miller et al. (2014) demonstrated that GARP together with TGF-β can convert TH1 cells to Treg cells in an animal model of feline immunodeficiency virus (FIV) infection (Figure 2.15). This can explain the maintained Treg cell number and function during HIV infection, even with depletion of CD4. It also sheds light on the beneficial role of Treg cells in terms of a suppressor-type function. This can also help explain the immune dysfunction of CD4 T cells that effectively limits the functioning of the antiviral CD4 T cell response. Others found that soluble GARP protein (sGARP) efficiently repressed the proliferation of naïve $CD4^+$ T cells into effector T cells and that it was involved in the induction of Treg cells (Hahn et al., 2013). This work also showed the potent anti-inflammatory role of sGARP that prevents T cell-mediated destruction of graft-versus-host disease.

2.5.8 The Role of SATB1 as Activated Markers for Regulatory T Cells

SATB-1 is ~ 800 amino acids long and contains three DNA-binding domains. It has the ability to bind matrix attachment regions (MARs) that control gene expression in maturing T cells. SATB-1 was identified as a nuclear protein that organizes the chromatin network and regulates expression of several genes and a key modulator in the development and differentiation of effector T cells. In particular, TH2 cell (by regulation of GATA-3) promotes and induces TH2 cytokine IL-4 secretion (Gottimukkala et al., 2012). Furthermore, SATB-1 is strongly upregulated and expressed in T effector cells (Thabet et al., 2012). There is an inverse expression pattern between FOXP3 and SATB-1 in maintaining Treg identity and function (Gottimukkala et al., 2012). Beyer et al. (2011) showed that SATB-1 repression in Tregs is necessary for suppressive activity and the inhibition of effector T cell differentiation. This means that FOXP3 negatively regulates SATB-1. By contrast, lentivirus can upregulate SATB-1 in Treg cells thereby blunting the ability of Treg cells to suppress the activation of effector T cells. Such SATB-1 overexpression skews its regulatory function towards the effector program and numerous pro-inflammatory genes are thus activated thereby increasing IL-4 and IFN-γ production (Thabet et al., 2012).

2.6 ANTIRETROVIRAL THERAPY IN THE HIV INFECTED-POPULATION

Globally more than 35 million individuals are infected with HIV, with ~7 million currently in South Africa (World Health Organization. 2015). However, cART has been successfully rolled out over the last decade with ~17 million (50%) of the global infected population now receiving such treatment (UNAIDS, Report on the Global AIDS Epidemic, Geneva: UNAIDS, 2015). The introduction of cART has created significant changes by decreasing mortality among HIV-infected patients, prolonging life expectancy, suppressing HIV-1 replication, restoring immune function and reducing transmission. There are currently six cART classes that can be employed with each respective one targeting a particular process in the viral cycle:

1. Nucleoside reverse transcriptase inhibitors (NRTIs)
2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
3. Protease inhibitors (PIs)

4. Integrase inhibitors (IIs)
5. Fusion inhibitors (FIs)
6. Chemokine receptor antagonists (CRAs)

In the South African context, cART is usually initiated when CD4 counts are less than 350 cell/mL (Mberi et al., 2015) although in some cases it is initiated within one week of HIV diagnosis. The latter group can include pregnant women, individuals displaying CD4 counts of less than 200 cells/mL, patients exhibiting Stage 4 of HIV disease progression (WHO classification) and individuals suffering from co-morbidities such as tuberculosis (Arg et al., 2016).

For first line treatment the following regimen is usually employed: TDF (Tenofovir) + FTC (Emtricitabine) or 3TC (Lamivudine) + EFV (Efavirenz). If a patient does not respond to such treatment and VL remain relatively high (HIV RNA > 1, 000 copies) then the treatment is escalated to second line therapy, i.e. AZT (Zidovudine) + 3TC (Lamivudine) + LPV/r (Lopinavir/ Ritonavir). For third line treatment, the following regimen can be used: Raltegravir/ Darunavir/Etravirine (Arg et al., 2016). Recently, US clinics have begun to initiate cART when CD4 cell numbers fall below 500 cells/mL, while in middle- and low-income countries the counts were raised from 200 to 350 cell/mL (Fox et al., 2010). This should increase the number of HIV-positive individuals receiving treatment and improve the immunological state as CD4 T cell recovery is a slow process that requires years and also depends on CD4 T cell number prior to cART initiation (Wilson and Sereti. 2013).

Although the role of ART in suppressing disease onset in HIV-infected patients is a cornerstone of therapeutic strategies, complete immune recovery is needed to prevent immune activation and to restore immune function and thus limit the onset of non-AIDS-related co-morbidities such as CVD.

Treatment Strategies

The primary focus was initially to suppress HIV replication and to also manage drug toxicity and side-effects. However, the current emphasis is to improve the general health of the HIV-infected population and also how best to extend life spans to match that of non-infected individuals. The focus has therefore shifted to the management of immune activation/inflammation and is based on three parameters, namely: a) reducing T cell activation, b) decreasing cytokine production and c) improving the underlying risk factor profile. Here the various cART combinations are most effective to reduce T cell activation and to also decrease immune activation markers (Rajasuriar et al., 2013). Some researchers suggested that cART initiation during the early stages of infection could limit viral reservoirs and decrease immune activation to normal levels (Sellers et al., 2014). However, others found that cART initiation during the acute stages of HIV infection failed to prevent persistent immune activation (Vinkoor et al., 2013). These results suggest that the early pathogenesis of HIV disease leaves more permanent, detrimental effects on overall immune function.

As a result of such associated pathologies, anti-inflammatory agents and statins have also been employed as co-treatments, with the latter successful as it lowered harmful circulating lipid levels and decreasing CVD risk (Funderburg et al., 2014). Thus, it is clear that pharmacologic approaches such as cART cannot lead to the total eradication of this debilitating disease and/or cure individuals burdened with HIV infection. Thus efforts to develop novel therapeutic interventions to help combat HIV and associated co-morbidities should be continually pursued. For example, Younan et al. (2013) demonstrated that hematopoietic stem cell (HSC) transplantation may be a prospective alternative to conventional cART as treated animals demonstrated increased CD4 counts, a higher CD4/CD8 ratio and also improved CD4 T cell function. Such preclinical, pilot studies offer early hope that HIV-positive individuals will eventually receive improved therapies that should enhance their overall well-being and also blunt the onset of various co-morbidities such as CVD.

2.7 RESEARCH QUESTION, MOTIVATION, AIMS AND OBJECTIVE OF THE STUDY

Antiretroviral treatment prolongs and improves the quality of life of HIV-infected individuals by viral suppression and by decreasing HIV/AIDS complications. However, despite its efficacy full-scale immunological recovery is not achieved and low grade inflammation and immune activation persist and induce changes in immune cell function and phenotype. Such changes can in turn lead to an increased risk for CVD onset. Although some studies explored the phenomenon of on-going inflammation and immune activation with chronic HIV infection, simultaneous evaluation of changes to monocytes, CD8 T cells (CTL) and Treg cells and their relationship to CVD risk markers has not been previously performed. This will also be the first time – as far as we are aware – that such a study has been pursued within the South African context.

The hypothesis of this study is therefore that HIV-1 infection induces several changes in the phenotype and function of immune cells, particularly in the case of monocytes, CD8 T cells, CD4 T cells and Treg cells, and those such changes pre-dispose infected individuals to the future development of atherosclerosis and CVD.

The current project set out to answer the following research questions:

1. Does HIV infection affect monocyte subset expression and changes in phenotype?
2. Does HIV infection affect FOXP3 cells? In addition, what is the relationship between activation markers (GARP, SATB-1, and FOXP3) and disease progression?
3. What is the correlation between immune activation and coagulation markers in T cells?
4. What is the correlation between immune markers and metabolic profile?

The aims of this study were to assess: a) both coagulation and immune activation marker expression on monocytes and T cells (CD4 and CD8), b) expression of novel activation markers (SATB-1, GARP) on Tregs with chronic HIV infection and c) the relationship of immune activation markers to CVD risk.

To achieve these aims, the following objectives were set:

1. Assess monocyte subpopulation expression in both treatment-naïve and HIV-treated individuals - classic versus intermediate versus non-classic monocytes
2. Evaluate markers for TF (CD142) and CD38 expression on the different monocyte subsets
3. Determine CD38 and CD142 markers on CD8 and CD4 T cells
4. Evaluate and compare the frequency of (CD4+FOXP3+, GARP and SATB-1) in CD25+ and CD25- cells and their relationship with other markers
5. Correlate the above-mentioned immune markers with: a) well-known CVD risk markers (CRP, lipid profile) and b) markers of disease progression (CD4 and VL).

CHAPTER 3 METHODOLOGY

3.1 STUDY POPULATION AND DESIGN

This thesis forms part of a larger longitudinal investigation, namely the Cape Winelands HAART to HEART Study that is set within the Cape Winelands region of the Western Cape (South Africa). This particular thesis is a cross-sectional study that investigated the association between immune activation and the onset of cardiac diseases in HIV patients on cART. Ethical approval was obtained from the Human Research Ethics Committee of Stellenbosch University and the Department of Health (Western Cape Government, South Africa) reference number RP090/2013. Prior to the study, all participants were informed about various procedures and the consent form was signed by all study subjects included. After completion of informed consent, blood samples (fasted) were obtained from participants together with the completion of various evaluations (detailed later in this section). The inclusion criteria were HIV-positive and HIV-negative participants 18-55 years, while the HIV/tuberculosis infection patients were excluded from the study. The subject recruits were divided into three groups: HIV-negative subjects (N= 13), HIV-positive cART naïve (N= 26), and HIV-positive on cART (N= 41) (Figure 3.1).

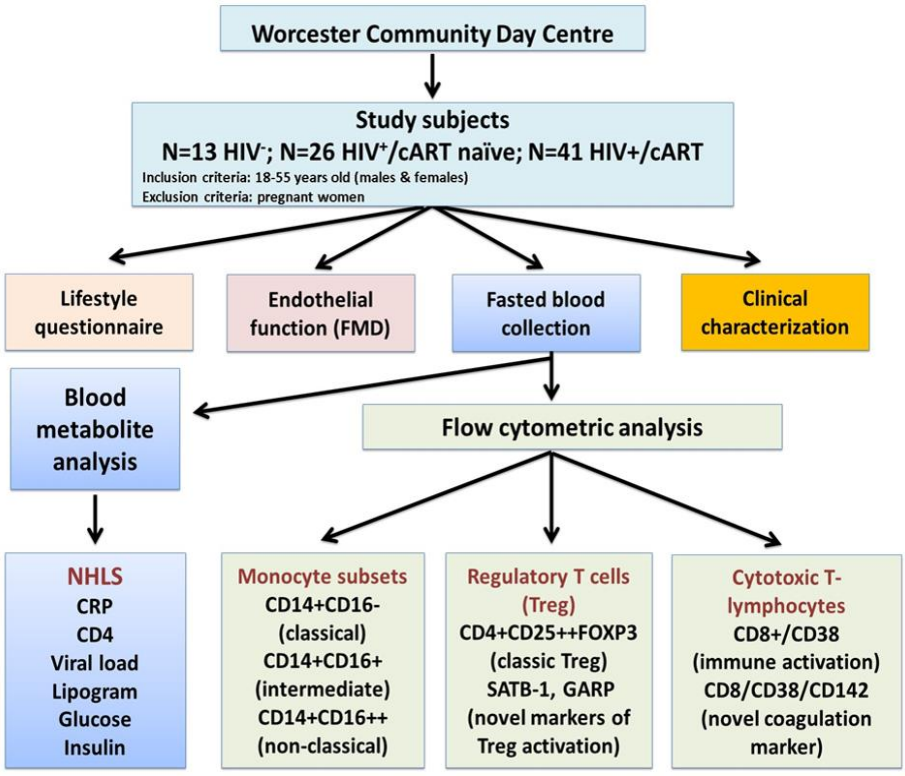

Figure 3-1: Layout of research methodology. Summary of study recruits and various assessments completed: flow mediated dilatation (FMD) for endothelial function assessment, analysis of blood metabolite profile and the assessment of immune activation

3.2 CLINICAL VISITATIONS

The study was conducted at Worcester Community Day Care Center (CDC) located within the Worcester Hospital (Western Cape, South Africa) that is a public referral facility that forms part of the metro and district health services. This facility also provides primary health care services and is responsible for treatment/ management of HIV-positive patients in the region.

3.2.1 Clinical History and Patient Characterization:

All HIV-positive and HIV-negative participants were recruited from the same communities (Worcester region, South Africa) to ensure that they shared similar CVD risk and socio-economic factors; the main history and characterization profile of participants were:

- Age
- Ethno-racial composition (Mixed Ancestry [‘’Colored’’], Caucasoid, Black)
- Duration of disease since HIV diagnosis
- History of cART - including duration, first or second line and regularity of treatment
- Viral load
- CD4 count
- Blood pressure
- CVD history (including family history)
- Record of any other drugs taken
- Information regarding smoking and diabetes mellitus
- Regularity of follow-ups

3.2.2 Blood Collection

Whole blood was collected by a research nurse and 5 tubes (20 mL) were collected from each patient. For CD4 counts, lipid profile and CRP evaluations, blood samples were sent to the nearby Worcester National Health Laboratory Service (NHLS, South Africa) laboratory within 30 minutes after collection. In addition, 1 mL of blood was collected into an EDTA tube and placed in a 15-mL polypropylene tube containing BD FACS lysing solution (BD Biosciences, San Jose CA) (1:9; lysing solution: distilled water) for the later evaluation of two flow cytometric panels (refer section 3.6.1.1).

Of the fresh whole blood samples collected, 1x EDTA-containing and 1x red-top (clotted blood) tubes were immediately centrifuged at 500–1000 *g* for 10 minutes (Biocom Biotech Centrifuge, Centurion South Africa). Plasma and serum were isolated and samples were subsequently stored at - 80°C until thawed for various analyses such as viral load (VL) assessments and lipoprotein sub-class assessments.

3.3 CD4 COUNT

TruCOUNT tubes (BD Biosciences, San Jose CA) together with MultiTESTTM CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC Reagent (BD Biosciences, San Jose CA) were used to perform CD4 counts. MultiTEST CD3-FITC/ CD8-PE/ CD45-PerCP/ CD4-APC reagent was added to the TruCOUNT tube followed by addition of 50 µL EDTA blood and gentle mixing. The samples were then incubated for 15 minutes at room temperature (in the dark); whereafter FACS lysing solution was added to the tube. This was followed by 15 minutes incubation at room temperature (in the dark), after which the cells were analyzed on a Becton Dickinson FACS Calibur four-color flow cytometer with designated software (BD Biosciences, San Jose CA). This is the standard CD4 count methodology employed by the Worcester NHLS (South Africa) laboratory and testing was done within 30 minutes after collection.

3.4 VIRAL LOAD (HIV-1 QUANTITATIVE ASSAY)

The evaluation of VL is an important assessment of HIV status and disease progression and here ~ 1 mL of frozen plasma (previously collected) was decanted into a 1.7 mL Axygen micro centrifuge tube (Axygen Inc, Union City CA) and the sample tested using the NucliSensEasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands). It is a quantitative test based on nucleic acid sequence based-technology with a combination of real-time molecular beacons and has a detection range of 350 to 10^6 copies/ml. These tests were completed at a SANAS-accredited laboratory based within the Division of Medical Virology, Faculty of Medicine and Health Sciences (Stellenbosch University, South Africa).

3.5 FLOW-MEDIATED DILATATION

Endothelial dysfunction represents an early event in atherosclerosis progression and FMD is a useful non-invasive test that can be employed in this instance. It is based on the ability of the endothelium to release nitric oxide (NO) in response to a stress stimulus (Solages et al., 2006). Here the brachial arterial diameter can be measured by ultrasound (high-resolution camera). This test initially evaluates the baseline brachial artery diameter (at rest) whereafter the maximum diameter is taken after 5 minutes of ischemia (pressure cuff placed around the forearm inflated to suprasystolic pressure for 5 minutes) once the blood pressure has been deflated (Figure 3.2). The subsequent vasodilation can be imaged and quantitated as an index of vasomotor function (Cornetti et al., 2002; Jarrete et al., 2016). The percent FMD reflects the percentage change in diameter and is an indicator of NO-mediated vasodilatation and endothelial function. Such procedures were performed (in parallel) at the Worcester CDC by one of our collaborators from the Division of Medical Physiology (Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa).

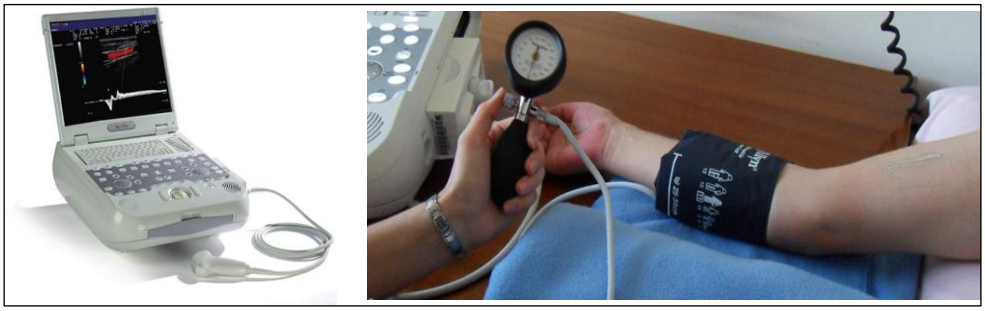

Figure 3-2: The ultrasound equipment used for FMD evaluation

3.6 CRP LEVELS:

CRP levels were measured by the immunoturbidmetric method, with relatively high levels defined as > 10 mg/L (Lau et al., 2006). Assays are based on the reaction between a soluble analyte and the corresponding antigen or antibody bound to polystyrene particles. For CRP quantification, particles consisting of a polystyrene core and a hydrophilic shell were used in order to covalently link anti-CRP antibodies. A dilute solution of test sample was mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP in the test sample will form an antigen-antibody complex with latex particles. Light scattering (measured by a nephelometric procedure after 6 min) is proportional to analyte concentration in the sample. An automatic blank subtraction was performed and CRP concentrations were calculated by using a calibration curve.

3.7 LIPID TESTS:

3.7.1 Traditional lipid tests

A Total cholesterol test:

Cholesterol is measured enzymatically in serum or plasma in a sequence of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3´-OH group of cholesterol (Hopkins. 2004). H_2O_2 is one of the reaction by-products and levels were quantified by a peroxidase-catalyzed reaction that produces a color. Absorbance was measured at 500 nm with color intensity being proportional to cholesterol levels.

B TG test:

Serum/plasma samples are exposed to a sequence of coupled reactions that result in triglycerides being hydrolyzed to produce glycerol (Hopkins. 2004; Fawwad et al., 2016). Glycerol is then oxidized using glycerol oxidase and a reaction by-product (H_2O_2) is measured as described above for cholesterol.

C HDL test:

HDL is measured directly in serum where the apoB-containing lipoproteins in specimens are reacted with a blocking reagent (Hopkins. 2004). This renders them non-reactive with the enzymatic cholesterol reagent

(under assay conditions). ApoB-containing lipoproteins are thus effectively excluded from the assay and only HDL-chol is detected. How measured though?

D LDL test:

Most of the circulating cholesterol is found in three major lipoprotein fractions: very low-density lipoproteins (VLDL), LDL and HDL.

[Total chol] = [VLDL-C] + [LDL-C] + [HDL-C]

LDL-cholesterol is calculated from the measured values of total cholesterol, triglycerides and HDL-cholesterol according to the relationship:

[LDL-C] = [Total C] - [HDL-C] - [TG]/5, where [TG]/5 is an estimate of VLDL-cholesterol (Fawwad et al., 2016). Values are expressed in mg/dL. Desirable levels of LDL-chol are below 130 mg/dL (converted to mmol/L by dividing values by 38.67) for adults and 110 mg/dL (converted to mmol/L by dividing values by 38.67) for children.

3.7.2 Lipoprint LDL System (lipoprotein sub-fractions)

Fasting blood sample was obtained from study recruits in the morning. Total cholesterol and triglycerides in serum were analyzed using the enzymatic CHOD-PAP method. This is an electrophoretic method that allows for the separation of lipoproteins on polyacrylamide gels by using the Lipoprint LDL System (Quantimetrix, Redondo Beach CA) (Dutheil et al., 2014). This method enables the analysis of 12 lipoprotein sub-fractions, i.e. VLDL, intermediate-density lipoprotein (IDL) 1-3, LDL 1-7 and HDL.

3.8 FLOW CYTOMETRY

We performed these studies at the CAF Fluorescent Imaging Unit at Stellenbosch University. Here we used the BD FACS Aria I cell sorter (BD FACS Aria I, BD Biosciences, and San Jose CA) and employed the following strategy (Figure 3.3):

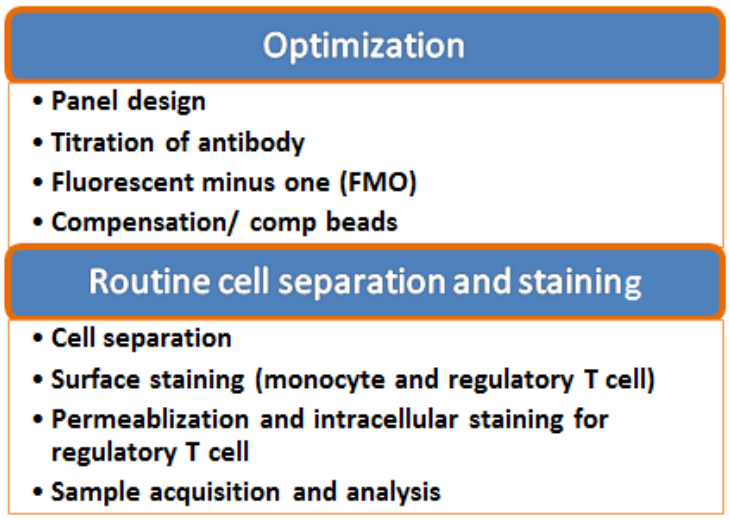

Figure 3-3: Flow diagram of flow cytometry protocol. This is a broad road map of the strategy employed and will be explained in detail for the rest of this chapter.

3.8.1 Optimization of Flow Cytometry Set-up

A Panel design and gating strategy for flow cytometry work

Multicolor flow cytometry is a powerful tool to detect and analyze multiple parameters at the cellular level. However, quality of the results depends on proper panel design and optimization of the instrument set up and here we employed the following panels (Table 3.1):

Panel #1: immune activation and coagulation markers on monocyte and lymphocyte T cells:
CD3, CD8, CD38, CD142, CD14, CD16.

Panel #2: regulatory T cell markers:
CD3, CD4, CD25, FOXP3, GARP, SATB-1.

Table 3-1: Antibody panels employed for flow cytometric analyses

MARKERS		
Immune activation	Monocyte	Regulatory T cell
CD3 APC-H7	CD3 APC-H7	CD3 APC-H7
CD38 PE	CD16 PE-CY7	CD4 FITC
CD8 BV 421	CD14 PE CF 594	CD25 PE-CY7
CD142 APC (HTF)	CD142 APC (HTF)	FOXP3 HU V450
	CD38 PE	GARP PE
		SATB-1 ALEXA fluor 647

Gating strategy for CD8 activation markers:

One hundred thousand events were collected for each tube and cells were gated according to lymphocyte population. The side scatter (SSC) was plotted against forward scatter (FSC) to identify and differentiate lymphocytes. T cells were extracted from lymphocytes by plotting CD3 APC-H7 Vs SSCA, while the doublets were excluded using forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). We then identified the $CD3^{+}CD8^{+}$ cells and determined the expression of CD38 and CD142 on $CD8^{+}$ T cells (refer Figure 3.4 for flow cytometry gating strategy).

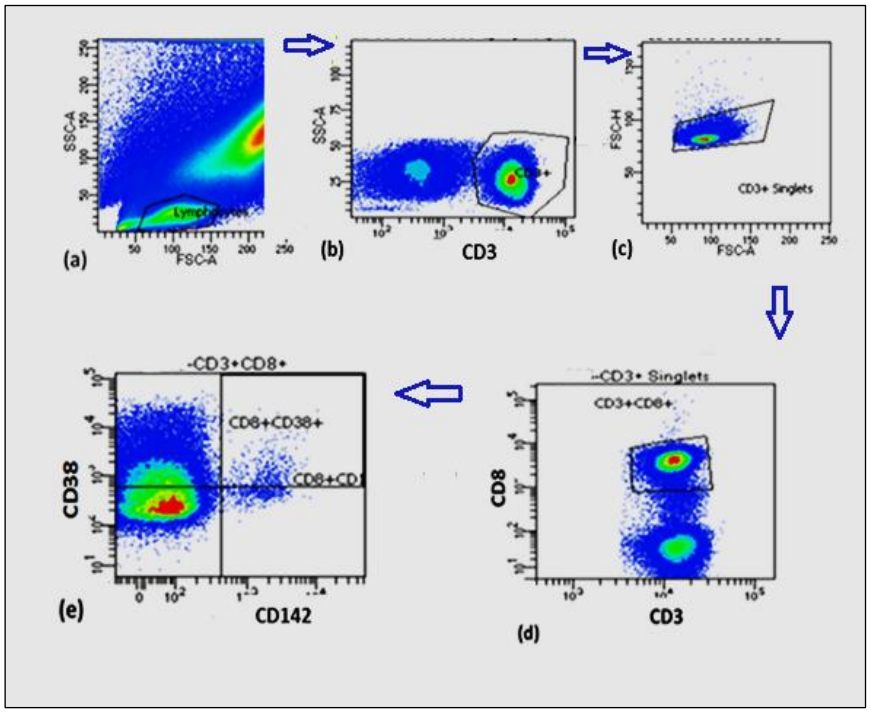

Figure 3-4: Flow cytometry-gating strategy to determine expression of CD38 and CD142 on $CD8^{+}$ T cells. (a) SSC versus FSC to identify lymphocytes; b) Dot plot shows SSC versus CD3 APC-H7 to identify T lymphocytes; c) CD3 singlets dot plot - FSC-height versus FSC-area; d) CD8 BV421 versus CD3 APC-CY7; and e) Expression of CD38 andCD142 on $CD8^{+}$ T cells.

Monocyte sub-population

The identification of monocyte sub-populations was based on the expression of CD14 and CD16 markers. Furthermore, fluorescent minus one (FMO) for CD16 was used to differentiate between $CD16^+$ positive and $CD16^-$ negative populations. The antibodies employed to stain for the monocyte panel included: APC-H7 mouse anti-human CD3, BV412 mouse anti-human CD8 and PE-CY 7 mouse anti-human CD16 –refer Figure 3.4 for flow cytometry gating strategy.

Gating strategy for monocyte panel

We began with CD14 versus SSC to identify (separate) individual monocyte populations. The single cell monocyte (singlets) was generated using FSC-H and FSC-A. This was followed by $CD14^+CD3^-$ versus SSC to ensure that the $CD3^+$ population was excluded from the monocyte population. Lastly, we performed $CD16^+$ versus SSC (Figure 3.5).

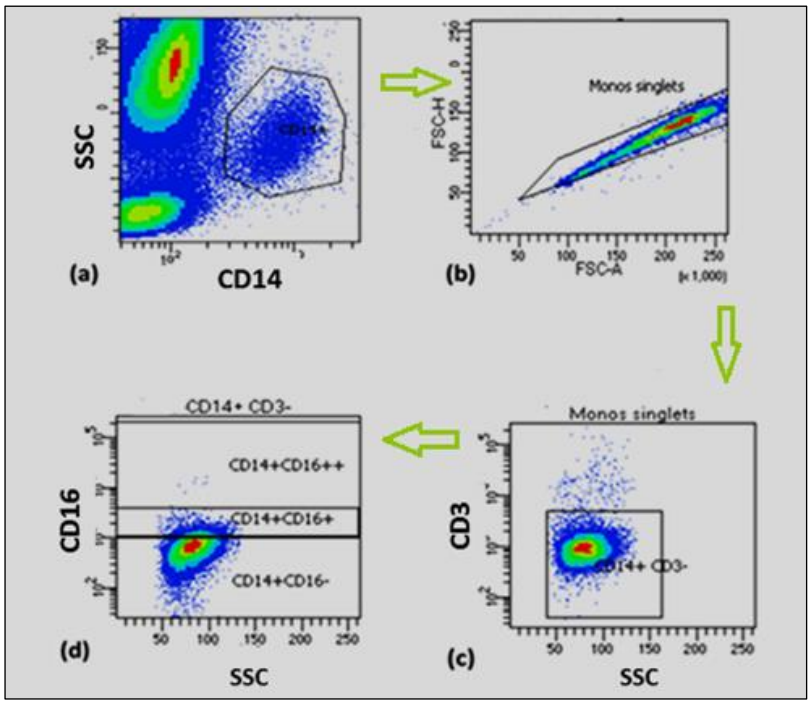

Figure 3-5: The gating strategy used to acquire monocyte subsets. (a) SSC versus CD14; (b) Monocyte singlet for CD14, FSC-H versus FSC-A; (c) Gate for CD14+ and CD3- (SSC) versus CD3 APC-CY7 to exclude CD3 positive cells from monocyte subsets; (d) CD16 PE-CY7 vs SSC

Treg cells

The identification of Treg cells was based on the expression of classical Treg $CD3^+CD4^+CD25^{++}FOXP3^+$ and the identification of activated markers (GARP, SATB-1) on Treg cells. For this panel, the gating strategy was based on the expression of $CD4^+$ $FOXP3^+$ cells and $CD4^+$ CD25 variability ($CD25^-$, $CD25^+$ and $CD25^{++}$). We assessed both intra- and extracellular markers for Treg cell evaluation. Extracellular markers included APC-H7 mouse anti-human CD3, FITC mouse anti-human CD4, PE CY7 anti-human CD25 and

PE mouse anti-human GARP. For intracellular marker assessment, additional steps were required as detailed in section 3.6.2.

Gating strategy for Treg cells

We started with SSC versus FCS to gate the lymphocyte population, then CD3 single cell (singlet) using FCS-H versus FCS-A, followed by CD3APC-CY7 versus FCS and CD4 FITC versus FCS. For Treg identification and differentiation, $CD4^{+}FOXP3^{+}$ (V450) was evaluated versus CD25 PE-CY7 variable ($CD25^{++}$, $CD25^{+}$ and in $CD25^{-}$) (Figure 3.6).

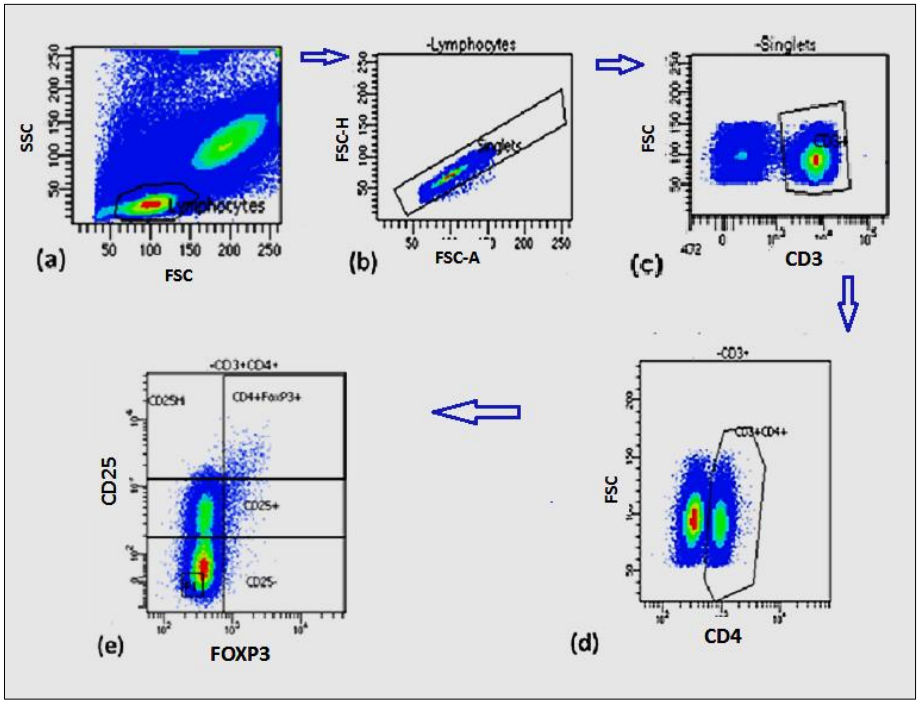

Figure 3-6: Representative flow cytometry dot plots demonstrating Treg cells gating. (a) SSC versus FSC to gate lymphocytes; (b) FCS-H versus FCS-A for CD3 singlets; (c) CD3 versus FSC; (d) CD4 versus FSC; (e) $CD4^{+}$ $FOXP3^{+}$ versus $CD4^{+}$ CD25 variable ($CD4^{+}CD25^{-}$, $CD4^{+}CD25^{+,}$ $CD4^{+}CD25^{++}$) was used to identify and differentiate Treg cells.

B Antibody titration

Titrations were done to determine optimal volumes that generate a bright enough signal to allow for precise measurement of antibody flouresence. Furthermore, this also minimizes non-specific binding and also ensures that the most cost-effective approach is employed. The first titration experiment was performed with the highest recommended antibody volume (manufacturer instructions), whereafter it was titrated downwards (doubling dilution). For example, for CD25 PE-CY7 we started with the recommended 5 µL volume in tube #1 that was followed by 2.5 µL, 1.25 µL and 0.625 µL, respectively, for the next set of tubes. During titrations, various factors may influence this procedure, e.g. antibody concentration, time of incubation,

temperature and the number of cells. Thus to ensure accurate results all titrations should be performed under similar conditions, see (table 3.2) for titrated antibody volumes, manufacturer information.

Titration of CD8 activation markers ($CD3^+$, $CD8^+$, $CD38^+$)

Titrations were started after washing of cells. Here four tubes were prepared with different antibody concentrations, e.g. for CD3 APC-H7 we started with the recommended volume of 5 µL /test in tube #1, followed by 2.5 µL, 1.25 µL and 0.625 µL, respectively, for the next set of tubes. A similar procedure was adopted for CD8 BV421 titration. However, for CD38 PE the titration started with 20 µL in the first tube, followed by 10 µL, 5 µL and 2.5 µL, respectively, in the next set of tubes. Subsequently, 100 µL of cells (after thawing and washing steps) were added to each respective tube. Samples were then incubated for 30 minutes at room temperature (in the dark), where after 2 mL of PBS was added to each sample. Samples were then centrifuged at 500 *g* for 5 minutes (Lab Centrifuge, Biocom Biotech, Centurion, South Africa) where after the supernatant was decanted and the pellet re-suspended in 300–400 µL PBS. Samples were thereafter stored at 4°C and analysed by flow cytometry within 24 hours.

Titration of monocyte activation markers

Four tubes were labelled with different volumes, i.e. 5 µL, 2.5 µL, 1.25 µL and 0.625 µL. For CD3 APC-H7, CD14 PE-CF594, CD16 PE-CY7 and CD142 APC each antibody titration started with 5 µL (as recommended) and then titrated downwards, i.e. 2.5 µL, 1.25 µL and 0.625 µL, respectively. After cells were thawed and centrifuged, 100 µL aliquots were added to the respective tubes (with antibody dilutions) and incubated for 30 minutes in the dark at room temperature. Subsequently, 2 mL PBS was added and samples centrifuged at 500 *g* for 5 minutes (Lab Centrifuge, Biocom Biotech, and Centurion, South Africa). The supernatant was then removed and the pellet re-suspended in 0.3 mL PBS. Samples were stored at 4°C and analysed by flow cytometry within 24 hours.

Titration of regulatory T cell activation markers

For this panel titrations were done for surface and intracellular markers. Here four tubes were labelled with different volumes, e.g. for CD25 PE-CY7 we started with the recommended volume (5 µL) followed by downward titration as before.

After the staining, incubation and washing of such samples, data were acquired using the BD FACS Aria (BD Biosciences, San Jose CA) and post-acquisition analysis was performed using FACS Diva software (BD Biosciences, San Jose CA). Thus we generated a histogram for each antibody titration (Figure 3.7). Here both negative and positive populations were identified for each antibody. For example, peaks on the right side of the histogram represent the positive population. Population statistics of the histogram were generated and the values were exported to excel sheets (for each antibody) and used for a standard curve. Additionally, the staining index (SI) value for each titration was calculated using the equation 3-1 (Telford et al., 2009):

$$SI = \left(\frac{\mathbf{MFI_{pod}} - \mathbf{MFI_{neg}}}{\mathbf{SD_{neg}}} \right) \quad (3\text{-}1)$$

The equation used to calculate the staining index and to generate the titration curve. MFI= mean fluorescence intensity, SD= standard deviation.

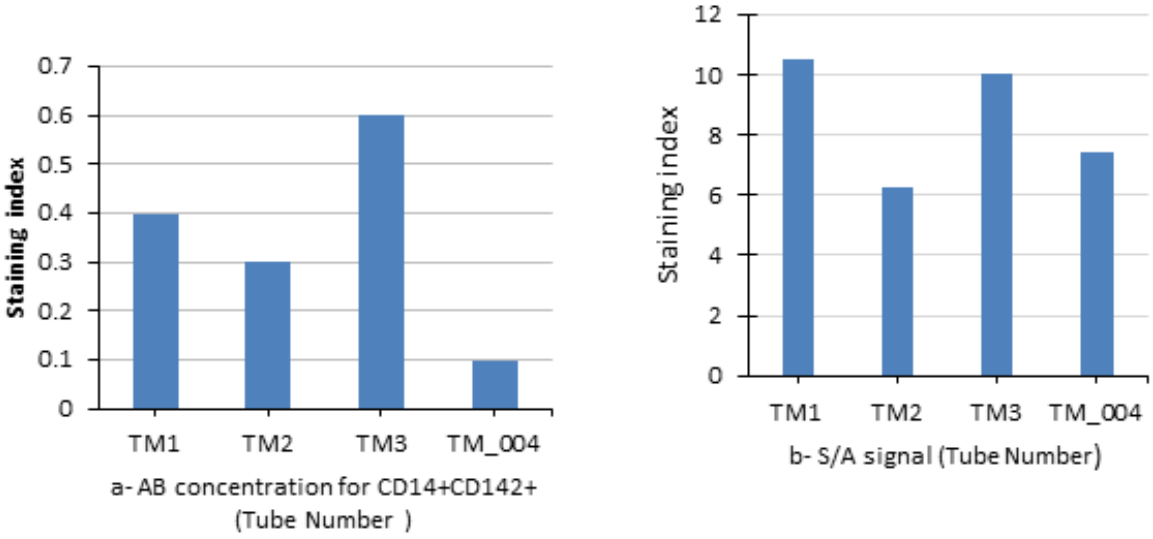

Figure 3-7: Staining index and different antibody concentrations for antibody titration. (a) four dilutions (TM1 - 5 µL; TM2 – 2.5 µL; TM3 – 1.25 µL; and TM4 - 0.625 µL) to obtain optimal concentration for TF (CD142). a) Staining for CD142$^+$ using different dilutions; and (b) Brightness of signal. Optimal concentrations were determined according to relatively high SI values with a value that was roughly less than half the recommended concentration. Thus the titration provides an intense signal to be detected with a relatively low antibody concentration. TM: Tube Monocyte (label for the different sample tubes).

Table 3-2: Titrated antibody volumes, manufacturer information and isotypes employed.

Antibodies	Manufacture	Volume (µL)	Isotype (clone)
CD8 BV 421	BD Biosciences, San Jose CA	2 µL	Ms IgG1, κ (RPA-T8)
CD142 APC (HTF)	Bio Legend Inc. , San Diego CA	2 µL	Ms IgG1, κ (NY2)
CD14 PE CF 594	BD Biosciences, San Jose CA	4 µL	Ms (BALB/c) IgG2b,κ(MϕP9)
CD4 FITC	BD Biosciences, San Jose CA	8 µL	Ms IgG1, κ (RPA-T4)
CD3 APC-H7	BD Biosciences, San Jose CA	3 µL	Ms (BALB/c) IgG1, κ (leu-4)
CD16 PE-CY7	BD Biosciences, San Jose CA	3 µL	Ms IgG1, κ (3G8)
FOXP3 HU V450	BD Biosciences, San Jose CA	3 µL	Ms IgG1 (259D/C7)
SATB-1 ALEXA fluor 647	BD Biosciences, San Jose CA	3 µL	Ms IgG1 (14/SATB1)
GARP PE	BD Biosciences, San Jose CA	3 µL	Ms IgG2, κ (7B11)
CD25 PE-CY7	BD Biosciences, San Jose CA	2 µL	N/A
CD38 PE	BD Biosciences, San Jose CA	5 µL	N/A

C Flow cytometry color compensation

Multiple staining flow cytometry has the advantage of identifying and characterizing very low cell frequencies in the periphery. However, a major problem with this method is the overlap between fluorescent dyes. Thus where emission spectra of flourchromes may overlap, compensation is made to limit spill-over of fluorescent signals from one detector channel to another. Some fluorochromes can also be excited by more than one laser and thereby contribute to spillover. The main goal of compensation is therefore to eliminate spillover, especially when considering the technical limitation of tandem dyes, which are susceptible to damage upon exposure to light or high temperatures.

Procedures:

BD Comp beads (BD Biosciences, San Jose CA) were vortexed before usage. Falcon tubes (BD Biosciences, San Jose CA) were labeled for each respective fluorochrome antibody and 100 µL of staining buffer added per sample. BD comp beads for negative and positive controls were then vortexed. One full drop (~60 µL) of BD comp beads (negative control) and one full drop (~60 µL) of BD comp beads (positive; anti-mouse Igκ) were added to each respective tube. This was followed by a vortexing step. Subsequently, the titrated antibody volume was added to labeled tubes, followed by vortexing and incubation for 30 minutes in the dark at room temperature. Thereafter we added 0.5 mL staining buffer to each tube. This procedure was completed to ensure that the instrument is indeed functioning optimally – thus a control step for the instrument (Figure 3.8).

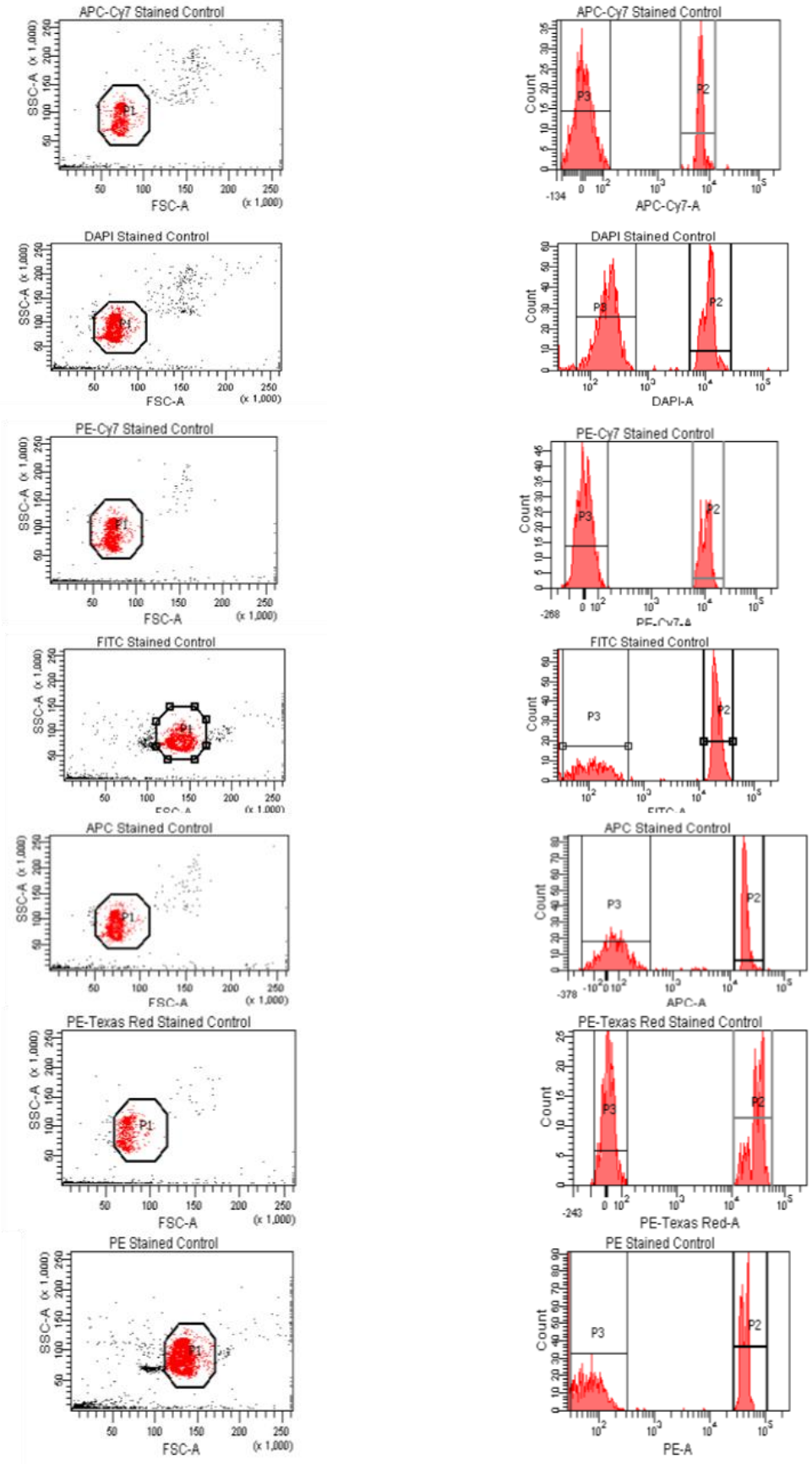

Figure 3-8: Instrumental set up by comp beads compensation. Compensation controls must consist of a negative and a positive population for each single color.

D Fluorescence minus one (FMO)

The gating control was an additional parameter required for optimization. Fluorescence minus one technology was performed in order to differentiate between positive and negative events. This procedure involves the addition of all fluorescent reagents to a cell sample except for one. It is an important procedure especially for antibody markers where positive signals are expressed at relatively low levels, e.g. GARP and SATB-1. It is also necessary to differentiate between positive and negative populations, e.g. for CD16 where 11 antibody colors were employed in an experiment.

Procedure:

Eleven FMO tubes were prepared, i.e. each tube with all antibodies except the antibody that the respective tube was labeled with. After the appropriate antibodies were added to the respective tubes, 100 µL of FACS-lysing blood was added and tubes gently mixed and incubated for 30 minutes in the dark at room temperature. The staining protocol used during the antibody titration experiment was detailed earlier and was followed for cellular surface and intracellular markers; a) Treg panels; b) activation markers; and c) monocyte panels (Addendum A Figures).

3.8.2 Routine Cell Separation and Cell Staining

We usually completed 10 blood samples at a time and employed stained as indicated (Table 3.3).

Table 3-3: Composition of three flow cytometric panels used and placement of the 11 antibodies into the relevant panels

Immune activation markers	Monocyte population markers	Regulatory T cell markers
CD3 APC-H7	CD3 APC-H7	CD3 APC-H7
CD38 PE	CD16 PE-CY7	CD4 FITC
CD8 BV 421	CD14 PE CF 594	CD25 PE-CY7
CD142 APC (HTF)	CD142 APC (HTF)	FOXP3 HU V450
	CD38 PE	GARP PE
		SATB-1 ALEXA fluor 647

Where: **APC**: Allophycoyanin; **PE**:Phycoerythrin; **FITC**:Fluorescein isothiocyanate; **APC-H7:**Allophycocyanin-Hilite®7; **PE-Cy7**:Phycoerythrin-cyanine 7; **BV421**:Brilliant violet 421; **SATB-1** Alexa Fluor 647: Special AT-rich sequence-binding protein 1; **GARP**: Glycoprotein A repetition predominant; **FOXP3 HU V450**: Fork head boxp3 human Violet 450.

Equipment

a. 15 mL polypropylene tube (BD Biosciences, San Jose CA)
b. 12 × 75 mm polyester Falcon tube (BD Biosciences, San Jose CA)
c. Vortex mixer
d. Centrifuge (Lab Centrifuge, Biocom Biotech, Centurion, South Africa).

e. Flow cytometry (BD FACS Aria, BD Biosciences, San Jose CA)

Reagents:

i. PBS phosphate buffer solution
ii. BD FACS-lysing solution (1 from fix-lysing: 10 water) (BD Biosciences, San Jose CA)
iii. FOXP3 buffer set (BD Biosciences, San Jose CA)
iv. Wash buffer (PBS with 0.5% BSA and 0.1% sodium azide).

Preparation of Antibody Staining Mixture

Antibody panels consisted of both cellular surface and intracellular markers. For monocyte and activation panels the antibody staining mixture was prepared for surface staining for 10 samples. For Treg cells the surface and intracellular staining protocol was prepared for 10 samples (refer Tables 3.4, 3.5 and 3.6).

Table 3-4: Monocyte staining mix panel for 10 samples.

AB	**µL/test**	**×12 (total/10 patients)**
CD3 APC-H7	3 µL	36 µL
CD8 BV421	2 µL	24 µL
CD38 PE	5 µL	60 µL
CD14 PECF594	4 µL	48 µL
CD16 PE-CY7	3 µL	36 µL
CD142 APC (HTF)	2 µL	24 µL
Buffer (PBS)	1 µL	12 µL
Total	20µL	240µL

Table 3-5: Treg surface staining mix for 10 samples.

AB	µL/test	(Total /10 patient)×12
CD3 APC-H7	3 µL	36 µL
CD4 FITC	8 µL	96 µL
CD25 PE-CY7	2 µL	24 µL
GARP PE	3 µL	36 µL
Buffer (PBS)	4 µL	48 µL
Total	20	240µL

Table 3-6: Treg Intracellular staining mix for 10 samples.

AB	µL/test	Total for 10 patient ×12
SATB- Alexa fluor 6471	3 µL	36 µL
FOXP3 HV450	3 µL	36 µL
Buffer (PBS)	4 µL	48 µL
Total	10µL	120µL

A Cell preparation

1 mL of blood from EDTA tube was collected in BD FACS lysing solution in a 15-mL polypropylene tube (BD Biosciences, San Jose CA). After mixing, the sample was allowed to stand for 10 minutes and then stored at - 80°C until the time of staining. The frozen sample was thawed at 37°C and then centrifuged at 400–500 *g* for 10 minutes (Lab Centrifuge, Biocom Biotech, Centurion, South Africa). The supernatant was then decanted and cells twice washed with PBS. We then added 100 µL of cells to two Falcon tubes (BD Biosciences, San Jose CA), respectively. Here tube #1 was prepared for a monocyte and CD8 T cell activation markers panel, while tube #2 was prepared for a Treg markers panel. The appropriate amount of surface staining reagents was then added to the respective tubes.

B Flow cytometry routine panel and acquisition

Monocyte panel with immune activation

Monocytes and T cells were identified by size and granularity. Monocytes were distinguished by expression of CD14 and CD16, while T-lymphocytes were identified by CD3 and CD8. Cell surface molecule expression was studied by staining cells with the following flourochrome-labeled antibodies: APC-H7 mouse anti-human CD3, BV412 mouse anti-human CD8, PE-CY 7 mouse anti-human CD16, PE-CF594 mouse anti-human CD14, APC anti- human CD142 (TF) and PE anti-CD38 (refer Figure 3.5. for gating strategy). Immune activation was measured using anti-CD38 PE, anti-CD3 APC-H7, anti-CD8 BV412 and TF CD142 (refer Figure 3.4).

After thawing frozen samples at 37°C, it was centrifuged at 500 *g* for 10 minutes (Lab Centrifuge, Biocom Biotech, Centurion, South Africa). The supernatant was thereafter decanted and the pellet re-suspended in 2 mL PBS. Cells were thereafter washed (2x) and then placed in a Falcon tube (BD Biosciences, San Jose CA). The appropriate antibody panel (titrated) was then added to the respective tubes (CD3, CD14, CD16, CD142, CD8, and CD38). This was followed by 30 minutes incubation at room temperature, whereafter cells were washed with 2 mL PBS. Samples were then centrifuged at 500 *g* for 5 minutes (Lab Centrifuge, Biocom Biotech, Centurion, South Africa), re-suspended in 0.3 mL PBS and stored at 4°C until flow cytometric analysis was performed within 24 hours.

Regulatory T cell panel

Here identification is based on expression of classical Treg markers (CD4+CD25+FOXP3+) and the identification of activated markers (GARP, SATB-1). This panel contains both extracellular surface and intracellular markers for antibody staining. We started with extracellular staining steps (as described before) with APC-H7 mouse anti-human CD3, FITC mouse anti-human CD4, PE CY7 anti-human CD25 and PE mouse anti-human GARP.

Intracellular markers antibody staining- panel 2

We included a permeabilization step for intracellular antibody staining (V450 mouse anti-human FOXP3 and Alexa Fluor 647 mouse anti SATB-1), where after standard staining procedures were followed as for the extracellular experiments (for CD3, CD4, GARP and CD25). For the permeabilization step (for intracellular staining of SATB-1 and FOXP3) the FOXP3 buffer set was employed (Human FOXP3 buffer set, BD Biosciences, San Jose CA). Here cells were re-suspended in wash buffer and then 0.5 mL of Buffer C (Human FOXP3 buffer set, BD Biosciences, San Jose CA) was added to each tube. After vortexing the samples were incubated for 30 minutes at room temp in the dark (some antibodies photosensitive), thereafter cells were washed with 2 mL of PBS, followed by centrifugation at 500 *g* for 5 minutes (Lab Centrifuge, Biocom Biotech, Centurion, South Africa). The wash buffer was then removed and the pellet washed for a second time. Subsequently, titrated concentrations of both FOXP3 and SATB-1 were added to the cells together with diluted PBS. With some gentle shaking this allowed for the re-suspension of the pellet. It was thereafter incubated for 30 minutes in the dark (room temperature). After incubation, the cells were washed and re-suspended in 0.3 mL wash buffer and analysed by flow cytometry within 24 hours.

3.9 SAMPLE ACQUISITION AND DATA ANALYSIS

The samples were acquired using the BD FACS Aria flow cytometer (BD Biosciences, San Jose CA) and analysed using FACS Diva software (BD Biosciences, San Jose CA). One hundred thousand events were collected per sample tube and expression for each marker determined based on total gated events. Data were exported from the FACS Diva software (BD Biosciences, San Jose CA) to an Excel spreadsheet to allow for further analyses.

3.10 STATISTICAL ANALYSIS

The percentage data were exported to an Excel spreadsheet and statistical analysis performed using Graph Pad Prism (Version 5, Graph Pad Software, San Diego CA). Here all data were derived from the parent percentage, except GARP and SATB-1 (from grandparent percentage). One-way ANOVA analyses were performed and comparisons made between four study groups distinguished by CD4 count (control, CD4 count > 500, CD4 count 200-500, and CD4 count < 200). In addition, we also compared data for control versus naïve versus HIV-treated groups. Correlations between the variables were also performed using the Spearman's correlation. All statistical analyses were performed under supervision of a qualified statistician – Prof. Daan Nel (Department of Statistical Sciences, Stellenbosch University). The statistical significance of results was determined by the respective P value obtained: if $P \leq 0.05$, the result is significant; for P value between 0.05 and 0.005, the result is highly significant; and for $P < 0.005$ the result is extremely significant. Data are reported as mean percentage ± SD (standard deviation).

CHAPTER 4 RESULTS

All the data presented in this chapter show the mean percentage of markers ± standard deviation (SD). One way-ANOVA was performed on expression markers to compare different study groups, while student t-test analyses were also performed to compare between two specific groups.

The Spearman correlation test was performed and such correlations are presented with linear regression XY data, together with significant coefficient r- and p-values. In this instance, all data are presented with standard error of the mean (SEM).

4.1 PATIENT DEMOGRAPHICS

A total of 80 participants were recruited for this study. This comprised a control group of 13 HIV-negative persons and 67 HIV-positive individuals (N=21 on first line treatment and N=20 on second line treatment); the age range the participants varied from 18 to 55, with a mean age of ~38 years. Here 64% (N=51) were females while males comprised the rest (Table 4.1). An evaluation of HIV-positive individuals using the WHO staging classification showed that 65% can be categorized into stage 1 (N=44), 11% into stage 2 (N=7), 15% (N=10) into stage 3 and 9% into stage 4 (N=6). Data collected from patient histories revealed that 51% of the HIV-positive participants (N=34) exhibited a family history of CVD while 59% were smokers (N=47).

Table 4-1: Demographics for the different study groups

Variable	(HIV-) control	(HIV+) naïve	HIV treated 1st line	HIV treated 2nd line	p-value
Number of donor	13	26	21	20	
Age (years) (mean ± SD) Median	45± 10 50	35 ±8 36	36± 7 37	38± 11 36	0.04
SBP (mmHg) (mean ± SD) Median	126± 12 120	118 ±14 120	125± 17 130	121±19 115	N/S
DBP (mmHg) (mean ± SD) Median	78± 6 80	73 ± 7 75	78± 6 80	71±10 70	0.03
HR (minutes) (mean ± SD) Median	77±8 80	76 ±7 80	75±7 80	74±7 75	N/S
CD4nadir (cells/µL) (mean ± SD) median	N/A	553 ±280 510	203±163 195	135±150 86	<0.01
CD4 count (cells/µL) (mean ± SD) Median	N/A	548 ±333 503	378±295 332	331±270 278	0.02
HIV/DX (month) (mean ± SD) Median	N/A	49 ±37 36	66 ± 40 63	104±45	<0.01
Time cART (month) mean ± SD Median	N/A	N/A	49 ± 31 48	78 ± 45 68	0.03
Smoking (number cigarettes per week: mean ± SD Median	0.6 ± 0.5 1	0.8 ± 0.3 1	0.5 ± 0.5 1	0.2 ± 0.4 0	<0.01

N/A - not applicable; **N/S** - not significant; **DBP** - diastolic blood pressure; **SBP** - systolic blood pressure; **HR** - heart rate; **CD4 nadir** - the lowest CD4 count; **HIV/DX** - time of HIV diagnosis; **Time/cART** - time since starting cART.

4.2 CD4 COUNT

HIV-positive participants were randomly selected and displayed CD4 counts ranging from 3–1, 800 cells/µL. For the HIV-naïve group the mean CD4 count was 548 ± 333 (median = 503 cells/µL), while for the HIV-treated group the mean CD4 count was 354 ± 280 (median = 300 cells/µL) (P = 0.006). When sub-dividing the HIV-positive patients into groups based on CD4 count, mean CD4 counts of the HIV-positive groups with CD4 > 500, CD4 count (200–500) and CD4 count < 200 were 756 ± 219, 349 ± 73 and 127± 76 cells/µL, respectively (Figure 4.1).

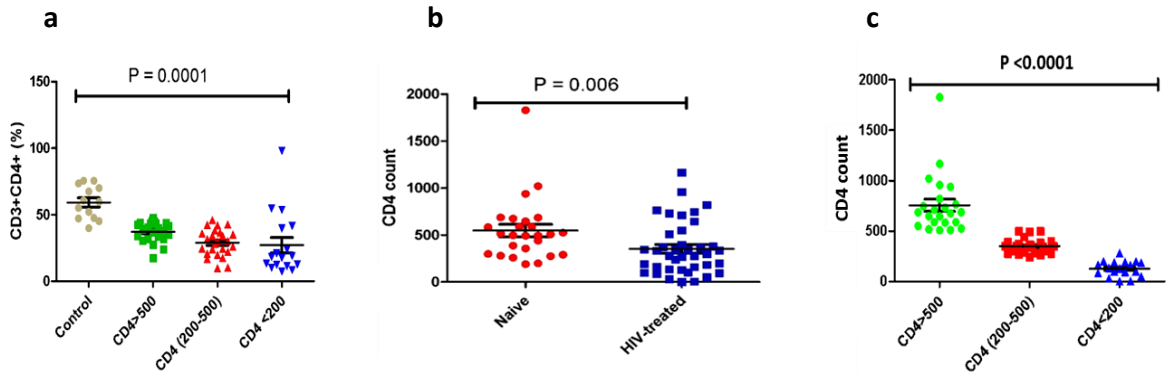

Figure 4-1: Summary of CD4 counts. (a) Expression of the percentage $CD3^+CD4^+$ between control and HIV positive individuals with varying CD4 counts (P=0.0001); (b) Comparison of CD4 counts between naïve and HIV-treated patients receiving cART (548 ± 333 vs. 354 ± 280; P=0.006); (c) HIV-positive subgroups constituted on the basis of CD4 counts (P<0.0001) (data presented as mean ± SEM).

Correlation analyses between CD4 counts and various immunological markers revealed that CD4 is negatively correlated with several of these markers (Table 4.2).

Table 4-2: Correlation between CD4 counts and various immunological markers

CD4 count versus;	R-value	p-value
$CD8^+CD38^+$ (%)	-0.34	0.005
$CD8^+CD142^+$ (%)	-0.29	0.02
$CD8^+142^+38+$ (%)	-0.37	0.001
$CD4^+FOXP3^+$ (%)	-0.52	0.0001
$CD25^{++}FOXP3^+SATB\text{-}1^+$ (%)	-0.03	0.7
$CD25^{++}GARP^+$ (%)	-0.44	0.0001
VL (C/mL)	-0.45	0.001

4.3 VIRAL LOAD

The mean VL of the HIV-positive group was 53, 532 copies/mL (median = 5, 675 copies/mL). For the three groups, i.e. CD4 count > 500, CD4 count (200-500) and CD4 count < 200, mean VL were 11.440 ± 31.5;

329 ± 431 and 527.300 ± 756 copies/mL, respectively (Figure 4.2). Moreover, the Spearman correlation performed between VL and CD4 counts showed a negative relationship (r = - 0.45; p-value = 0.001).

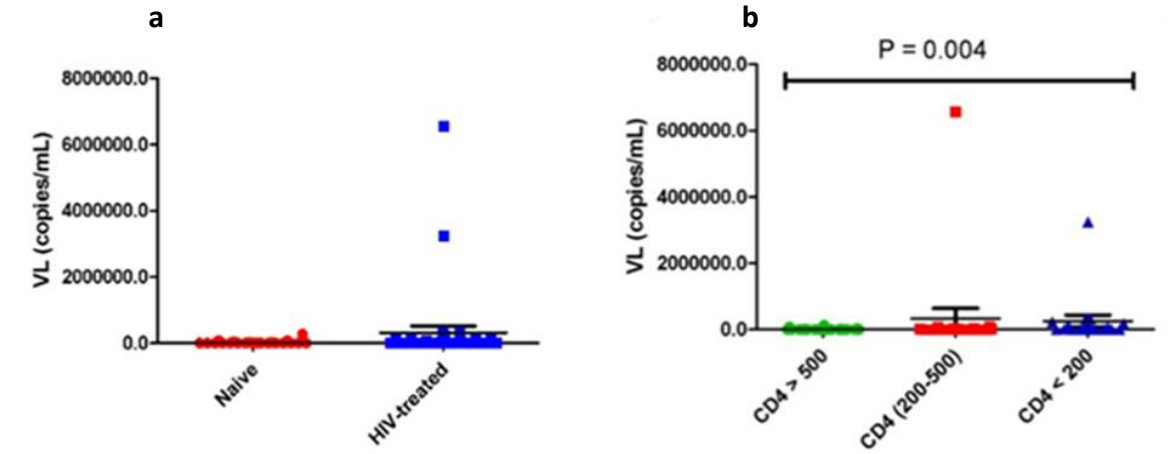

Figure 4-2: Assessment of VL in HIV-positive individuals. (a) Naïve vs. HIV-treated; (b) HIV-positive groups with varying CD4 counts (data presented as mean ± SEM).

4.4 CYTOTOXIC T LYMPHOCYTES

Cytotoxic T lymphocytes (CD8 T cells) are expanded and activated during HIV infection and thus express markers that allow for the identification of immune activation. Analysis of total lymphocytes (as percentage of total leukocytes) in the study groups revealed that it was 24 ± 6% for the control group versus 31 ± 9%, 22 ± 9%, and 15 ± 8% for groups with CD4 count > 500, 200–500 and < 200, respectively (Figure 4.3).

Cytotoxic T lymphocytes (CD8 T cells) are expanded and activated during HIV infection and thus express markers that allow for the identification of immune activation. Analysis of total lymphocyte percentages (of total leukocytes) in the study groups showed that the mean percentage of total lymphocytes in the control group was 24 ± 6 versus 31 ± 9, 22 ± 9, and 15 ± 8 for groups with CD4 count > 500, 200–500 and < 200, respectively (Figure 4.3)

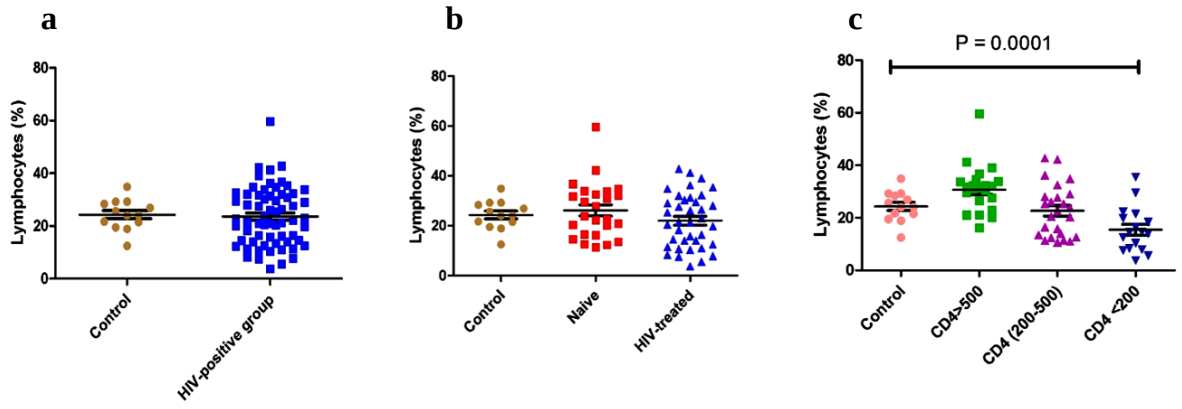

Figure 4-3: Percent lymphocytes with HIV infection. (a) Controls vs. HIV-positive individuals; (b) Controls vs. naïve vs. HIV-treated persons; and (c) Varying CD4 counts (data presented as mean ± SEM).

4.4.1 Immune Activation Status – $CD8^+CD38^+$

As CD38 expression on $CD8^+$ T lymphocytes is a robust marker of systemic immune activation (Paiardini et al., 2013), the degree of immune activation for different HIV stages was evaluated. Our data show that this was significantly higher in all HIV-positive groups compared to controls (Figure 4.4). Further analysis demonstrated that there was increasing immune activation with lower CD4 counts, with incremental changes in the sub-groups not being statistically different. For the control group the number of $CD8^+$ T cells expressing CD38 was 4.2 ± 2.8% compared to 26.8± 18.2% for the group with a CD 4 count > 500 cell/µL. For the group with a CD4 count between 200 and 500 this amounted to 36.5 ± 22.0% compared to the last group (CD4 count < 200) where the value was 46.7 ± 28.0% (p=0.0001).

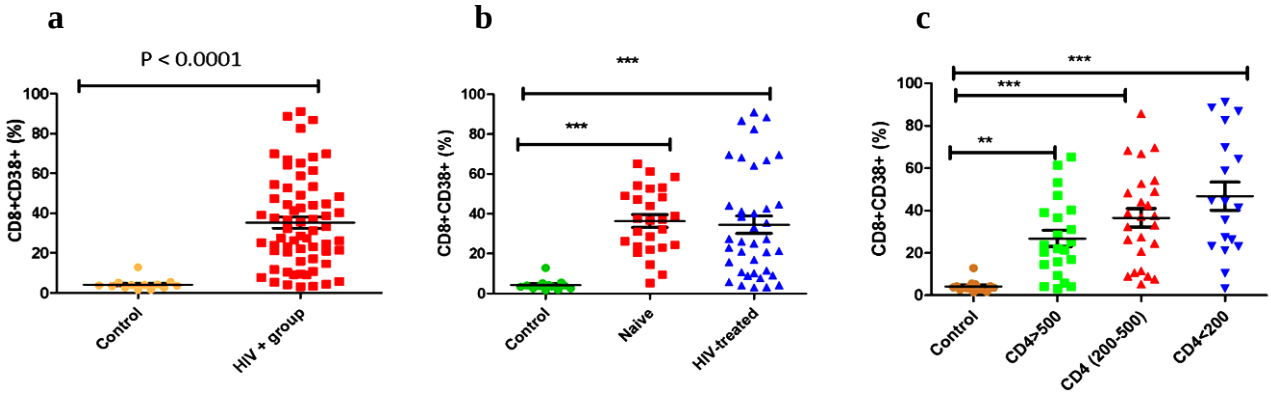

Figure 4-4: Immune activation ($CD8^+CD38^+$) in study groups. (a) Controls vs. HIV-positive individuals; (b) Control vs. naïve vs. HIV-treated; and (c) Controls vs. HIV-positive individuals with varying CD4 counts (data presented as mean ± SEM). *** P<0.0001 and ** P=0.001.

4.4.2 Immune Activation Markers on $CD3^+CD4^+$ T cells

The degree of immune activation expressed on $CD3^+CD8^-$ (CD4) between the control and HIV-positive groups revealed strong positive results. Here the value for the control group was 33 ± 11% versus 63 ± 16% for the HIV-positive group and 59 ± 12% for the naïve group. Further analyses based on CD 4 count showed a progressive increase with lower CD4 counts, i.e. 54 ± 11% for CD4 > 500, 61 ± 13% for the group with a CD4 count between 200 and 500 and 73 ± 14% for the group with CD4 counts<200) (P<0.0001).

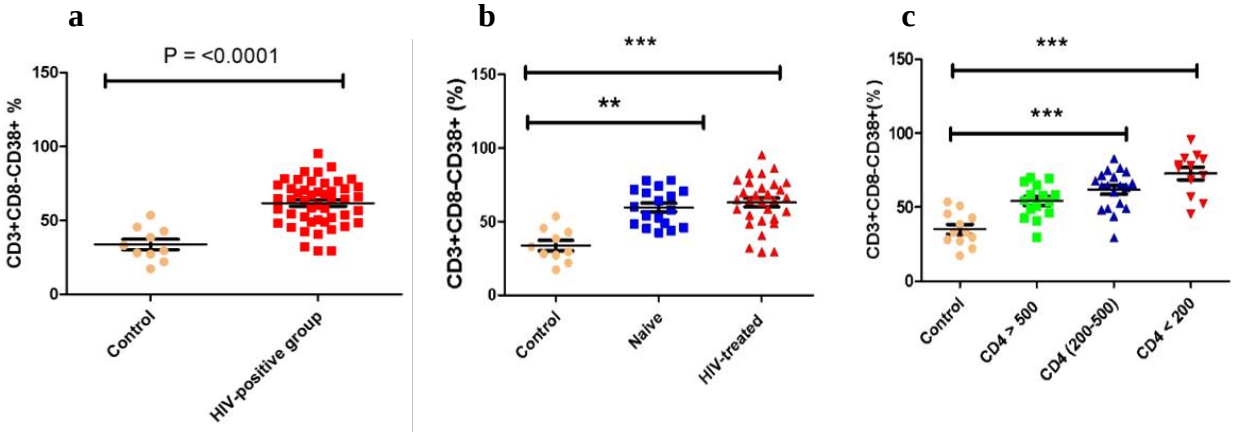

Figure 4-5: Immune activation ($CD3^+CD8^-CD38^+$) in study groups. (a) Controls vs. HIV-positive individuals (P < 0.0001); (b) Control vs. naïve vs. HIV-treated; and (c) Controls vs. HIV-positive individuals with varying CD4 counts (data presented as mean ± SEM). ***

4.4.3 Coagulation Markers (CD142) on CD8 T Cells

CD142 is an important marker related to the extrinsic coagulation pathway and is usually expressed on the surface of monocytes and platelets (Ferreira et al. 2010). Current literature indicates that a positive link between inflammation and coagulation exists. It is therefore very useful to evaluate CD142 on $CD8^+$ T cells. Here the data show that the highest CD142 levels were detected in the HIV-positive patients with relatively low CD4 counts (Figure 4.5).

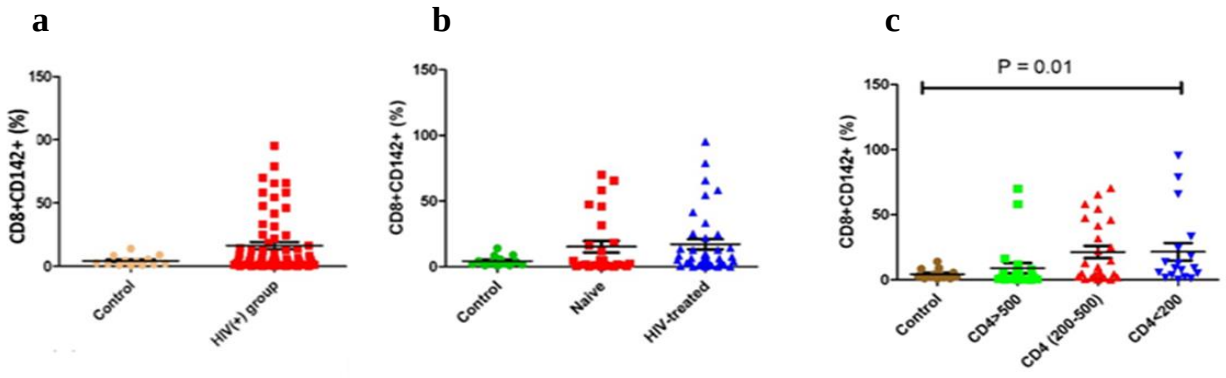

Figure 4-6: Expression of CD142 on CD8 T lymphocytes. (a) Control vs. HIV-positive persons; (b) Controls vs. naïve vs. HIV-treated individuals (P = 0.1); and (c) Comparisons with varying CD4 counts in HIV-positive individuals (P = 0.01) (data presented as mean ± SEM).

4.4.4 Coagulation Markers on $CD3^{+}CD8^{-}$ T cell (CD4)

Expression of coagulation markers on $CD3^{+}CD8^{-}$ T cell (CD4) showed significance when comparing the control versus the HIV-treated group, i.e. CD142 expression on $CD3^{+}CD8^{-}$ T cells (CD4) was 9 ± 6% for controls compared to 28 ± 25% for the naïve group (Figure 4.7). We also found a progressive increase in CD142 expression with lower CD4 counts.

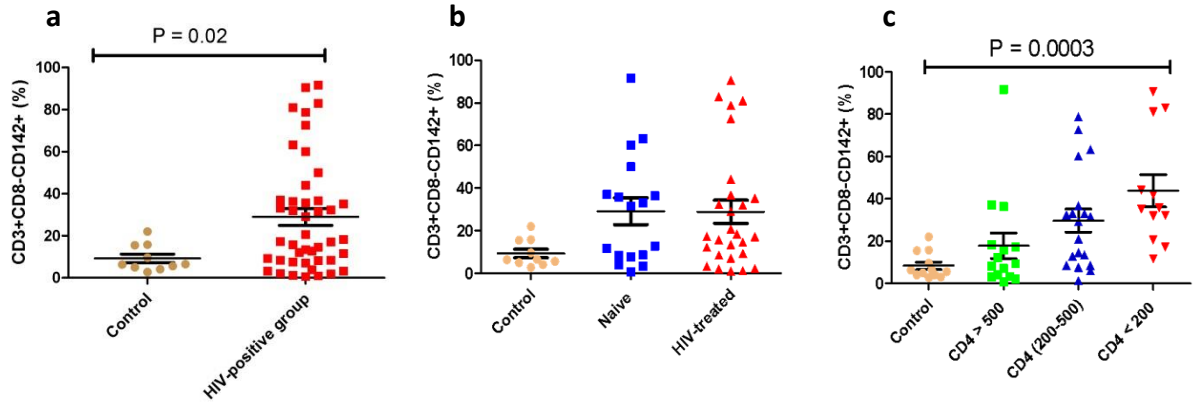

Figure 4-7: Expression of CD142 on CD3+CD8- (CD4) T lymphocytes. (a) Control vs. HIV-positive persons (P=0.02); (b) Controls vs. naïve vs. HIV-treated individuals; and (c) Comparisons with varying CD4 counts in HIV-positive individuals (P=0.0003) (data presented as mean ± SEM).

4.4.5 Co-expression of CD38 and CD142 on CD8 T Cells

For the current study, we combined the expression of an immune activation marker (CD38) with a coagulation marker (CD142) on CD8 T cells – for the first time as far as we are aware. These data show that such co-expression is extremely significant between controls and HIV-positive persons (P=0.0005) and also between controls and the group with the lowest CD4 counts (P =0.0001) (Figure 4.8).

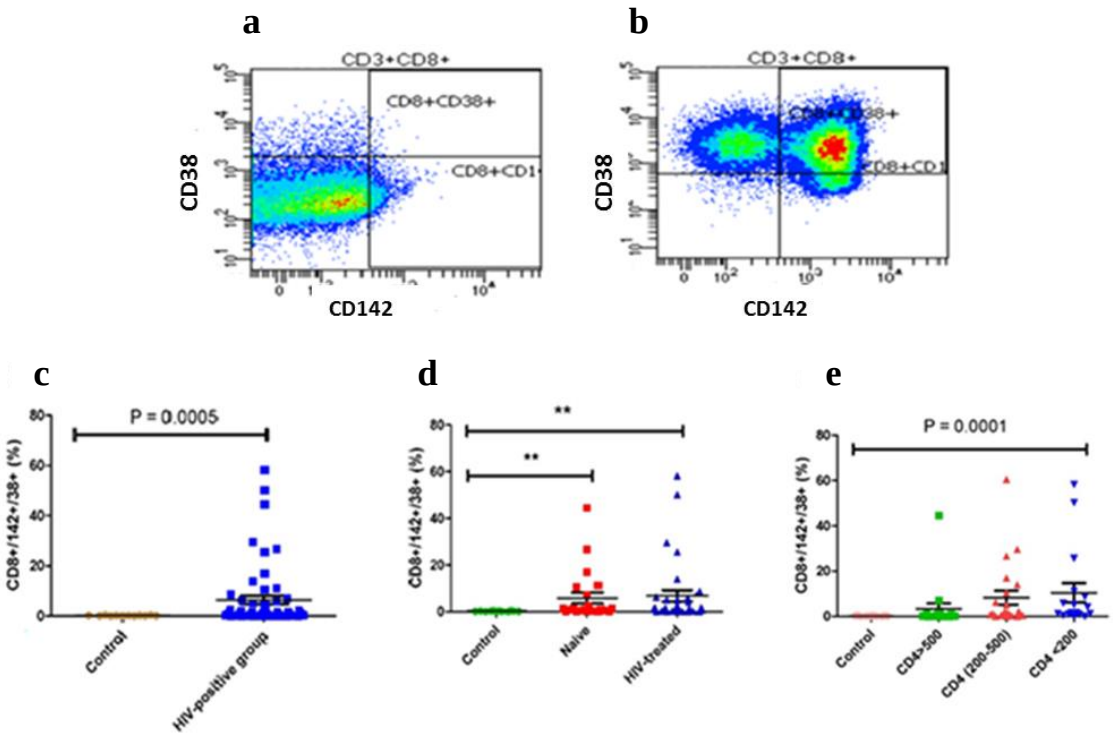

Figure 4-8: Immune activation and coagulation assessment in CD8 T cells. (a) Representative flow cytometry dot plot showing co-expression of CD38 and CD142 on CD8 T cells in an HIV-negative control sample; (b) Representative flow cytometry dot plot demonstrating co-eexpression of CD38 and CD142 on CD8 T cells in an HIV-positive sample with a relatively low CD4 count (CD4 count = 3 cells/µL); (c) Controls vs. HIV-positive; (d) Controls vs. naïve vs. HIV-treated patients (Controls vs. naïve P=0.002; Controls vs. HIV-treated P<0.002); and (e) CD38 and CD142 expression on CD8 T cells with varying CD4 counts (data presented as mean ± SEM). ** P=0.0001.

4.4.6 Co-expression of CD38 and CD142 on CD4 T Cells

Co-expression of immune activation and coagulation markers on $CD3^{+}CD8^{-}$ (CD4) T cells revealed significant results, i.e. 2.5 ± 1% for controls versus 20 ± 20% for the HIV-treated group (P=0.002) (Figure 4.9). For the group with the lowest CD4 count (<200) this was even higher, i.e. 31 ± 20% when compared with the control group (P<0.0001).

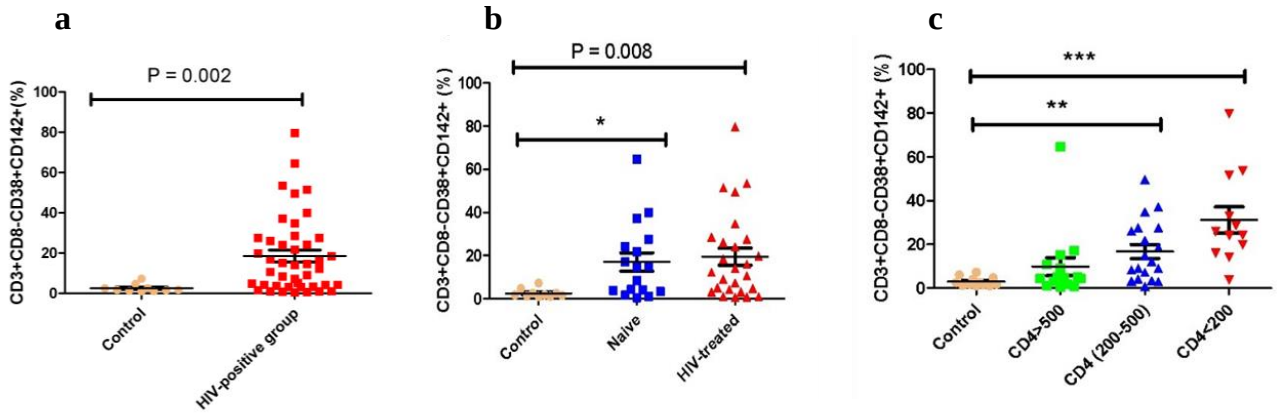

Figure 4-9: Co-expression of CD142 and CD38 on $CD3^+CD8^-$ (CD4) T lymphocytes. (a) Control vs. HIV-positive persons (P=0.002); (b) Controls vs. naïve vs. HIV-treated individuals (P=0.008); and (c) Comparisons with varying CD4 counts in HIV-positive individuals (P<0.0001) (data presented as mean ± SEM).

4.4.7 The Correlation between CD4 Counts and $CD8^+CD142^+$

A correlation between CD4 counts and TF (CD142) expressed on CD8 T cells was performed and showed a significant negative correlation (Spearman correlation r =- 0.29; p-value = 0.02) (Figure 4.10).

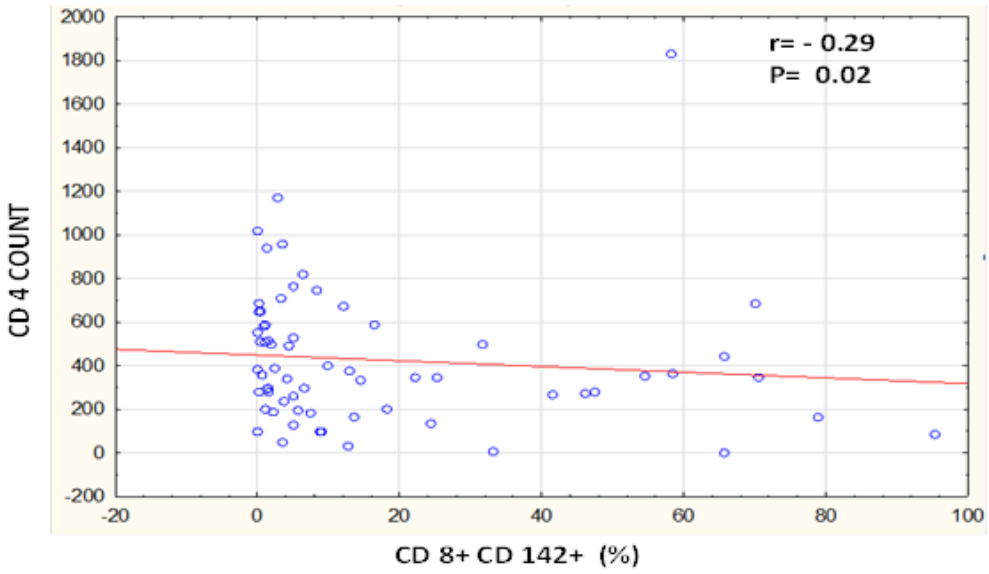

Figure 4-10: Negative correlation between CD4 count and CD142 expression on CD8 T cells

4.4.8 Correlation between Viral Load and Immune Activation and Coagulation Markers

Spearman correlations were also completed for VL versus $CD8^+CD142^+$ or $CD8^+CD38^+$ and here a positive correlation was found in both instances (Figure 4.11). Correlations were performed a comparison between immune activation and coagulation markers expressed on CD8 T cells and their relationship to disease

progression markers (CD4 and VL), monocyte subsets and activated markers of regulatory T cell (Table 4.3). Hence, increased immune activation and coagulation was positively correlated with all of these markers.

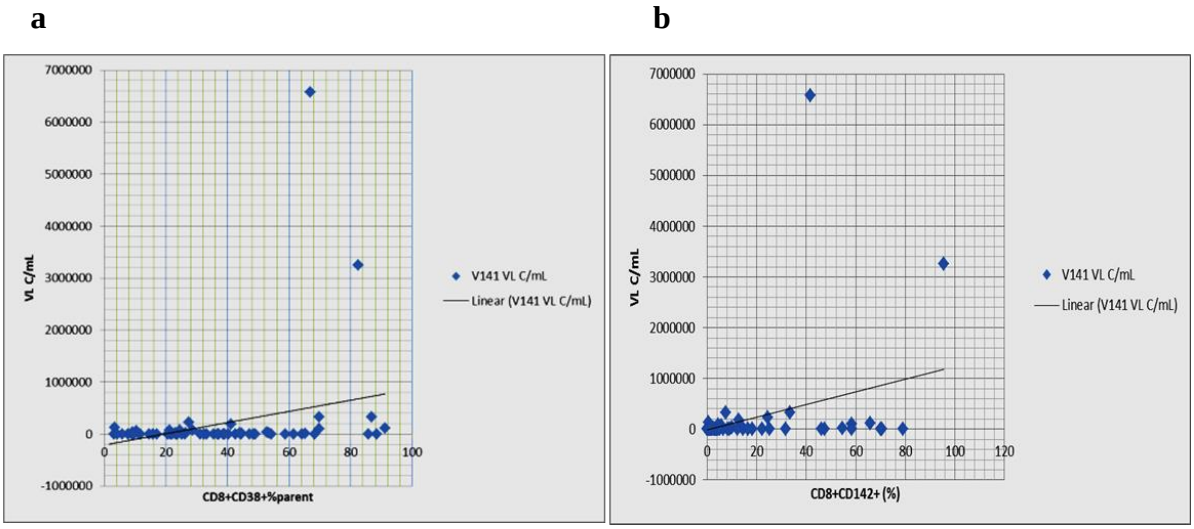

Figure 4-11: . Correlation and linear regression for VL and immune activation and coagulation. (a) VL and immune activation marker (CD38 on CD8 T cells) (r=0.27, P=0.04); and (b) VL and CD8+CD142+ (r = 0.26, P= 0.04).

Table 4-3. The correlation between immune activation and coagulation markers and other markers

Variable	*$CD8^+/142^+$*	*$CD8^+/38^+$*	*$CD8^+/142^+/38^+$*
CD4 count (cell/µL)	r = - 0.29 & P = 0.02	r = - 0.34 & P = 0.005	r = 0.37 & P = 0.004
VL (C/mL)	r = 0.26 & P = 0.04	r = 0.27 & P = 0.04	r = 0.30 & P = 0.03
$CD14^+CD16^{++}$ (%)	r = 0.31 & P =0.001	r = 0.51 & P = 0.001	r = 0.38 & P = 0.001
$CD14^+CD16^+$ (%)	r = 0.22 & P = 0.05	N/S	N/S
GARP (%)	r = 0.49 & P = 0.001	r = 0.43 & P = 0.001	r = 0.55 & P = 0.0001
SATB-1 (%)	r = 0.31 & P = 0.008	r = 0.39 & P = 0.001	r = 0.43 & P = 0.001
CRP (mg/L)	r = 0.25 & P = 0.03	N/S	N/S

N/S - not significant, **CRP** – C-reactive protein; **VL** - viral load; **$CD14^+CD16^{++}$** - non-classical monocytes; **$CD14^+CD16^+$** - intermediate monocytes; **GARP** - glycoprotein A repetition predominant**; SATB-1** - special AT-rich sequence-binding protein-1.

4.5 MONOCYTE SUBSETS

Monocytes can be identified by the expression of CD14 and form part of the total leukocyte population. It can be subdivided into three subpopulations distinguished by particular combinations of CD14 and CD16 expression on their surface (refer below).

4.5.1 Classical Monocyte Subset ($CD14^+CD16^-$)

Classical monocytes decrease during HIV infection and there is corresponding expansion of the intermediate and non-classical monocyte subsets in proportion to the total monocytes; this preferential expansion is significant in chronic HIV infection with low CD4 counts. As predicted, our data revealed a decrease in classical monocytes with HIV infection, especially for individuals with relatively low CD4 counts (Figure 4.12).

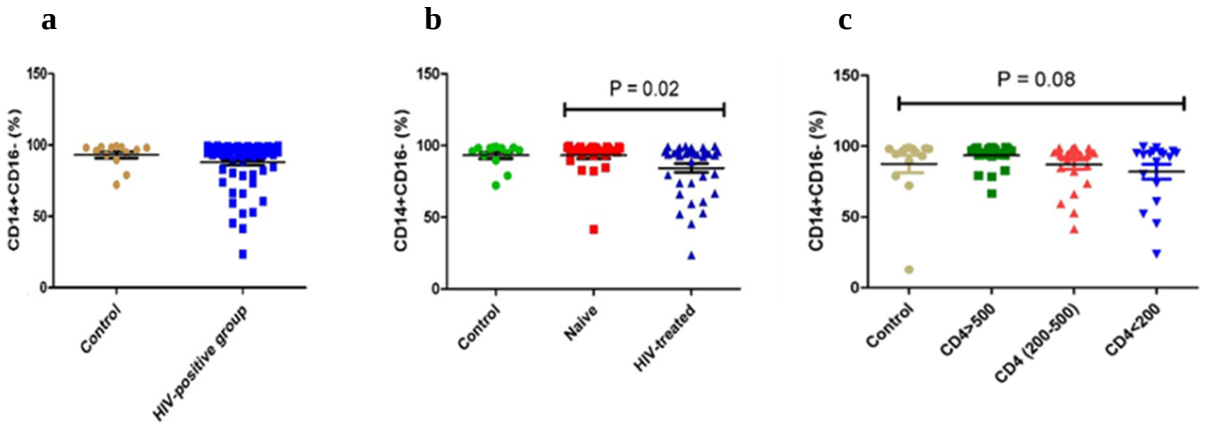

Figure 4-12: Analysis of classical monocyte subset as a percentage of total CD14+ monocyte events. (a) Controls vs. HIV-positive group; (b) Controls vs. naïve vs. HIV-treated (P=0.02); (c) Classical monocyte subset expression with varying CD4 counts (P=0.08) (data presented as mean ± SEM).

4.5.2 Intermediate Monocyte Subset ($CD14^+CD16^+$)

These data showed an increased number of $CD14^+CD16^+$ cells in the HIV-positive treated group versus the cART naïve and control groups (Figure 4.13). There was also a significant increase in $CD14^+CD16^+$ in the low CD4 count group versus controls.

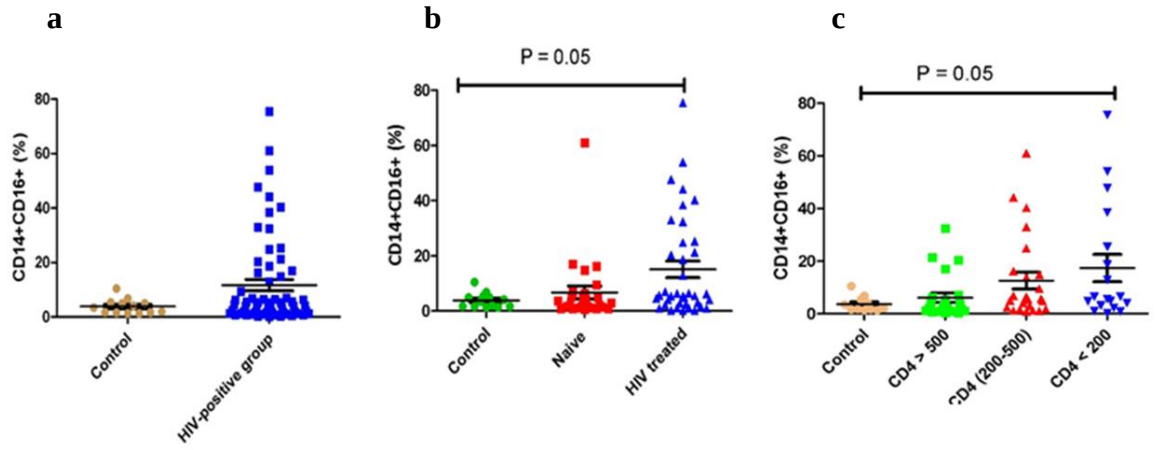

Figure 4-13: Evaluation of the intermediate monocyte subpopulation with HIV infection. (a) Controls vs. HIV-positive (4 ± 2.7% vs 10 ± 15%; P-value not significant); (b) Controls vs. naïve vs. HIV-treated patients (p=0.05); and (c) Intermediate monocyte expression between Controls and HIV-positive patients with varying CD4 count groups (P = 0.05) (data presented as SEM).

4.5.3 Non-classical Monocyte Subset ($CD14^{+}CD16^{++}$)

The expression of non-classical monocytes showed extremely significant results between the control and HIV-positive groups (P=0.0001). The percentage $CD14^{+}CD16^{++}$ monocytes showed an increase for groups displaying a relatively low CD4 count (P=0.0001) (Figure 4.14).

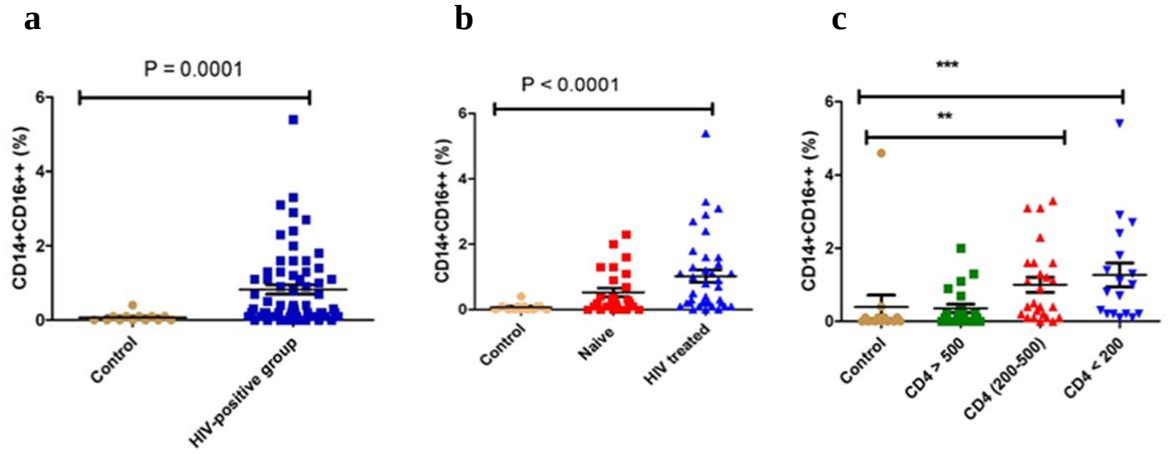

Figure 4-14: Expression of non-classical monocytes with HIV infection. (a) Control group vs. HIV-positive group; (b) Control vs. naïve vs HIV-treated (0.06 ± 0.1% vs. 0.5 ± 0.6% vs. 1 ± 1.1%; P<0.0001); and (c) Non-classical monocytes with varying CD4 counts(data presented as mean ± SEM). ** P=0.001; *** P=0.0001.

A Comparative analysis: expression of monocyte subsets in HIV-negative controls versus HIV-positive individuals (with relatively low CD4 counts)

Expression of monocyte subsets in HIV-negative individuals (control)

The expression of monocyte subsets in control group show the normal range of percentage, classical monocytes (80-95), intermediate monocyte (2-11%) and non-classic monocyte (2-8%)

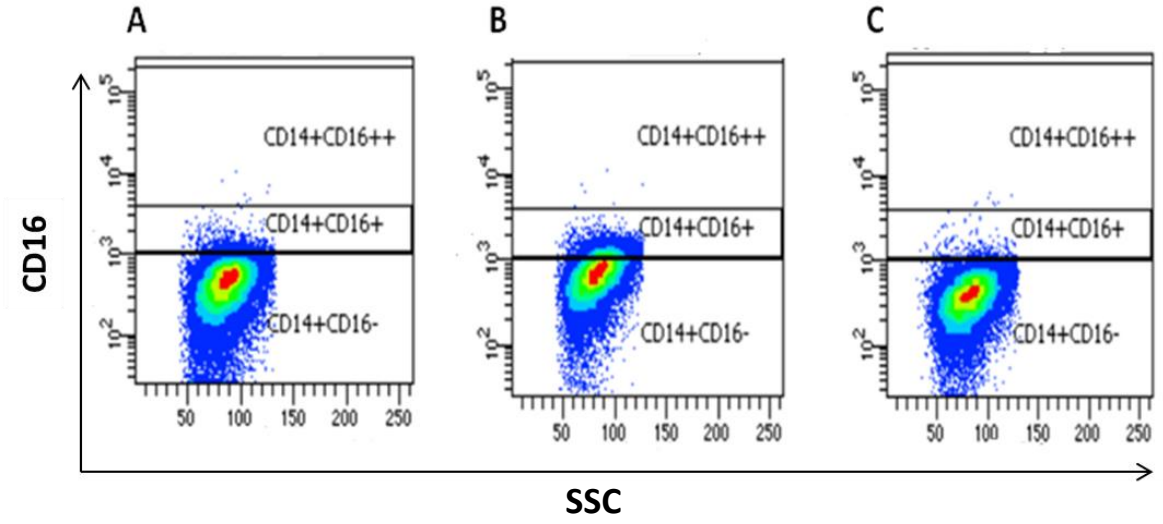

Figure 4-15: Images of the three monocyte subsets in an HIV-negative (control) sample. Panels A, B and C show the expression of three subsets, i.e. CD14++CD16-, CD14+CD16+ and CD14+CD16++, respectively.

Expression of monocyte subsets in individuals with low CD4 counts

The expressions of monocyte subsets show expansion of these subsets in intermediate and non-classical monocyte subsets.

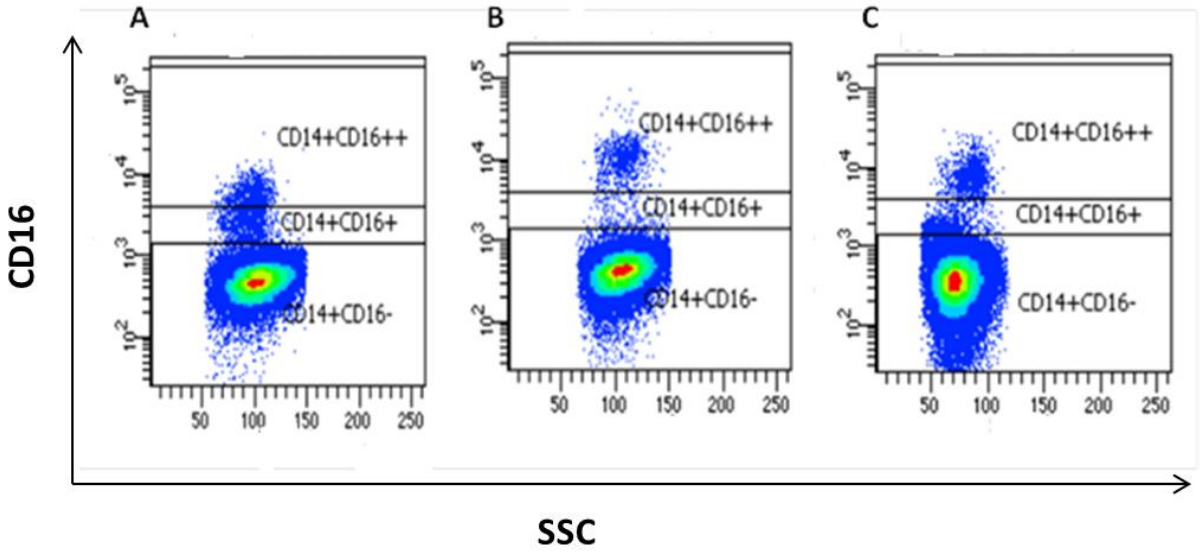

Figure 4-16: Images of three monocyte subsets in HIV-positive individuals with relatively low CD4 counts A, B, C – show expansion of percentage of CD14+CD16+ and CD14+CD16++ expression in these population

4.5.4 Monocyte Subsets and CD38 Expression

No significant differences were found for CD38 expression on monocyte subsets, except for the comparison of control versus HIV-positive group in classical monocyte (P=0.01) (Figure 4.17)

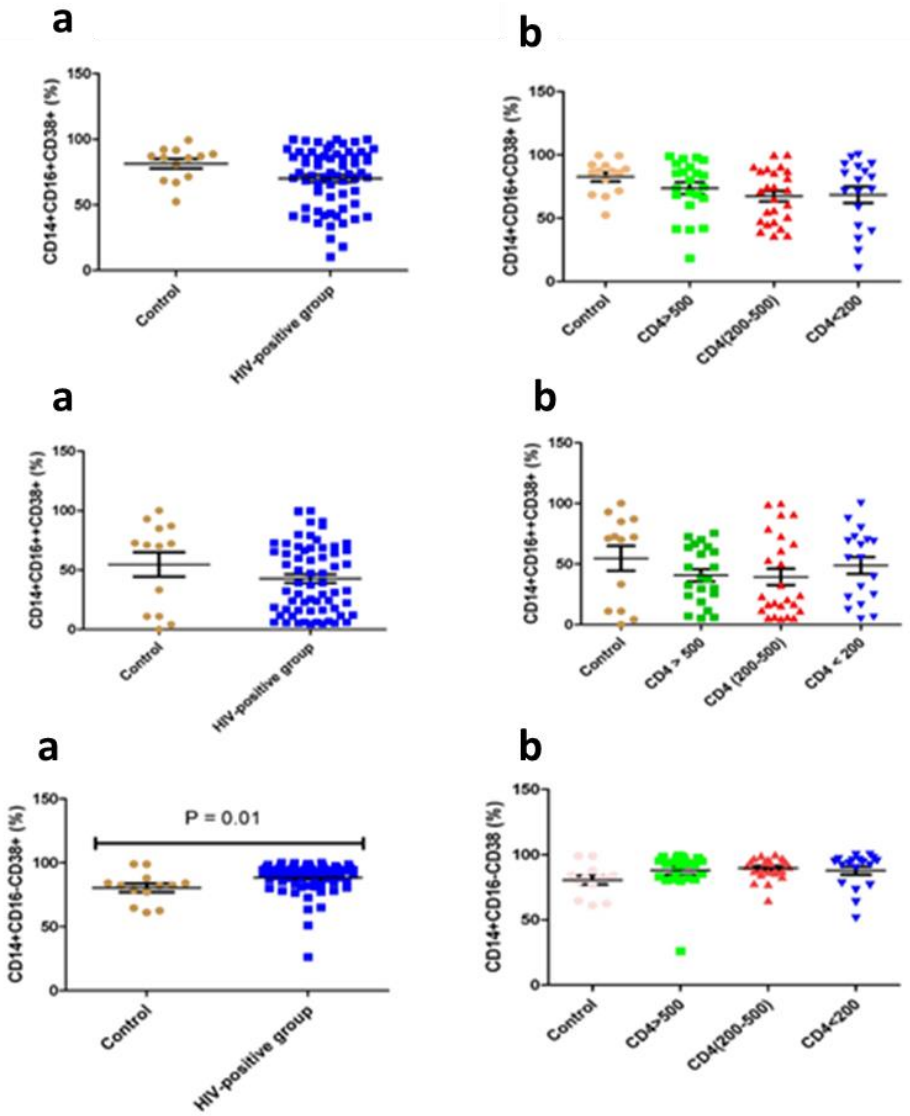

Figure 4-17: CD38 expression on the surface of monocyte subsets. (a) Comparison between controls and the HIV-positive group for three monocyte subsets (non-classical, intermediate and classical monocyte) showed limited changes except for expression on classical monocytes (80 ± 11% vs. 88 ± 12%; p=0.01); (b) Comparison between controls and HIV-positive persons with varying CD4 counts.

4.5.5 Monocyte Subsets and Expression of TF ($CD142^{+}$)

These results did not show any significant differences, except for TF expression on non-classical monocytes (Figure 4.18).

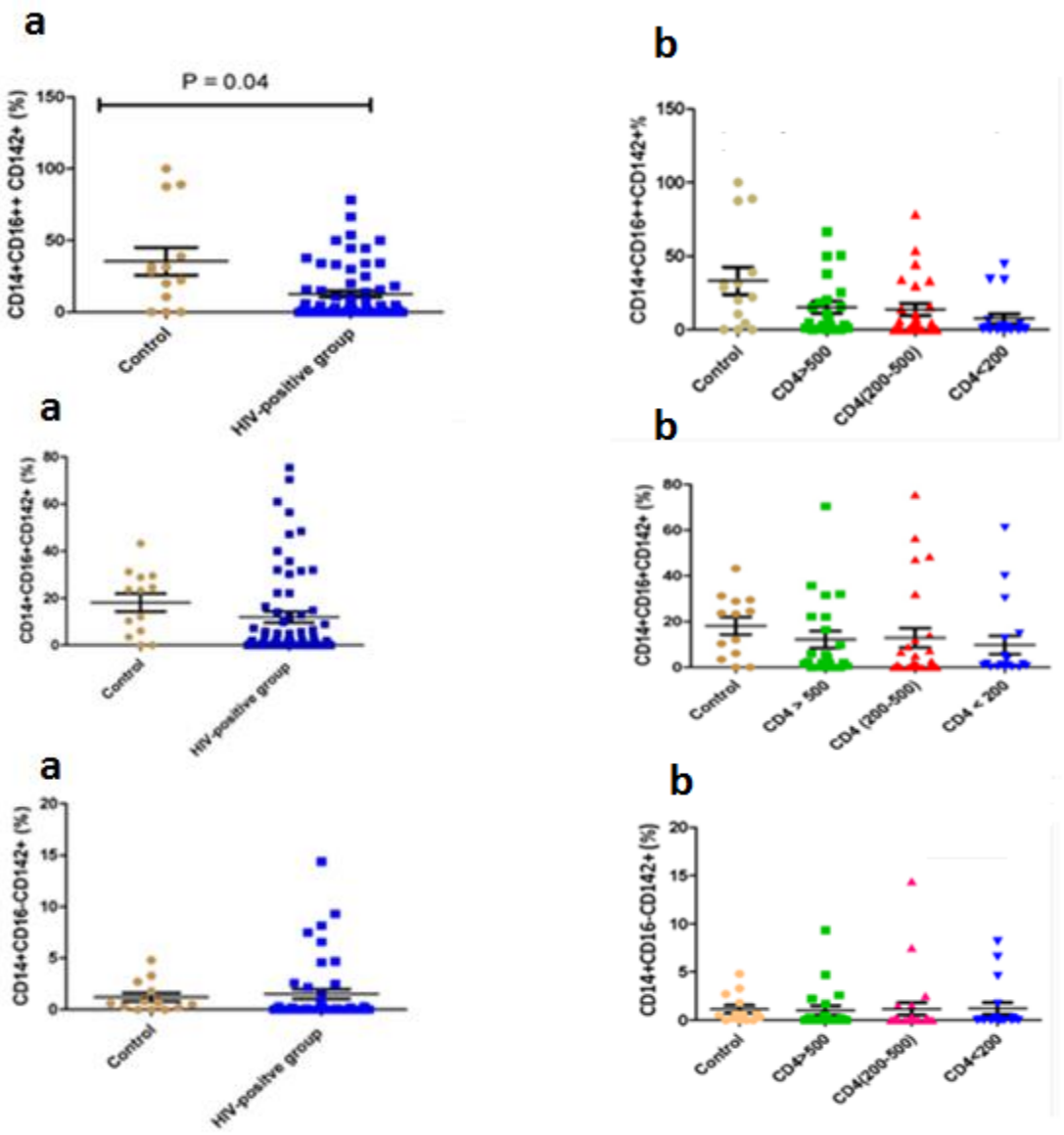

Figure 4-18: TF expression on monocyte subsets. (a) Controls vs. HIV-positive for three monocyte subsets (classical, intermediate and non-classical monocyte); and b) Control vs. varying CD4 counts for the three monocyte subsets. Data presented as mean ± SEM.

4.5.6 Correlations

A Monocyte subsets and TF expression on CD8 T cell

<u>$CD14^{+}CD16^{++}$ monocyte subset</u>

A correlation analysis was performed between the $CD14^{+}CD16^{++}$ monocyte subset and coagulation markers ($CD8^{+}CD142^{+}$) and also with coagulation plus immune activation ($CD8^{+}CD38^{+}CD142^{+}$). There was a positive correlation between the $CD14^{+}CD16^{++}$ monocyte subset and $CD8^{+}CD142^{+}$ (r=0.31; P=0.01) and also with $CD8^{+}CD142^{+}CD38^{+}$ (r=0.38; P=0.001) as shown in Figure 4.19.

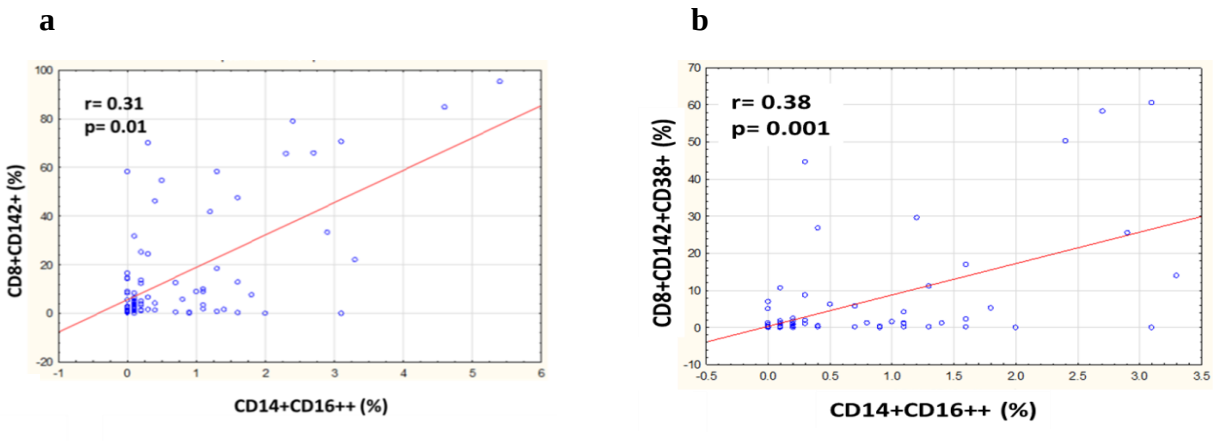

Figure 4-19: Correlation between non-classical monocyte subset and coagulation (a) CD8+CD142+ (r=0.31; P=0.01); and (b) with CD8+CD38+CD142+ (r=0.38; P=0.001).

CD14^{+}CD16^{+}monocyte subset

A correlation analysis was next performed between the CD14^{+}CD16^{+} monocyte subset and CD8^{+}CD142^{+} markers on CD8 T cells and here results showed a positive correlation (r=0.22; P =0.05) (Figure 4.20).

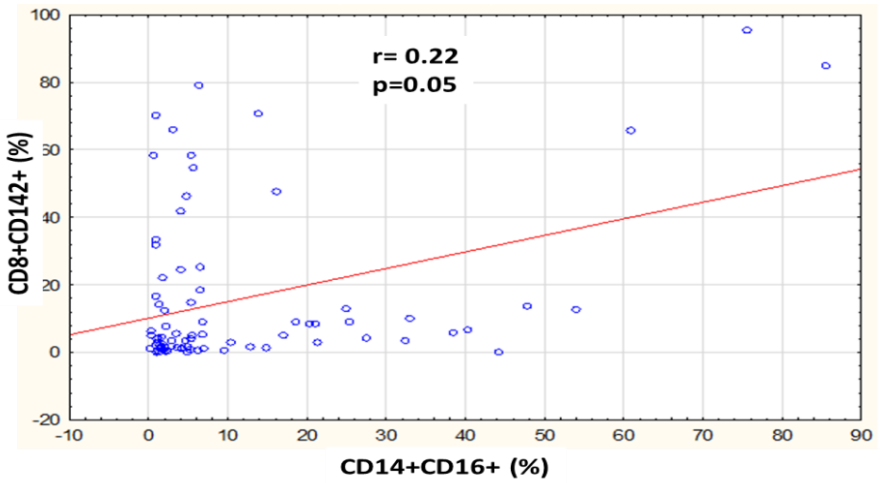

Figure 4-20: Correlation between the intermediate monocyte subset and a coagulation marker expressed on CD8 T cells (r .22; P=0.05).

CD14^{+}CD16^{-} monocyte subset

The classical monocyte subset (CD14^{+}CD16^{-}) showed a negative correlation with CD8^{+}CD142^{+} (r = - 0.24; P=0.03) (Figure 4.21).

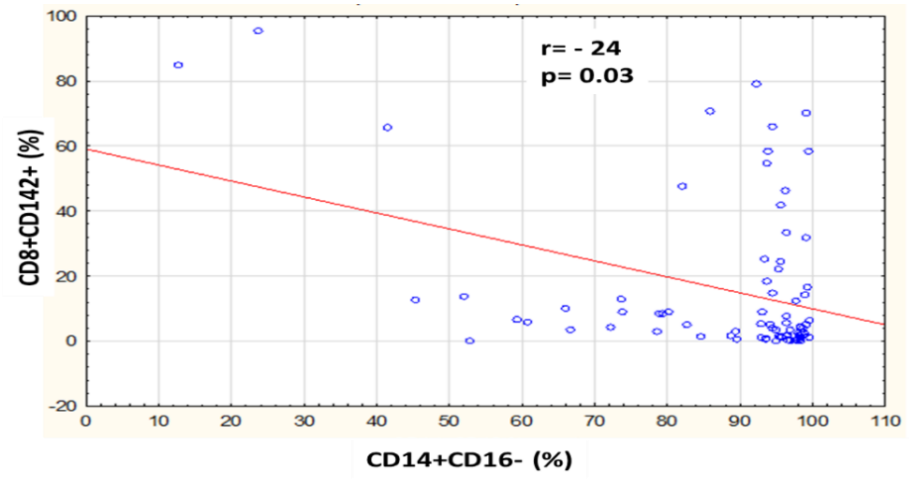

Figure 4-21: Correlation between classical monocytes and a coagulation marker. Linear regression analysis shows a negative correlation between the classical monocyte subset and the coagulation marker (r = - 0.24; P=0.03).

B Monocyte subsets and immune activation

$CD14^{+}CD16^{++}$

Correlation of non-classical monocytes with an immune activation marker ($CD8^{+}CD38^{+}$) was also performed and this showed an extremely significant positive correlation (Figure 4.22). Of note, there were no significant correlations with the other monocyte subsets – refer Table 4.4.

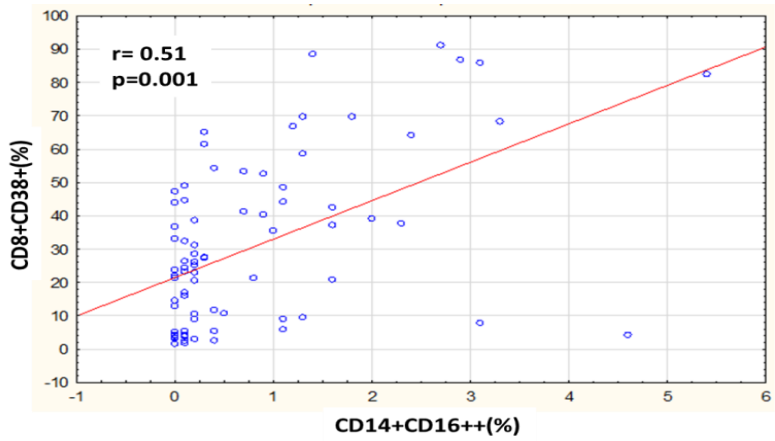

Figure 4-22: Correlation between non-classical monocytes and immune activation marker. Shows a positive correlation (r=0.51; P=0.0001).

C CD4 count and monocyte subsets

$CD14^{+}CD16^{++}$

The correlation performed between $CD14^{+}CD16^{++}$ and CD4 counts showed a significant negative correlation (r = - 0.40 and P=0.001) (Figure 4.23).

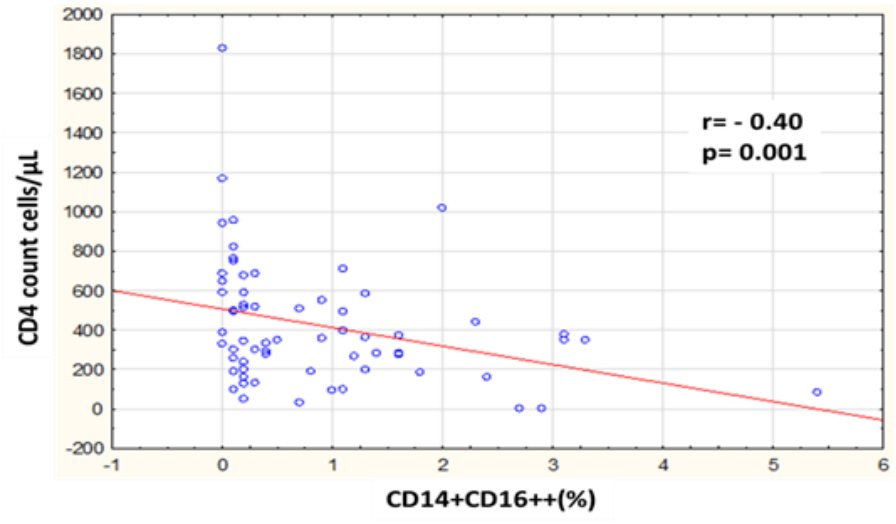

Figure 4-23: A negative relationship between non-classical monocytes and CD4 count. (r = - 0.40, P=0.001).

CD14$^+$CD16$^+$

A correlation was also performed between CD14$^+$CD16$^+$ and CD4 counts and showed a significant negative relationship (Figure 4.24).

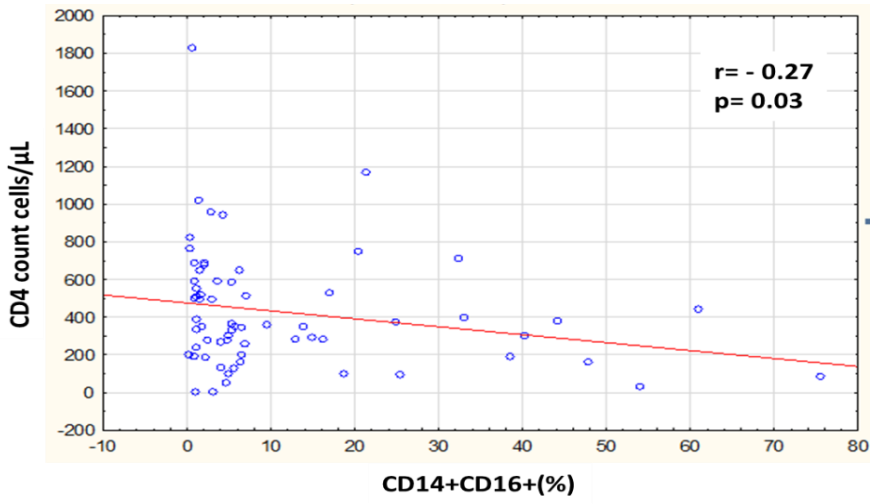

Figure 4-24: Negative correlation between CD4 count and the CD14+CD16+ monocyte subset

CD14$^+$CD16$^-$ subset

Figure 4.25 shows a significant positive correlation between classical monocytes and CD4 counts. Comparison between immune activation and coagulation marker is viewd in Table 4-4.

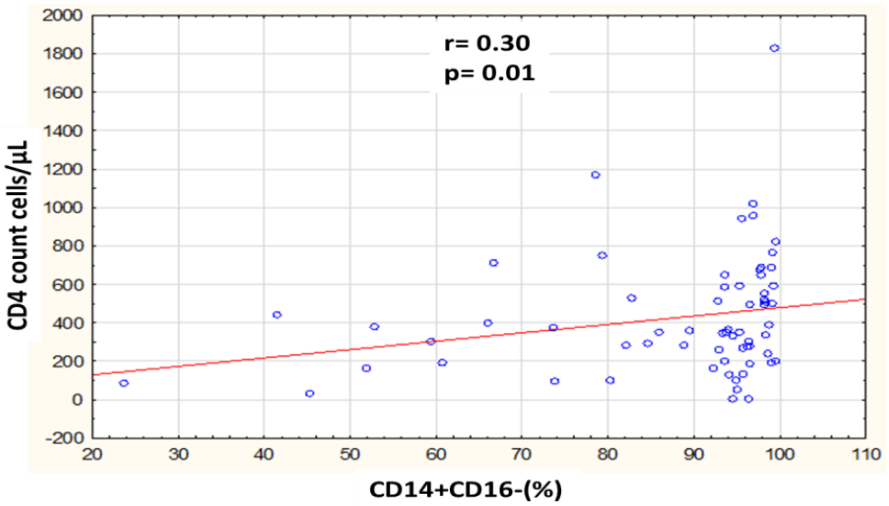

Figure 4-25: Positive correlation between classical monocytes and CD4 counts. (r=0.30; P=0.01).

Table 4-4: comparison between immune activation and coagulation marker

Variable 1	$CD14^+CD16^-$	$CD14^+CD16^+$	$CD14^+CD16^{++}$
Monocyte subsets	**Classical monocytes**	**Intermediate monocytes**	**Non-classical monocytes**
$CD8^+CD38^+$ (%)	N/S	N/S	r = 0.51, P = 0.001
$CD8^+CD38^+CD142^+$ (%)	N/S	N/S	r = 0.38, P = 0.001
$CD8^+CD142^+$ (%)	r = - 0.24, P = 0.03	r = 0.22, P = 0.05	r = 0.31, P = 0.01
$CD4^+FOXP3^+$ (%)	N/S	N/S	r = 0.23, P = 0.04
$CD25^{++}SATB\text{-}1^+$ (%)	r = - 0.28, P = 0.01	r = 0.27, P = 0.02	r = 0.32, P = 0.001
$CD25^{++}GARP^+$ (%)	r = -0.46, P = 0.001	r = 0.41, P = 0.001	r = 0.42, P = 0.001
$CD25^-$ $GARP^+$ (%)	r = - 0.31, P = 0.01	r = 0.25, P = 0.03	r = 0.43, P = 0.001
CD4 count (cells/µL)	r = 0.30, P = 0.01	r = - 0.27, P = 0.03	r = - 0.40, P = 0.001
VL (C/mL)		N/S	N/S
CRP (mg/L)	N/S	N/S	r = 0.23, P = 0.05

VL - viral load**;** **$CD4^+FOXP3^+$**- regulatory T cell**;** **$CD25^{++}GARP^+$**- glycoprotein A repetition predominant**;** **$CD25^{++}SATB\text{-}1$**- special AT-rich sequence-binding protein-1 in regulatory T cells; **$CD8^+CD38^+$**- immune activation marker; **$CD8^+CD142^+$**- coagulation marker; **$CD8^+CD38^+CD142^+$**- co-expression of inflammation and coagulation marker**;** **CRP**- C-reactive protein.

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4.6 REGULATORY T CELL SUBSETS

Tregs are a subset of CD4 T cells that normally make up less than 10 per cent of this group of cells

4.6.1 Regulatory T Cells ($CD4^+FOXP3^+$)

An increase in $CD4^+FOXP3^+$ expression in Treg cells with HIV infection is a key feature associated with such disease progression. Here the findings show that HIV-infected individuals possess a greater percentage of $CD4^+$ T cells that express FOXP3, especially with relatively lower CD4 counts (Figure 4.26). Here the percentage $CD4^+FOXP3^+$ cells in the control group was 6.8 ± 1.7% compared to 16± 16% and 19.7 ± 19% for CD4 counts (200-500 cells/µL) and CD4 counts less than 200 cells/µL. $CD4^+FOXP3^+$ Treg cells were also correlated with various immunological markers (Table 4.5), where it showed a positive correlation with immunological and coagulation markers while displaying a negative relationship with CD4 count.

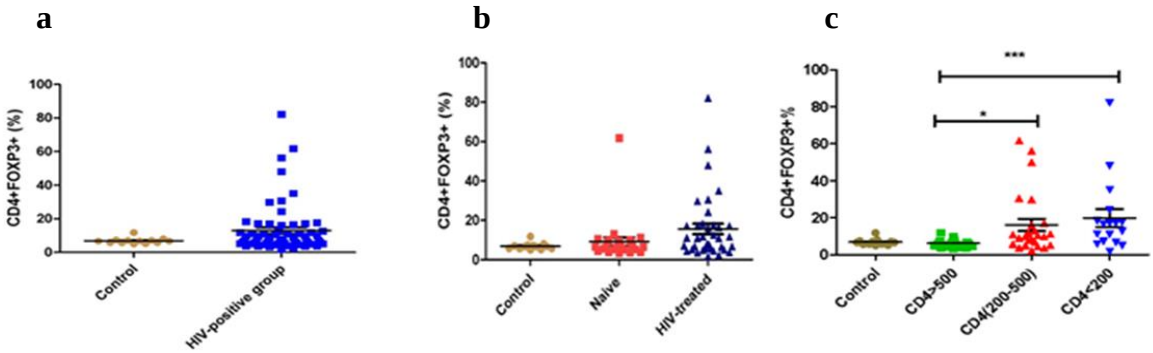

Figure 4-26: CD4+FOXP3+ expression in HIV-positive individuals. (a) Comparison between controls vs.the HIV-positive group; (b) Comparison between controls vs. naïve vs. HIV-treated; (c) Comparison with varying CD4 counts. Data presented as mean ± SEM. *** P 0.0005, * P = 0.01.

Table 4-5: Correlation of CD4+FOXP3+ with various immunological markers

$CD4^+FOXP3^+$ vs	r-value	p-value
$CD8^+CD38^+$ (%)	0.34	0.001
$CD8^+CD142^+$ (%)	0.17	0.13
$CD8^+142^+38^+$ (%)	0.20	0.08
$CD25^{++}SATB\text{-}1^+$ (%)	0.47	0.0001
$CD25^{++}GARP^+$ (%)	0.42	0.0009
CD4 count	-0.52	0.0001

4.6.2 Regulatory Markers in Cytotoxic T Cells ($CD3^+CD4^-FOXP3^+$)

We also evaluated the expression of FOXP3 on CD8 T cells and the results revealed a significant increase in FOXP3 expression in the HIV-positive group with relatively low CD4 counts (Figure 4-27) (P=0.005).

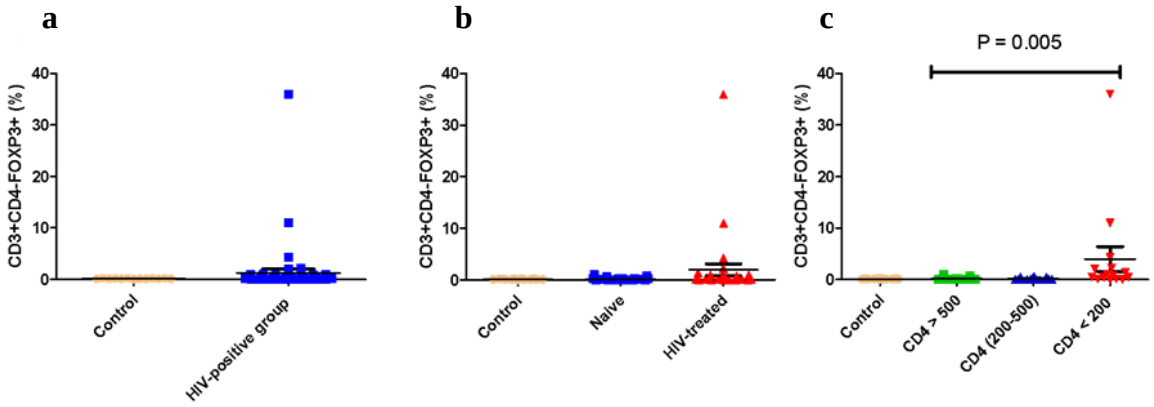

Figure 4-27: $CD8^+FOXP3^+$ expression in HIV-positive individuals. (a) Comparison between control and HIV-positive groups; (b) Comparison between control vs. naïve vs. HIV-treated groups; (c) Comparison with varying CD4 counts (P=0.005).

<u>$CD4^+CD25^{++}FOXP3^+$ subset</u>

As shown in Figure 4.28, a significant rise in the expansion of FOXP3 in $CD4^+CD25^{++}$ cells, particularly with relatively low CD4 counts; such upregulation is linked to disease progression (Bi, X. et al., 2009).

As shown in Figure 4.24, a significant rise in the expansion of FOXP3 in $CD4^+CD25^{++}$ cells, particularly with relatively low CD4 counts; such upregulation is linked to disease progression (Bi, X. et al., 2009).

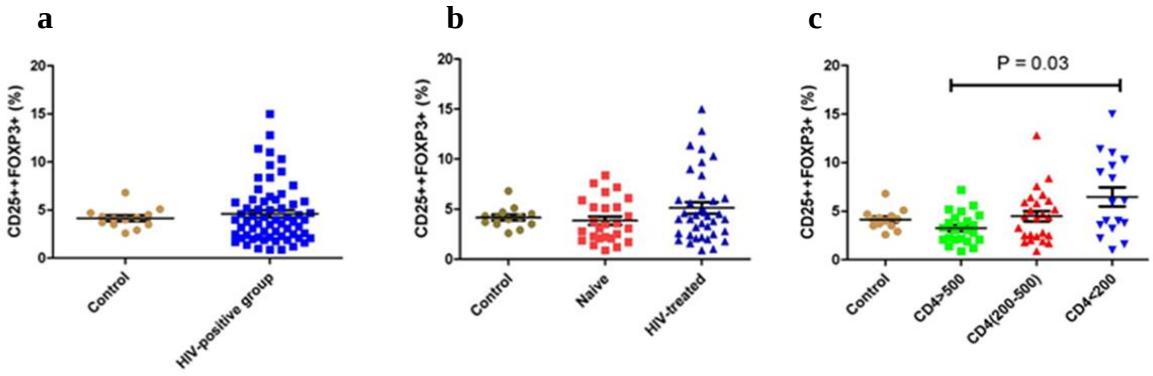

Figure 4-28: FOXP3 expression on $CD4^+CD25^{++}$ cells. (a) Comparison between control and HIV-positive groups; (b) Comparison between control vs. naïve vs. HIV-treated groups; and (c) Comparison of the expression of CD4+CD25++FOXP3+ with varying CD4 counts (P=0.03).

4.6.3 Activated Markers Special AT-rich Sequence-Binding Protein-1 (SATB-1)

A $CD4^{+}CD25^{++}$SATB-1

These data revealed increased SATB-1 expression in $CD4^{+}CD25^{++}$ cells with the lowest relative CD4 counts (Figure 4.29).

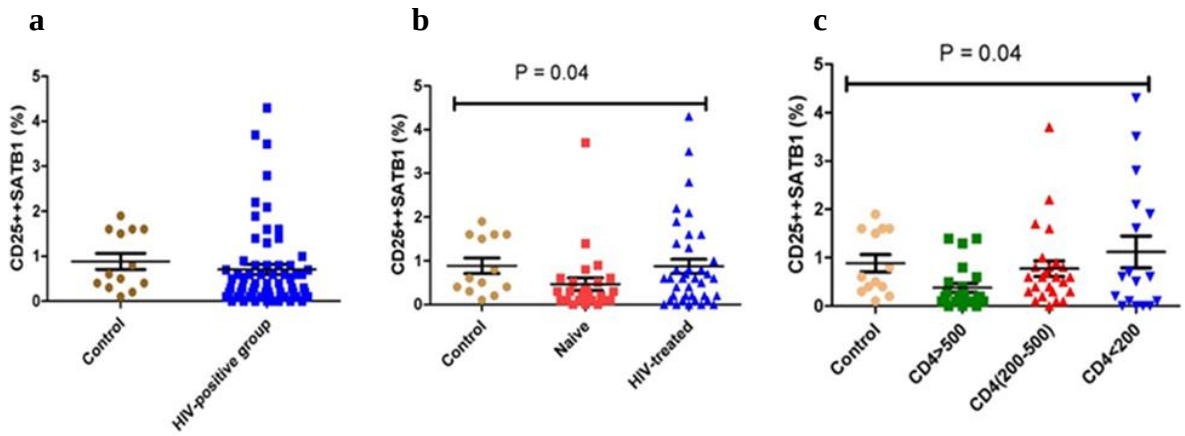

Figure 4-29: The expression of SATB-1 in $CD25^{++}$ cells with HIV infection. (a) Control vs. HIV-positive groups; (b) Controls vs. naïve vs. HIV-treated groups; and (c) SATB-1 expression with varying CD4 counts (data presented as mean ± SEM).

B $CD4^{+}CD25^{-}$ $FOXP3^{+}$SATB-1

A comparison of SATB-1 expression on $CD25^{-}$ $FOXP3^{+}$ cells showed an upregulation in HIV-infected groups versus controls, i.e. from 10 ± 18% to 23 ± 22% (Figure 4.30). There was also increased SATB-1 expression for the group with the lowest CD4 counts (<200).

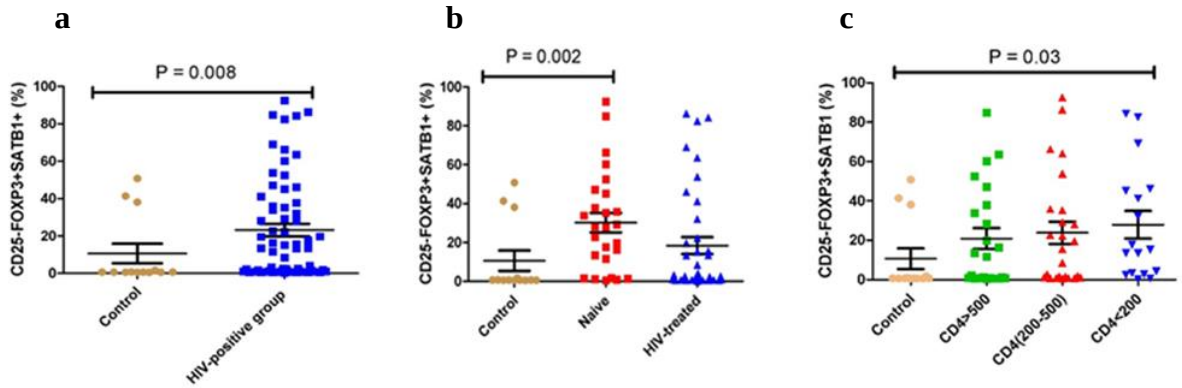

Figure 4-30: SATB-1 expression in $CD25^{-}FOXP3^{+}$ cells. (a) Control vs. HIV-positive group (P=0.008); (b) Control vs. naïve (P=0.002) vs. HIV-treated groups; (c) SATB-1 levels in HIV-positive individuals with varying CD4 counts (P=0.03) (data presented as mean ± SEM).

C CD3$^+$CD4$^-$FOXP3$^+$SATB1$^+$ (CD8$^+$FOXP3$^+$SATB-1$^+$)

SATB-1 expression on CD8 T cells showed no significant differences between study groups (Figure 4.31).

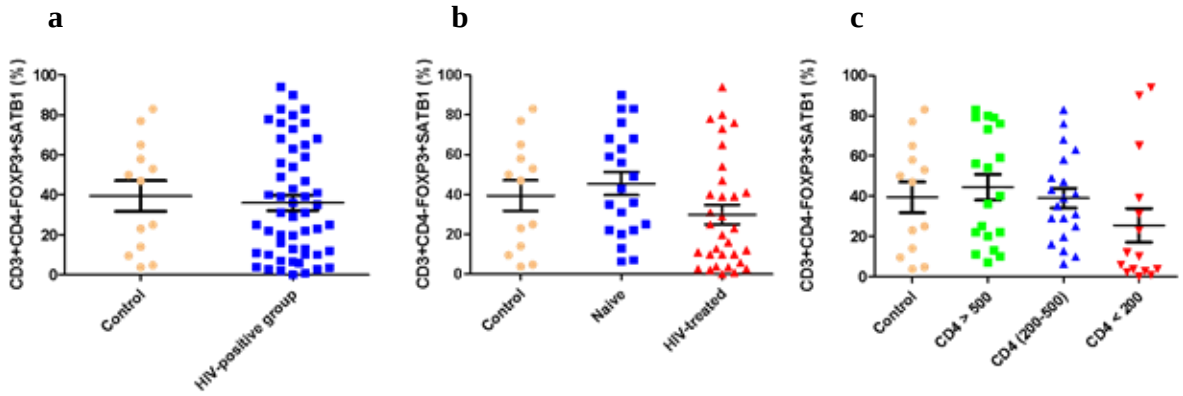

Figure 4-31: SATB-1 expression on CD8 T cells (CD8$^+$FOXP3$^+$). (a) Control vs. HIV-positive group; (b) Control vs. naïve vs. HIV-treated groups; (c) SATB-1 levels in HIV-positive individuals with varying CD4 counts.

4.6.4 Activated Markers Glycoprotein a Repetitions Predominant (GARP)

A CD4$^+$CD25^{++}GARP subset

Our data showed increased GARP$^+$ cells with chronic infection (Figure 4.31).

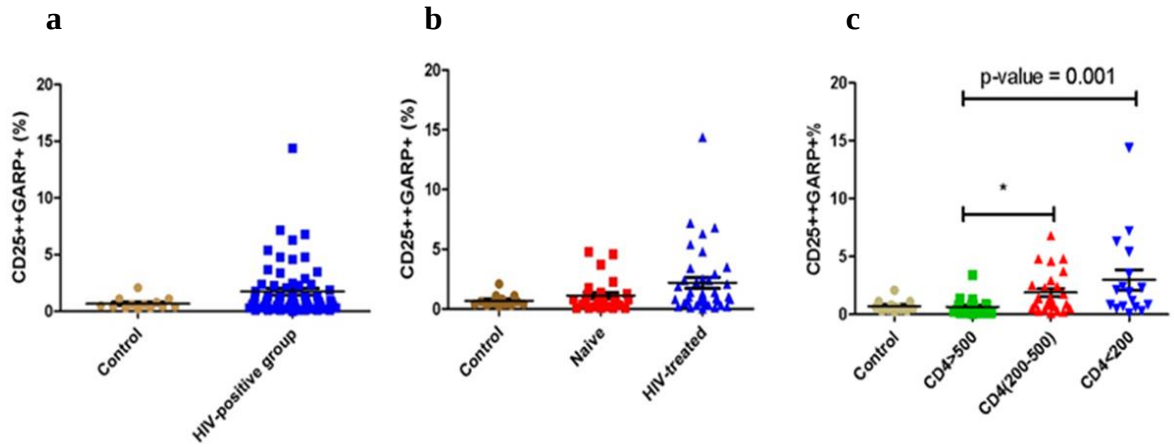

Figure 4-32: Expression of CD25^{++}GARP$^+$ for different groups. (a) Control vs. HIV-positive group; (b) Control vs. naïve vs. HIV-treated groups; (c) GARP expression with varying CD4 counts. , GARP increase between CD4 > 500 and CD4 200-500 (* P<0.04), and highly significant when comparing CD4 > 500 and CD4 < 200 (P=0.001) (data presented as mean ± SEM).

B CD4$^+$CD25$^-$GARP$^+$

The expression of GARP on CD25$^-$ cells was robustly increased with all HIV infection groups, with a relatively low CD4 count (P=0.003) (Figure 4.33). The mean percentage of the control group was 0.2 ± 0.1

versus 0.5 ± 0.5, 3.2 ± 6, and 4.5 ± 6 for the groups with CD4 counts > 500, 200–500 and < 200, respectively. The GARP expression in CD25- controls was 0.2 ± 0.1% compared to 2.5 ± 5% in the HIV-positive group (P=0.003).

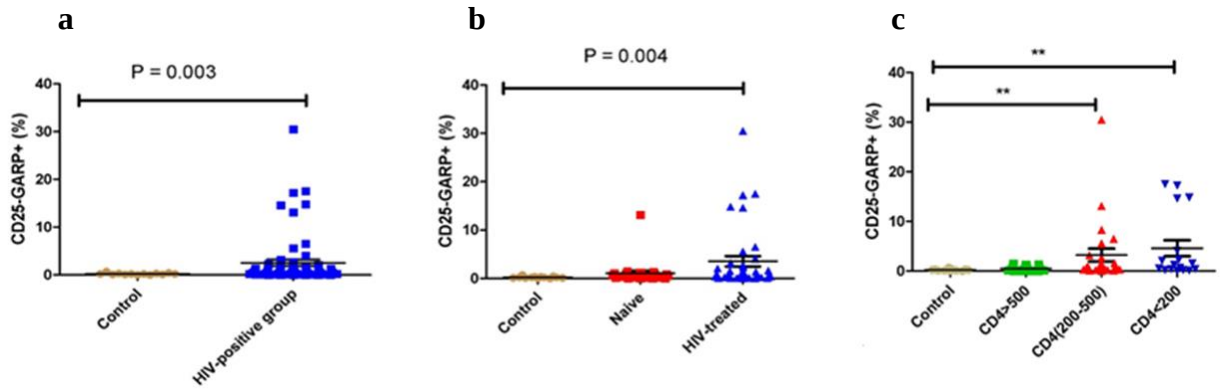

Figure 4-33: Expression of GARP in $CD4^{+}CD25^{-}$ cells. {(a) Control vs. HIV-positive individuals (P=0.003); (b) Control vs. naïve vs. HIV-treated groups (P=0.004); (c) Control group and HIV-positive with varying CD4 counts (data presented as mean ± SEM). ** P=0.003.

C $CD3^{+}CD4^{-}FOXP3^{+}GARP$ ($CD8^{+}FOXP3^{+}GARP$)

GARP expression in $CD8^{+}$ T cells revealed no significant results (Figure 4.34).

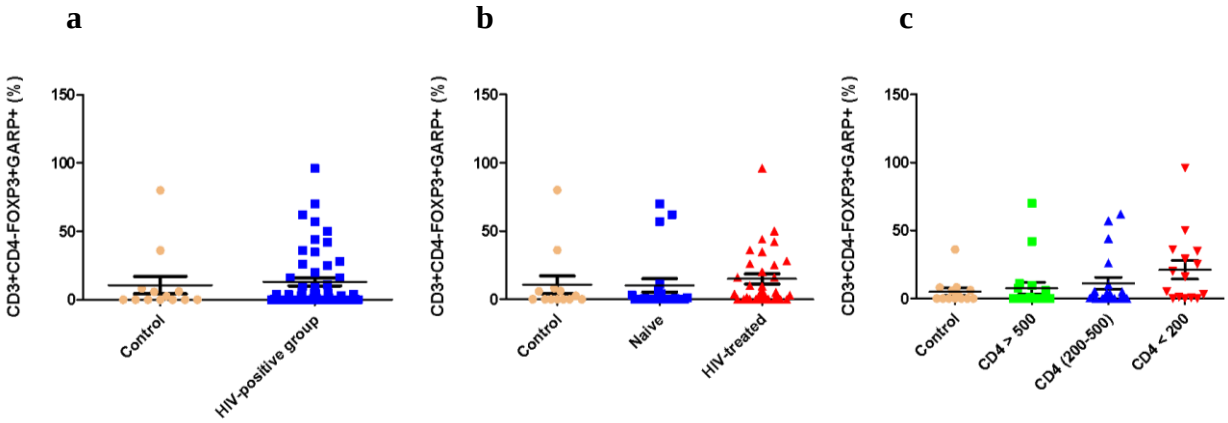

Figure 4-34: GARP expression in CD8+FOXP3+ T cells. (a) Control vs. HIV-positive individuals; (b) Control vs. naïve vs. HIV-treated groups; (c) Control group and HIV-positive individuals with varying CD4 count (P not significant).

4.6.5 Correlation between Treg Markers and Various Immunological Markers

To better understand the role of Treg cells during the different HIV stages, correlations with other immunologic markers were completed as detailed below:

A Correlations between monocyte and Treg subsets

i *$CD14^{+}CD16^{++}$ and $CD4^{+}CD25^{++}FOXP3^{+}SATB\text{-}1$*

The Spearman analysis showed a positive correlation between non-classical monocytes and SATB-1 expressed on $CD25^{++}$ $FOXP3^{+}$ cells (r=0.32; P=0.001) (Figure 4.35).

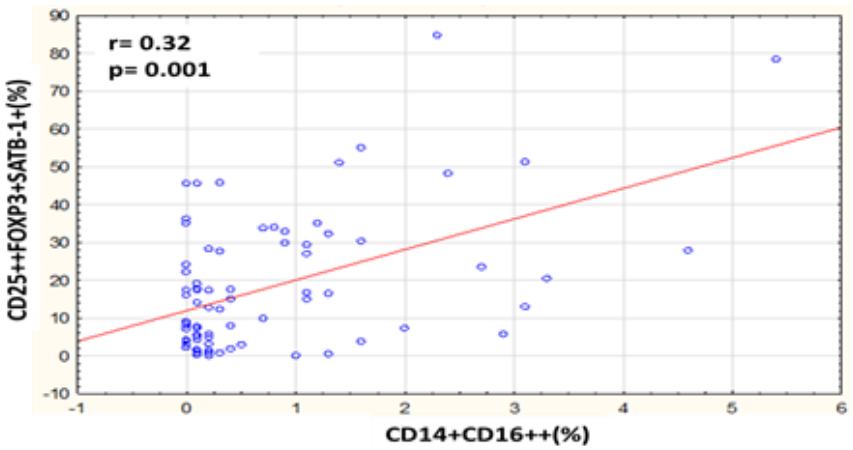

Figure 4-35: Correlation between non-classic monocyte and SATB-1. Linear regression analysis showing a significant positive relationship between $CD25^{++}FOXP3^{+}SATB\text{-}1^{+}$ and $CD14^{+}CD16^{++}$.

ii *$CD14^{+}CD16^{+}$ and $CD4^{+}CD25^{++}FOXP3^{+}SATB\text{-}1$*

The correlation of intermediate monocytes and activated marker SATB-1 expressed on $CD25^{++}FOXP3^{+}$ also showed a positive correlation (r=0.27; P=0.02) (Figure 4.36).

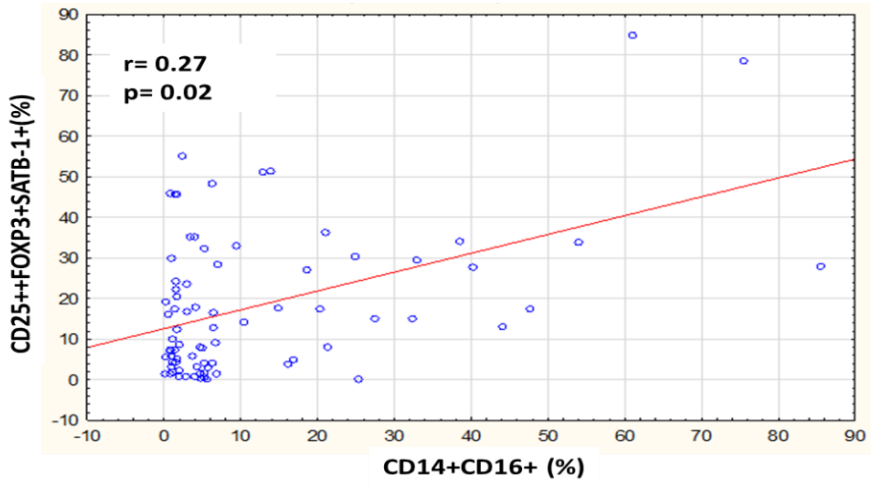

Figure 4-36: Correlation between intermediate monocytes and SATB-1. Linear regression showing a positive correlation between $CD14^{+}CD16^{+}$ and $CD25^{++}FOXP3^{+}$ SATB-1.

iii *$CD14^{+}CD16^{-}$ and $CD25^{++}FOXP3^{+}SATB\text{-}1$*

However, the findings show that the classical monocyte subset correlated negatively with the $CD25^{++}FOXP3^{+}$ SATB-1 subset (r = - 0.28; P=0.01) (Figure 4.37).

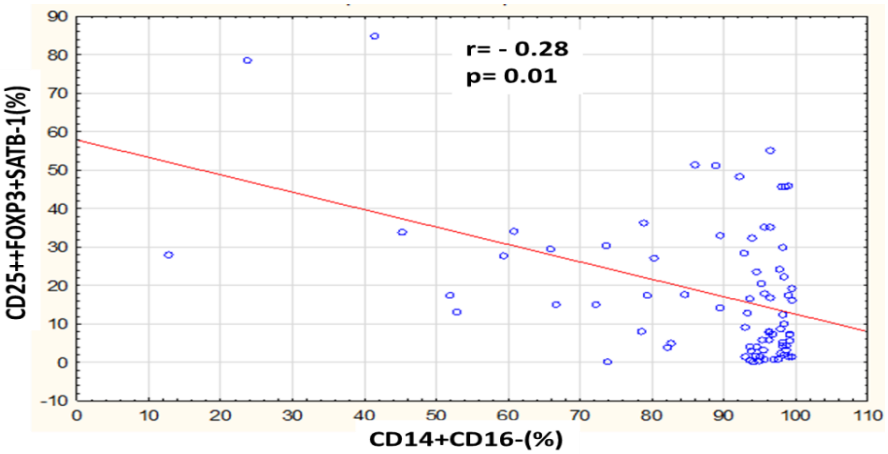

Figure 4-37: Negative correlation between $CD25^{++}FOXP3^{+}SATB\text{-}1$ and $CD14^{+}CD16^{-}$ cells.

iv Correlation between CD14+CD16++and CD25++GARP+

The correlation between non-classical monocytes and the activation marker GARP produced a positive correlation (r=0.42; P=0.001) (Figure 4.38).

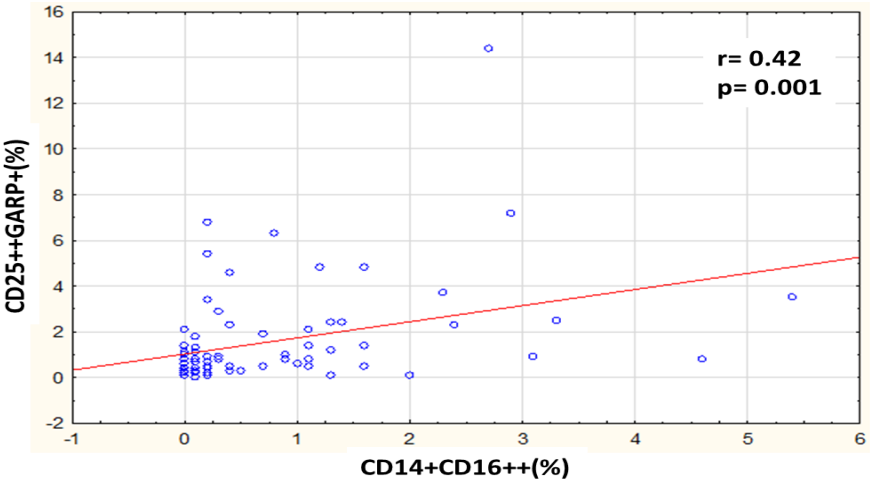

Figure 4-38: The correlation between non-classic monocytes and $CD25^{++}$GARP. Linear regression showing a positive relationship between non-classical monocytes and GARP expression on $CD25^{++}$.

v Correlation of $CD14^{+}CD16^{++}$ and $CD4+CD25^{-}GARP$

The correlation between non-classical monocytes and GARP ($CD4+CD25^{-}GARP$) also generated a positive correlation (r=0.43; P=0.001) (Figure 4.39).

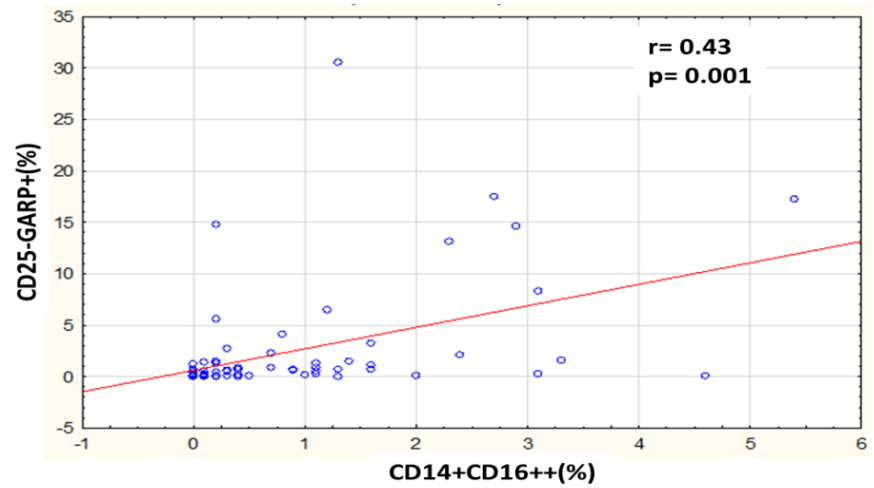

Figure 4-39: The correlation between non-classic monocytes and CD25$^-$ GARP. Linear regression with significant positive correlation between CD25$^-$ GARP$^+$ and CD14$^+$CD16^{++} (r=0.43; P=0.001).

vi Correlation between CD14$^+$CD16$^+$ subset and GARP expression

Correlations were performed between CD14$^+$CD16$^+$ and GARP expressed on CD25^{++} and CD25$^-$ cells (Figure 4.40). The results showed a positive correlation between CD14$^+$CD16$^+$ and CD25^{++}GARP$^+$ (r=0.41; P=0.001) and also between CD14$^+$CD16$^+$ and CD25$^-$GARP$^+$ (r=0.25; P=0.03).

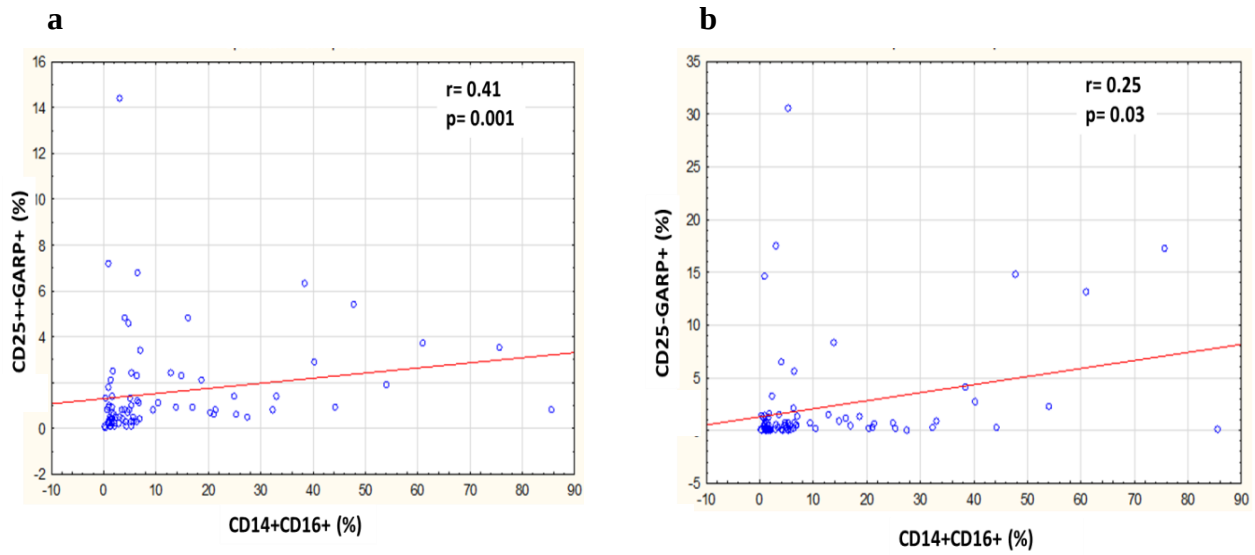

Figure 4-40: The correlation between intermediate monocytes and GARP expression. Positive linear regression between the monocyte subset, CD14$^+$CD16$^+$ and GARP expression on (a) CD25^{++} and on (b) CD25$^-$ cells.

vii Correlation between classical monocytes and GARP

Spearman correlations between classical monocytes ($CD14^{+}CD16^{-}$) and GARP expression were also performed on $CD25^{++}$ $CD4^{+}$ and $CD25^{-}$ $CD4^{+}$ cells, respectively (Figure 4.41). Here data revealed a negative relationship between $CD14^{+}CD16^{-}$ and $CD25^{++}$GARP (r = - 0.46; P=0.001) and also with $CD25^{-}$GARP (r = - 0.31; P=0.01) (table 4.6).

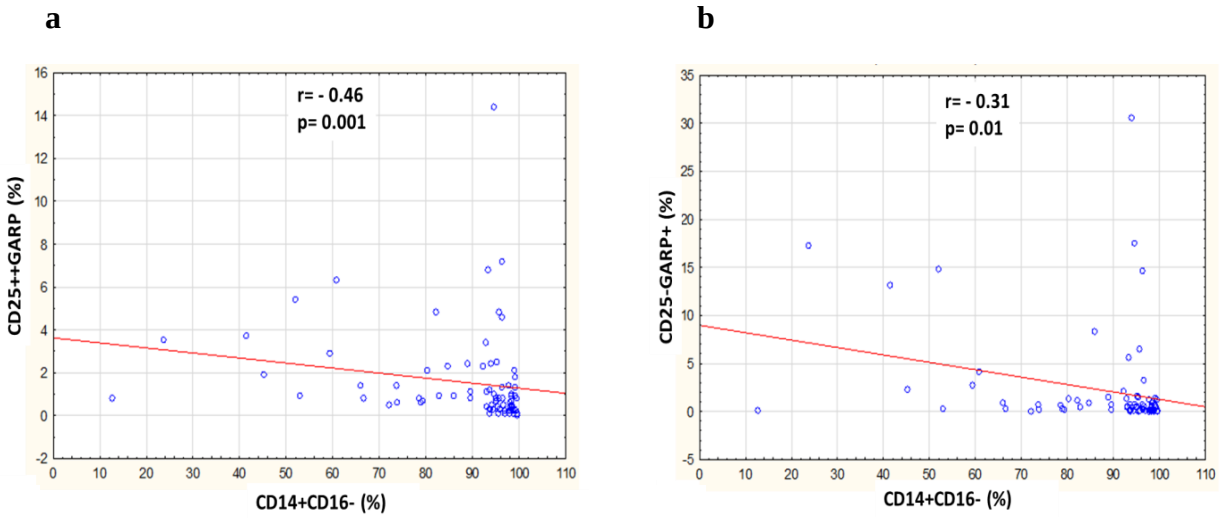

Figure 4-41: Correlation between CD14+CD16- and the expression of GARP. (a) $CD25^{++}GARP^{+}$ and (b) $CD25^{-}GARP^{+}$.

Table 4-6: Comparison between Treg subset (SATB-1, GARP and FOXP3) correlationsand HIV infection

Variable	SATB-1	GARP	FOXP3
VL (C/mL)	N/S	r = 0.40 & P = 0.001	N/A
CD4 (cell/µL)	N/S	r = - 0.44 & P =0.0002	r = -0.52 & P = 0.00006
$CD8^{+}38^{+}$ (%)	r = 0.39 &, P = 0.001	r = 0.43 & P = 0.001	r = 0.34 & P = 0.001
$CD8^{+}142^{+}$ (%)	r = 0.31 & P = 0. 008	r = 049 & P = 0.001	N/S
$CD8^{+}142^{+}38^{+}$ (%)	r = 0.43 & P = 0.001	r = 0.55 & P =0.0001	r = 0.20 P = 0.08
$CD14^{+}CD16^{++}$ (%)	r = 0.41 & P = 0.001	r = 0.42 & P = 0.001	r = 0.23 & P = 0.04
$CD14^{+}CD16^{+}$ (%)	r = 0.27 & P = 0.02	r = 0.41 & P = 0.001	N/S
$CD14^{+}CD16^{-}$ (%)	r = - 0.28 & P = 0.01	r = - 0.46 & P = 0.001	N/S

VL - viral load; **CD8/38 -** immune activation markers; **CD8/142 -** coagulation marker; **CD8/38/142 -** immune activation and coagulation markers; **$CD14^{+}CD16^{++}$** - non-classical monocytes; **$CD14^{+}CD16^{+}$**- intermediate monocytes; **$CD14^{+}CD16^{-}$** - classical monocytes; **FOXP3 -** fork head boxp3.

4.7 C-REACTIVE PROTIEN COMPARISONS

As CRP is robust marker linked to acute myocardial infarction in HIV pathogenesis (Triant et al., 2009), we also evaluated its levels in the different groups. Here we found a significant increase in CRP levels in the group with a relatively low CD4 count, i.e. 3.3 mg/L and 23 mg/L for the control and the group with a CD4 count < 200, respectively (Figure 4.42).

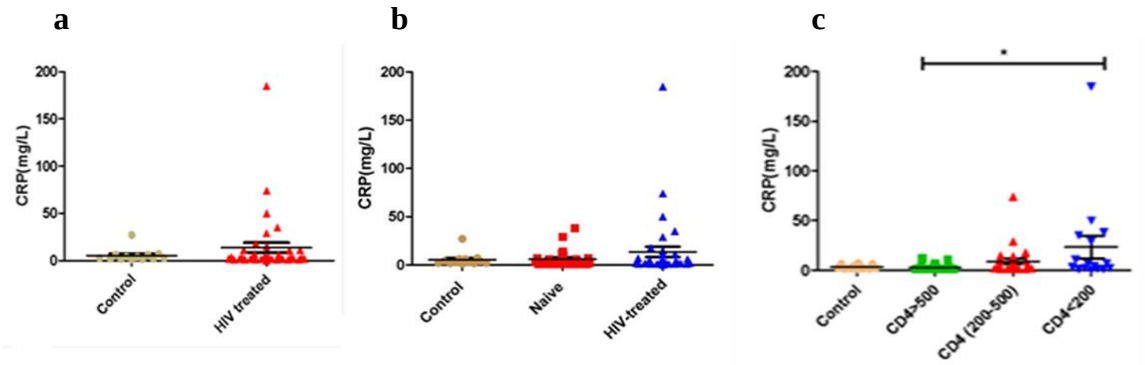

Figure 4-42: Evaluation of CRP levels in study cohort. (a) Controls vs. HIV-positive group; (b) Control vs. naïve vs. HIV-treated groups; (c) Comparison of CRP levels with varying CD4 counts (data presented as mean ± SEM). * P=0.01.

4.8 FLOW MEDIATED DILATATION

FMD is defined as the percentage change in diameter and is employed an indicator of nitric oxide (NO)-mediated vasodilatation (endothelial function). There are no standard cut-off a value available in the literature, but generally an FMD of 6% or higher is regarded as a normal value. Our data revealed that FMD percentages between study groups were not significant (Figure 4.43). The results did, however, show a slight improvement in endothelial function in the HIV-positive group versus controls. In addition, there was a small improvement in the HIV-positive group with a CD4 count > 500 versus controls (not statistically significant). The mean FMD percentage in the control group was 4.7 ± 3 compared to 7.3 ± 4 for the HIV-positive group (refer Addendum B for complete data set).

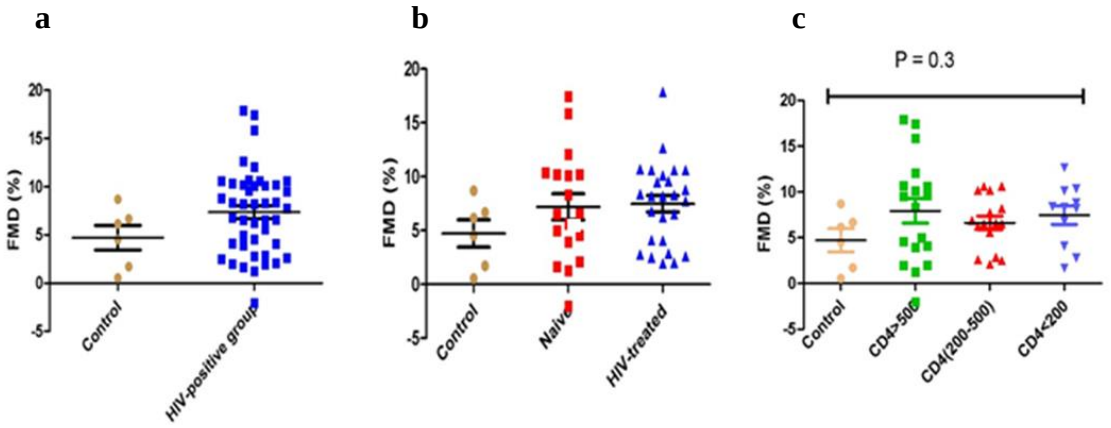

Figure 4-43: FMD (%) with HIV infection. (a) Control vs. HIV-positive patients; (b) Control vs. naïve vs. HIV-treated groups; (c) Control and HIV-positive groups with varying CD4 counts.

4.9 TRADITIONAL LIPID PROFILE

Plasma levels of a traditional lipid panel (total cholesterol, triglycerides, LDL and HDL) were determined using standard methods.

4.9.1 Total Cholesterol

Evaluation of total cholesterol (TC) in the study groups showed a significant decrease with HIV infection (P=0.005) (Figure 4.44). Further comparisons demonstrated a decrease in TC levels for individuals with a CD4 count < 200 versus the control group (P=0.02).

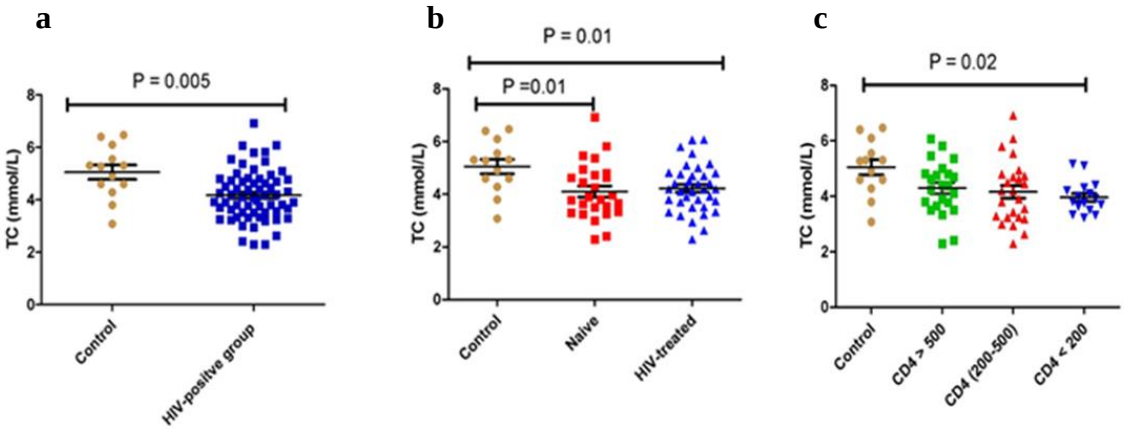

Figure 4-44: Evaluation of TC in study groups. (a) Control vs HIV-positive patients (P = 0.005); (b) Control vs. naïve vs. HIV-positive treated groups (5 ± 0.9 mmol/L vs. 4.1 ± 1 mmom/L vs. 4.2 ± 0.8 mmoml/L;P =0.01); (c) TC levels with varying CD4 counts (P=0.02) (data presented as mean ± SEM).

4.9.2 Triglyceride Levels

The results revealed a decrease in TG levels for the HIV-positive group versus controls (P=0.001) (Figure 4.45). Further analyses showed highly significant results between the controls and the groups with varying CD4 counts (200-500) and CD4 counts < 200 (Figure 4.45).

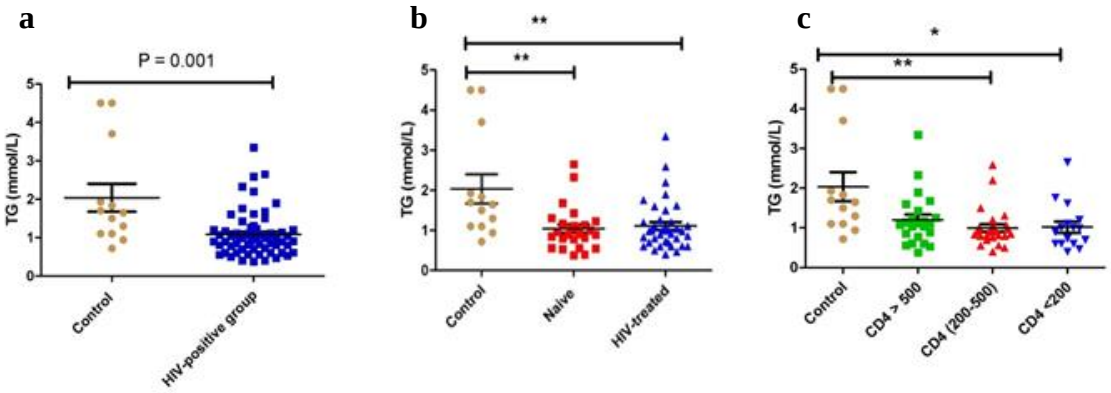

Figure 4-45: Assessment of TG levels with HIV infection. Controls vs. HIV-positive group (P=0.001); (b) Control vs. naïve vs. HIV-treated groups (P=0.004); (c) Comparisons with varying CD4 counts (data presented as mean ± SEM). ** P=0.003 and * P=0.01.

4.9.3 High-density lipoprotein Levels

We found no significant differences for HDL or LDL levels for any of the analyses completed (Figures 4.46 and 4.47).

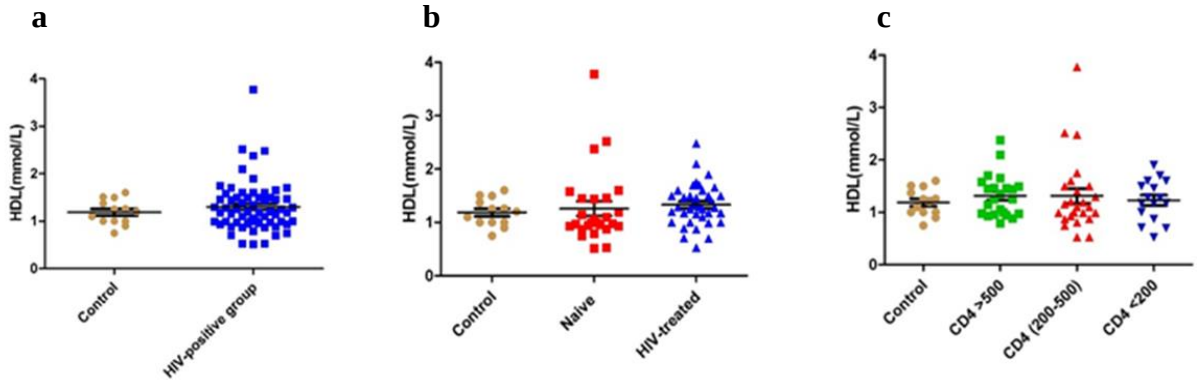

Figure 4-46: Evaluation of HDL levels with HIV infection. (a) Controls vs. HIV-positive group(P=0.2); (b) Control vs. naïve vs. HIV-treated groups (P=0.08); (c) Comparison of HDL levels for HIV-positive individuals with varying CD4 counts.

4.9.4 Low-density lipoprotein levels

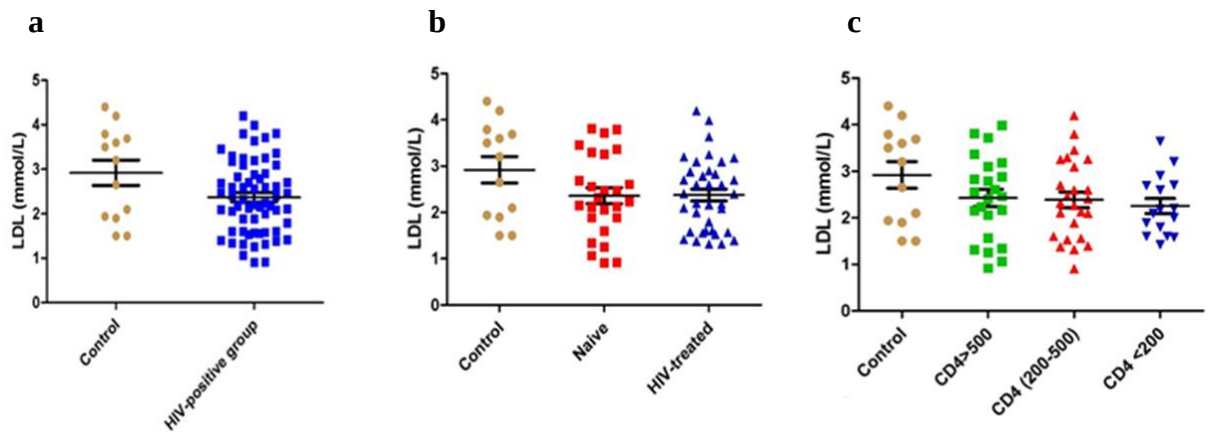

Figure 4-47: Evaluation of LDL levels with HIV infection. (a) Controls vs. HIV-positive group (P=0.09); (b) Control vs. naïve vs. HIV-treated groups (P=0.2); (c) Comparison of LDL levels for HIV-positive individuals with varying CD4 counts

However, more detailed analyses revealed that LDL and total cholesterol levels were decreased in individuals not on treatment yet and also for those receiving second line treatment (Tables 4.7 and 4.8).

Table 4-7: Summary of lipid data

Marker	Control (N=21)	HIV naïve (N=18)	HIV therapy (N=45)
Body mass index (kg/m^2)	29.1 ± 1.5	21.5 ± 1.6**	25.5 ± 1.0#
HDL-C (mmol/L)	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
LDL-C (mmol/L)	3.0 ± 0.2	2.4 ± 0.2	2.4 ± 0.1*
Triglycerides (mmol/L)	1.4 ± 0.2	1.0 ± 0.2*	1.2 ± 0.1
Total cholesterol (mmol/L)	4.8 ± 0.2	4.3 ± 0.2*	4.3 ± 0.2*

Table 4-8: Summary of the lipid data

Marker	Control (N=21)	HIV first line therapy (N=21)	HIV second line therapy (N=24)	HIV naïve (N=18)
Body mass index (kg/m^2)	29.1 ± 1.5	24.3 ± 1.5*	26.6 ± 1.4	21.4 ± 1.6**
HDL-C (mmol/L)	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
LDL-C (mmol/L)	3.0 ± 0.2	2.6 ± 0.2	2.3 ± 0.2*	2.4 ± 0.2
Triglycerides (mmol/L)	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.1	1.1 ± 0.2*
Total cholesterol (mmol/L)	4.8 ± 0.2	4.5 ± 0.2	4.2 ± 0.2*	4.3 ± 0.2*

* p<0.05 vs. control; ** p<0.005 vs. control

Correlations were next performed between the traditional lipid biomarkers (HDL and LDL) and various immunological markers. However, no significant correlations were found in this instance (Tables 4.8 and 4.9).

Table 4-9: Correlation between HDL and immune markers.

HDL vs	r- value	p-value
$CD14^{+}CD16^{++}$	-0.10	0.39
$CD14^{+}CD16^{+}$	0.17	0.15
$CD14^{+}CD16^{-}$	-0.13	0.27
$CD8^{+}CD142^{+}$	-0.12	0.29

Table 4-10: Correlation between LDL and immune markers

LDL vs	r-value	p-value
$CD14^{+}CD16^{++}$	-0.13	0.25
$CD14^{+}CD16^{+}$	0.14	0.22
$CD14^{+}CD16^{-}$	-0.15	0.18
$CD8^{+}CD142^{+}$	-0.09	0.46

4.10 EVALUATION OF HDL AND LDL SUBCLASSES

4.10.1 HDL Subclasses

To gain additional insight into lipid profiles an electrophoretic method was employed (Lipoprint LDL System, Quantimetrix, Redondo Beach CA) that allows for the analysis of 12 lipoprotein sub-fractions, i.e. VLDL, IDL 1-3, LDL 1-7 and HDL. Here there was an increase in the large HDL subclass for all HIV-infected individuals together with decreased small HDL subclasses (Figures 4.48 and 4.49).

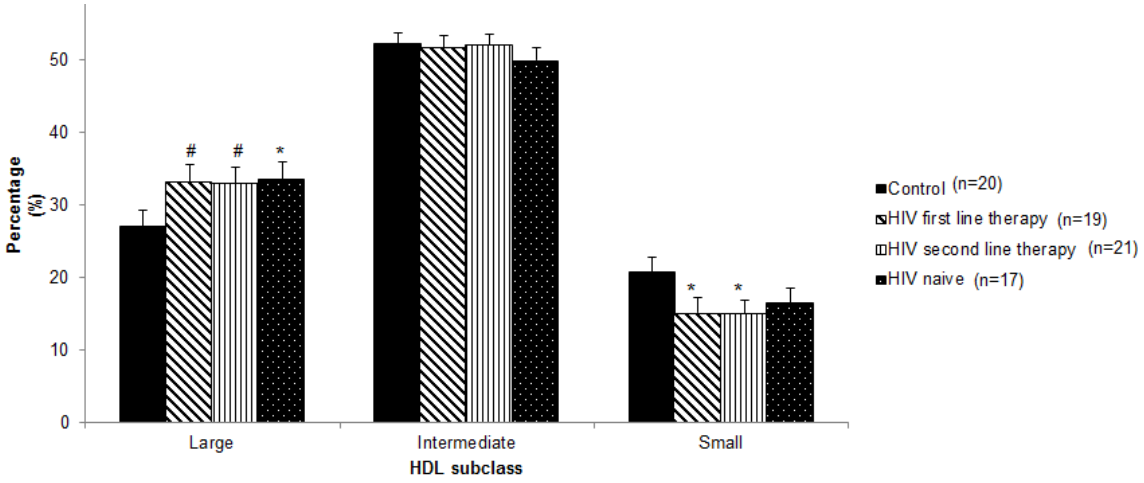

Figure 4-48: .Distribution of HDL subclasses in control and HIV individuals. Results represent means ± SEM. # p=0.05 and * p<0.05 compared to controls.

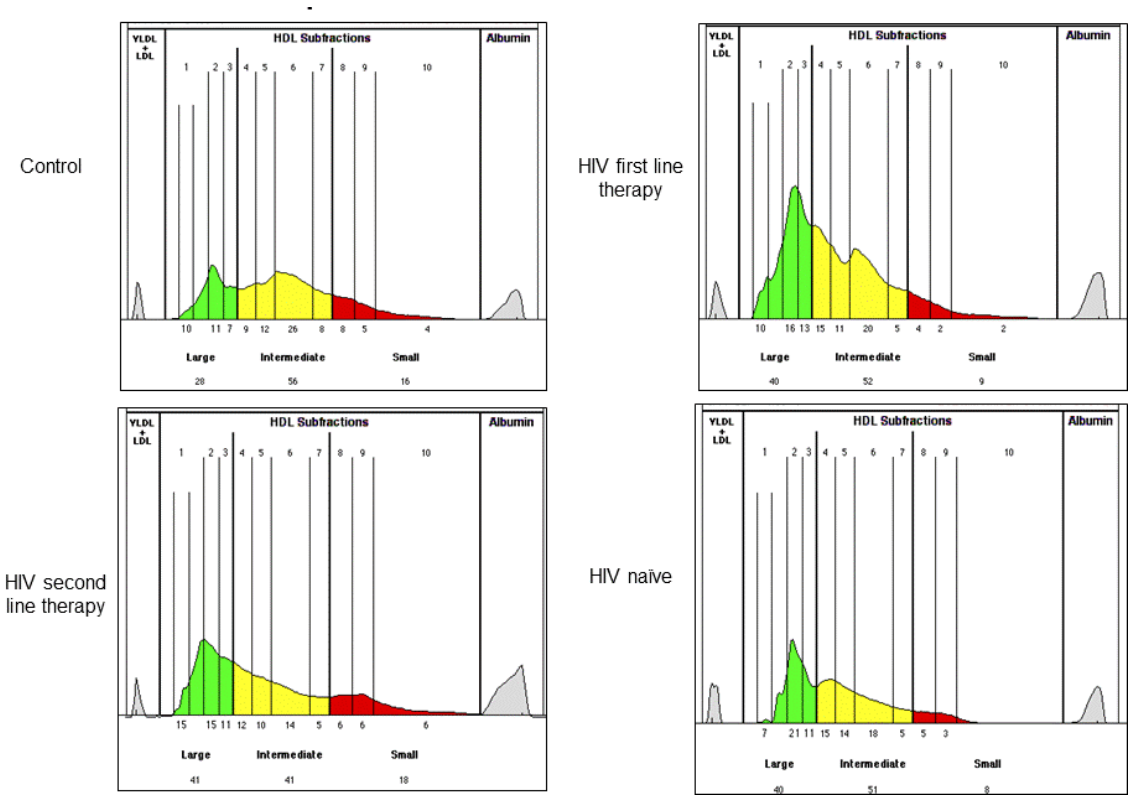

Figure 4-49: Representative HDL subclasses scan results

Table 4-11: HDL subclass data

V		Age	SBP	DBP	HR	TC	TG	HDL-C	LDL-C	CD4-N	CD4-C	BMI	WC
L-HDL	C	-0.10	-0.27	-0.08	0.25	0.28	-0.49*	0.51*	0.35	--	--	-0.42	-0.47*
	1st line-T	0.00	0.13	0.14	0.32	-0.17	-0.35	0.67**	-0.41	-0.47*	0.20	-0.09	-0.18
	2nd line-T	-0.06	-0.36	-0.39	-0.10	-0.11	-0.21	0.40	-0.26	-0.31	-0.43	-0.60*	-0.64**
	N	-0.14	0.05	-0.02	0.28	0.27	-0.40	0.72**	-0.16	0.35	0.33	-0.28	-0.35
I-HDL	C	0.42	0.14	-0.19	-0.03	0.26	0.24	0.05	0.14	--	--	-0.22	-0.12
	1st line-T	-0.11	0.14	-0.13	-0.31	0.12	0.30	-0.50*	0.26	0.64**	-0.18	0.23	0.25
	2nd line-T	0.17	0.50*	0.38	-0.01	0.24	0.29	-0.36	0.38	0.18	0.41	0.46*	0.49*
	N	-0.29	-0.26	-0.02	-0.27	-0.50*	-0.01	-0.69**	-0.04	-0.28	-0.20	0.33	0.26
S-HDL	C	-0.14	0.16	0.17	-0.20	-0.40	0.30	-0.48*	-0.39	--	--	0.50*	0.48*
	1st line-T	0.12	-0.35	-0.08	-0.13	0.11	0.18	-0.44	0.30	0.00	-0.13	-0.13	-0.01
	2nd line-T	-0.06	0.05	0.19	0.14	-0.08	0.03	-0.23	0.01	0.28	0.23	0.45*	0.47*
	N	0.44	0.17	0.04	-0.10	0.13	0.49	-0.25	0.23	-0.18	-0.23	0.04	0.18

V – variable; **L-HDL** - large- HDL subclass; **I-HDL** - intermediate-HDL subclass; **S-HDL** - small-HDL subclass; **C** - control; **1st line-T** - first line therapy; **2nd line-T** - second line therapy; **N** - naïve; **WC** - waist circumference; **BMI** - body mass index; **CD4-N** = CD4nadir; **CD4-C** - current CD4 count; **SBP** - systolic blood pressure; **DBP** - diastolic blood pressure; **HR** - heart rate; **TC** - total cholesterol; **TG** - triglyceride; **HDL-C** - high density lipoprotein-cholesterol; **LDL-C** - low density lipoprotein –cholesterol; $P < 0.05$, * $P < 0.005$**.

These correlations show that the large HDL subclass is generally associated with beneficial risk factor profiles, i.e. with increased HDL-C levels and decreased TG levels together with lowered BMI and waist circumference. The intermediate and small HDL subclasses in HIV-positive individuals were associated with more harmful risk profiles, i.e. with lowered HDL-C levels, increased BMI and waist circumference, greater CD4 nadir counts and higher systolic blood pressures (Table 4.11). Additional analyses were performed and showed a significant positive correlation between I-HDL and immune activation markers in naïve patients (r=0.54; P=0.02). I-HDL also correlated positively with classical monocytes (r=0.54; P=0.02) and negatively with intermediate monocytes (r = - 054; P=0.02) as illustrated in Table 4.12.

Table 4-12: Correlation of HDL subclasses with immunological markers

V		CD8+ CD142+%	CD8+ CD38+%	CD14+ CD16++%	CD14+ CD16+%	CD14+ CD16-%	CD8+ CD142+ CD38+%	CD4+ FoxP3+ %	CD25++ SATB-1+ %	CD25++ GARP+ %
L-HDL	C	.0167	-.2507	-.0076	-.1487	.1483	-.4016	.2913	-.1576	.1098
	N	.1033	-.3294	.1239	.0643	-.0939	-.1199	.0698	.2140	-.1305
	T	.1217	.1075	.0762	.1478	-.1462	-.0612	.0428	.0149	.2484
I-HDL	C	.1618	-.7753	.2629	.3754	-.3731	-.2828	-.0709	.1883	-.2071
	N	.0740	.5403	-.1147	-.5308	.5486	.3842	.1570	.0599	.2709
	T	-.1163	-.0066	-.0146	-.2397	.2377	.0139	.0617	-.0079	-.1304
S-HDL	C	-.1126	.6828	-.1544	-.1050	.1039	.5113	-.2001	.0183	.0359
	N	-.1876	-.0822	-.0421	.4048	-.3843	-.1829	-.2183	-.3136	-.0835
	T	-.0597	-.1474	-.0906	.0299	-.0302	.0726	-.1181	-.0010	-.2250

L-HDL - large high density lipoprotein; **I-LDL** - intermediate high density lipoprotein; **S-HDL** - small high density lipoprotein; **C** - control; **N** - naïve; **T** - treated; **$CD8^+CD38^+$** - immune activation marker; **$CD8^+CD142^+$** - coagulation marker; **$CD14^+CD16^{++}$** - non-classical monocytes; **$CD14^+CD16^+$** - intermediate monocytes; **$CD8^+CD142^+CD38^+$** - co-expression of immune activation and coagulation markers on CD8 T cells; **$CD4^+FOXP3^+$** - regulatory T cell; **$CD25^{++}SATB\text{-}1$** - activation markers for Treg cells; **$CD25^{++}GARP$** - activation markers of Treg cells.

4.10.2 LDL Subclasses Evaluation

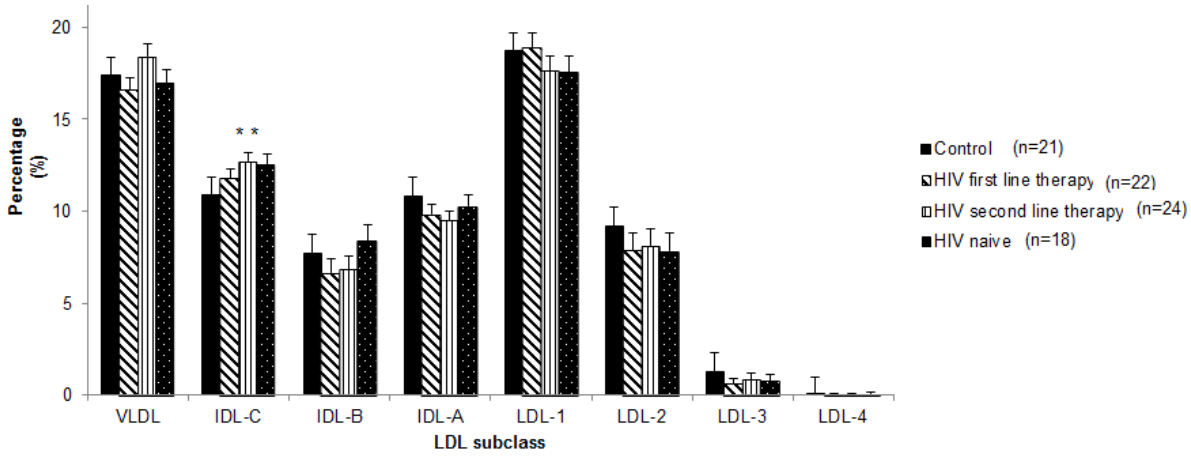

Figure 4-50: Distribution of LDL subclasses in control and HIV-infected individuals. Results are presented as mean ± SEM. #P = 0.05, *P < 0.05 compared to controls.

For the LDL subclass analysis, the results show limited changes except for significantly higher IDL-C levels in the HIV-positive groups, with this specifically manifesting in the naïve and second line treatment groups (Figures 4.50 and 4.51). More correlations were performed by comparing the different LDL subclasses with various parameters (refer Table 4.13). Focusing on the IDL subclass, these data show a significant positive correlation with total cholesterol and LDL for the control group. For the HIV-positive groups there was also a positive correlation with LDL and a concomitant negative correlation with HDL.

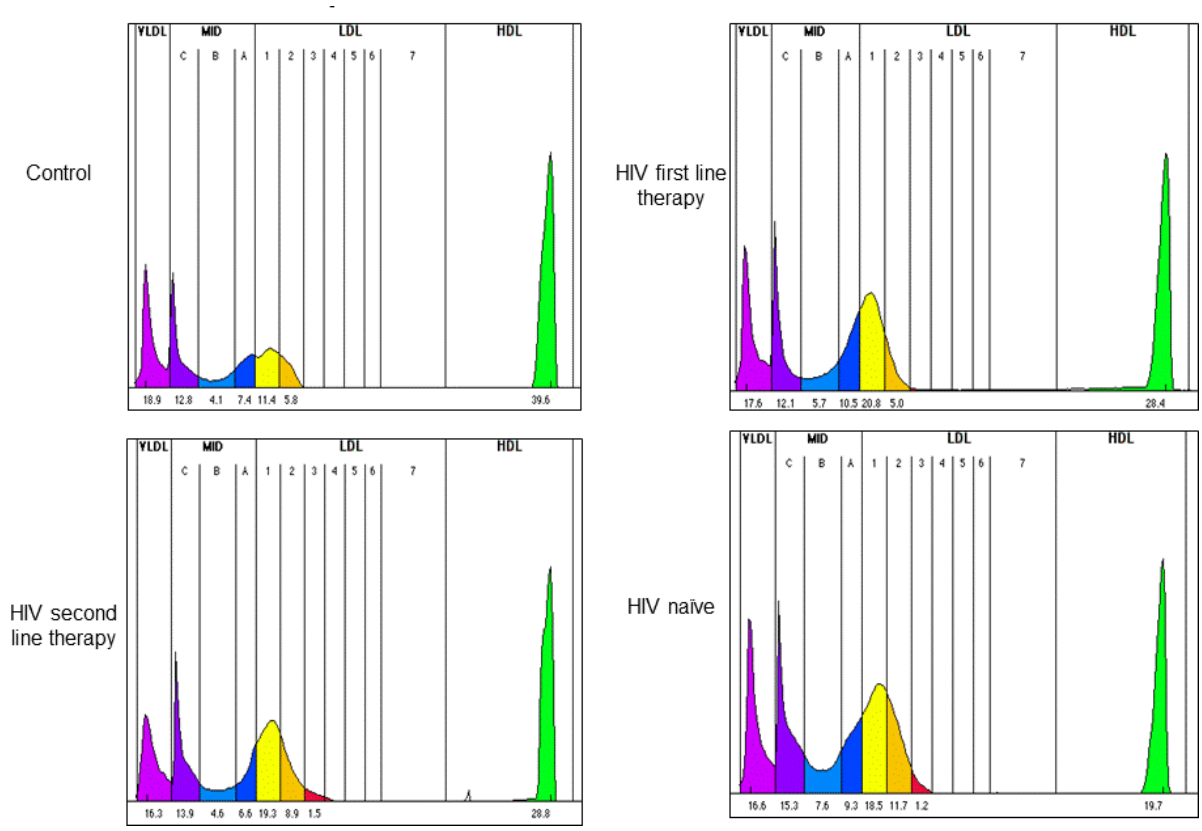

Figure 4-51: Representative LDL subclass scans results.

Table 4-13: Correlations between LDL subclasses versus various parameters

V		Age	SBP	DBP	HR	TC	TG	HDL-C	LDL-C	CD4-N	CD4-C	BMI	WC
VLDL	C	0.26	0.13	-0.19	-0.24	0.11	0.68**	-0.22	-0.13	--	--	0.01	-0.02
	1st line-T	0.08	-0.06	-0.19	-0.17	0.14	0.62**	-0.01	-0.25	0.34	0.51*	0.52*	0.35
	2ndline-T	0.19	0.00	-0.07	0.10	-0.12	0.58*	-0.18	-0.28	0.25	-0.17	0.03	0.23
	N	-0.05	0.41	0.45	-0.14	-0.23	0.40	-0.56*	0.08	-0.29	-0.18	0.39	0.44
IDL-C	C	0.23	-0.25	-0.39	-0.27	0.47*	0.14	-0.22	0.50*	--	--	-0.05	-0.14
	1st line-T	-0.06	0.09	0.25	-0.07	0.19	0.24	-0.61**	0.44*	0.20	-0.16	0.00	0.20
	2nd line-T	-0.15	-0.15	-0.27	-0.04	-0.37	-0.34	-0.48*	-0.11	-0.32	-0.32	-0.12	-0.27
	N	0.01	-0.51*	-0.30	-0.13	0.23	0.10	-0.45	0.61*	0.32	0.32	-0.07	-0.02
IDL-B	C	-0.05	-0.19	-0.20	0.03	-0.12	-0.22	0.08	-0.04	--	--	-0.20	-0.11
	1st line-T	-0.08	0.02	-0.44*	-0.22	-0.30	-0.28	-0.06	-0.11	-0.24	-0.16	-0.18	-0.29
	2nd line-T	-0.15	-0.03	-0.11	-0.08	-0.42*	-0.37	-0.39	-0.21	-0.33	-0.14	0.27	0.12
	N	0.07	-0.28	0.12	-0.44	0.14	0.25	-0.40	0.43	0.10	0.06	0.63*	0.65**
IDL-A	C	-0.49*	0.16	0.08	0.14	-0.13	-0.53*	0.35	0.00	--	--	0.07	-0.08
	1st line-T	0.07	0.01	-0.23	-0.17	-0.29	-0.54*	0.35	-0.18	-0.33	-0.20	-0.21	-0.30
	2nd line-T	-0.14	0.02	-0.10	-0.13	-0.01	-0.40	-0.03	0.16	-0.25	0.23	0.18	0.04
	N	-0.24	-0.29	-0.13	-0.27	-0.02	0.02	-0.24	0.17	-0.07	-0.06	0.21	0.13
LDL-1	C	-0.12	0.03	0.05	0.02	0.39	-0.29	-0.07	0.57*	--	--	0.13	0.08
	1st line-T	-0.14	0.00	0.19	-0.24	0.20	-0.42	-0.02	0.51*	-0.06	-0.12	-0.22	-0.07
	2nd line-T	-0.16	0.10	0.05	0.04	0.28	-0.23	0.08	0.42*	-0.13	0.28	0.11	0.08
	N	-0.13	-0.05	-0.24	0.14	-0.19	-0.13	-0.31	0.05	-0.07	-0.05	-0.61*	-0.55*
LDL-2	C	0.48*	0.06	0.05	-0.24	0.37	0.51*	-0.41	0.27	--	--	0.09	0.23
	1st line-T	0.06	-0.19	0.10	0.08	0.35	0.54*	-0.62**	0.41	0.28	0.01	0.15	0.19
	2nd line-T	-0.04	-0.01	0.09	0.30	0.47*	0.42*	-0.06	0.49*	0.26	0.19	-0.16	-0.01
	N	0.26	0.07	-0.12	0.23	0.21	0.36	-0.14	0.27	-0.09	-0.09	-0.35	-0.23
LDL-3	C	0.39	0.18	0.03	-0.31	0.26	0.66**	-0.39	0.09	--	--	0.15	0.29
	1st line-T	-0.16	0.06	0.32	0.15	0.11	0.16	-0.41	0.26	0.36	0.01	0.07	-0.03
	2nd line-T	-0.26	-0.12	0.07	0.00	0.36	0.11	-0.05	0.45*	-0.01	-0.16	-0.26	-0.22
	N	0.23	-0.06	-0.09	0.15	0.01	0.08	0.01	-0.02	-0.15	-0.10	-0.28	-0.20
LDL-4	C	--	--	--	--	--	--	--	--	--	--	--	--
	1st line-T	-0.36	-0.04	0.15	0.20	0.01	-0.07	-0.24	0.20	0.11	-0.08	-0.17	-0.23
	2nd line-T	-0.16	-0.12	0.36	0.14	-0.05	-0.16	0.07	-0.06	0.10	0.09	0.18	0.01
	N	0.03	-0.29	-0.08	-0.20	-0.20	-0.28	-0.11	-0.08	-0.06	0.03	-0.34	-0.27

V - variable; **IDL** - intermediate density lipoprotein; **VLDL** - very low density lipoprotein; **LDL** - low density lipoprotein; **C** - control; **1st line-T** - first line therapy; **2nd line-T** - second line therapy**; N** - naïve; **WC** - waist circumference; **BMI** - body mass index; **CD4-N** - CD4 nadir; **CD4-C** - current CD4 count; **SBP** - systolic blood pressure; **DBP** - diastolic blood pressure; **HR** - heart rate; **TC** - total cholesterol; **TG** - triglyceride; **HDL-C** - high density lipoprotein- cholesterol; **LDL-C** - low density lipoprotein –cholesterol; *P < 0.05, **P < 0.005

Correlations between LDL subclasses and immunological markers revealed that IDL-C in HIV-positive individuals correlated positively with immune activation (r=0.45; P=0.009) and Treg markers (r=0.35; P=0.04). In addition, IDL-B in HIV-infected patients correlated positively with Treg markers (r=0.45; P=0.009). However, the correlation in naïve patients showed a positive result between the IDL-A and immune activation (r=0.48; P=0.04) and highly significant results with $CD8^+CD38^+CD142^+$ (r=0.76; P=0.001) (refer Table 4.14).

Table 4-14: Correlations between LDL subclasses and immunological markers

V		CD8+ CD142+ %	CD8+ CD38+ %	CD14+ CD16++ %	CD14+ CD16+ %	CD14+ CD16- %	CD8+ CD142+ CD38+ %	CD4+ FoxP3+ %	CD25++ SATB-1+ %	CD25++ GARP+ %
VLDL	C	-.0148	-.0635	-.0114	.1066	-.1070	.4216	-.0432	.6498	-.3595
	N	-.0270	.1594	.0076	-.0943	.0845	.1434	-.0923	.0742	.1182
	T	.1208	.1926	-.0441	-.3103	.3175	.2875	.1255	.1013	.0433
IDL-C	C	-.2231	-.2626	-.2134	-.2154	.2173	-.2358	.4762	-.1476	.0133
	N	.2472	.1616	-.0632	-.1342	.1431	.1672	.0304	.0317	-.1359
	T	.2653	.4567	.3348	.0322	-.0452	.3236	.3578	.2398	.2631
IDL-B	C	.1276	-.0948	.1001	.1471	-.1461	.1651	.1583	.3647	-.2224
	N	.0385	.0205	-.1163	-.1390	.1318	.1840	-.0569	-.1262	-.0452
	T	.1842	.3150	.2320	-.0448	.0539	-.0400	.4563	.2474	-.0023
IDL-A	C	.4892	.2238	.4096	.3220	-.3215	.2372	-.1322	-.0931	.2445
	N	.1618	.4826	-.0862	-.2610	.2488	.7632	-.2358	.0183	-.1016
	T	-.0289	-.0623	.1289	.2759	-.2724	-.2716	.1846	.0981	-.1482
LDL-1	C	.0179	.0034	-.0200	-.0535	.0556	-.3792	.2025	-.6769	.3142
	N	.2847	.4575	.4010	.3715	-.3440	.0306	.3864	.2812	.2924
	T	-.0969	-.1462	-.0071	.1817	-.1917	.0178	-.3077	-.1455	-.1519

V = variable; **C** = control; **N** = HIV-naïve; **T** = HIV-treated

IDL – intermediate-density lipoprotein; **VLDL** - very low-density lipoprotein; **C** - control; **N** - naïve; **T** - treated**; V** - variable; $CD8^+CD38^+$ - immune activation marker; $CD8^+CD142^+$ - coagulation markers; **$CD14^+CD16^{++}$** - non-classical monocyte; **$CD14^+CD16^+$** - intermediate monocyte; **$CD8^+CD142^+CD38^+$** - co-expression of immune activation and coagulation on CD8 T cell; **$CD4^+FOXP3^+$** - regulatory T cell; **$CD25^{++}SATB\text{-}1$** - activation markers for Treg; **$CD25^{++}GARP$** - activation markers for Treg cells.

CHAPTER 5 DISCUSSION AND CONCLUSION

The hypothesis of this study was that chronic HIV-1 infection induces changes in monocytes, CD8, CD4 and Treg cells that are associated with risk factors for the development of atherosclerosis. Here markers for immune activation and coagulation, monocyte subsets and Treg cells expressing classical phenotypic ($FOXP3^{+}$) and novel activation markers (SATB-1, GARP) were investigated in a South African cohort. These parameters were then correlated with classic CVD markers (CRP, lipid profile), markers of disease progression (CD4, VL) and FMD. The most original findings of this study are: a) the identification of a unique coagulation marker (CD142) expressed on CD8 and CD4 T cells and its relatively early expression in HIV-infected individuals (naïve). More importantly, it is co-expressed with immune activation and strongly correlates with disease progression markers; b) changes in lipid subclasses that significantly correlated to HIV immunological markers despite a decrease in terms of the traditional lipid profile expected. Such subclass changes may also be a driver for CVD onset, although further research is needed to pursue this interesting notion; and c) upregulation of both anti-inflammatory GARP and pro-inflammatory SATB-1 in Treg cells in HIV-treated individuals (with immune dysregulation) may alter the balance of Treg cell function and also potentially contribute to CVD onset (refer Figure 5.1).

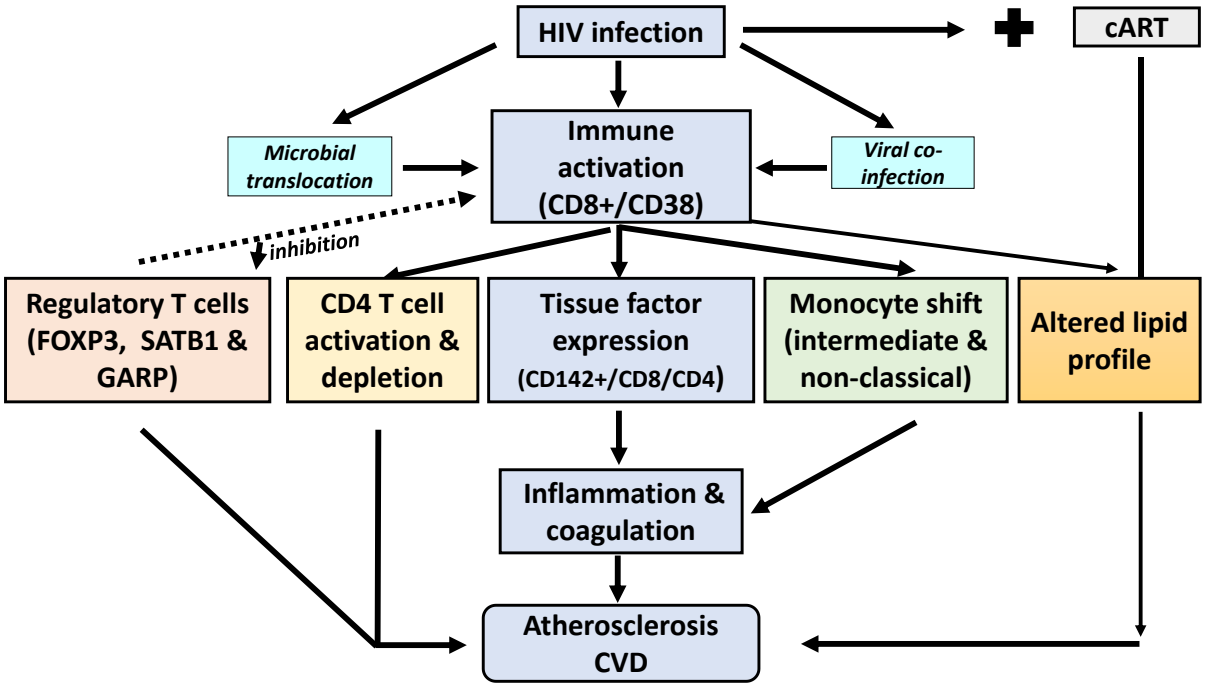

Figure 5-1: Novel findings generated by this study and integration into master model. Our data reveal enhanced immune activation and inflammation (in persons with decreased CD4 counts) together with increased coagulation marker presentation. In addition, there is an upregulation of Treg cell markers and inflammatory monocyte subsets with altered lipid profile. We propose that such effects, together with previously described cART toxicity, may in the longer-term result in the onset of CVD in HIV-infected individuals.

5.1 INCREASED CD8 T CELL-ASSOCIATED IMMUNE ACTIVATION MARKER EXPRESSION

This study confirmed the phenomenon of decreased CD4 counts and higher levels of immune activation (measured by CD38/8 expression) which occur relatively early-on during infection. The results demonstrated significant immune activation in the HIV-positive group with a relatively low CD4 count (< 200). Such correlations are supported by previous studies (Sousa et al., 2002; Bandrea et al., 2010; Paiardini and Muller-trutwin. 2013). In addition, immune activation is highly significant between controls and the HIV-positive group with a CD4 count > 500 (most of the patients in this group are cART naïve). Thus even with viral suppression by cART, high levels of immune activation persist – these findings are in agreement with others (Lederman et al., 2013; Paiardini and Muller-trutwin. 2013). Thus our findings link the persistence of immune activation to CD4 depletion (activation-induced cell death) in HIV-infected individuals. In support, Lederman et al. (2013) recently described a syndrome characterized by increased T cell activation together with high inflammation and coagulation in HIV-treated patients that is referred to as the residual immune dysregulation syndrome (RIDS). RIDS is more common in persons who fail to increase circulating $CD4^+$ T cells to normal levels and is linked to individuals with relatively low CD4 counts. Our results are consistent with the latter notion, i.e. increased inflammation/immune activation in HIV-positive patients that is linked with immune dysregulation and the subsequent activation of coagulation pathways (Baker et al., 2013).

5.2 THE ORGINAL EXPRESSION OF COAGULATION MARKERS (CD142) ON CYTOTOXIC T LYMPHOCYTES (CD8) AND T-HELPER CELLS (CD4)

The current study showed – for the first time as far as we are aware - significant expression of TF (CD142) on CD8 and CD4 T cells during HIV infection. Moreover, this was co-expressed with the immune activation marker $CD38^+$. Such expression in both HIV-naïve and HIV-treated groups demonstrates the role of HIV *per se* in inducing coagulation and inflammation early-on and increasing the risk for both subsets investigated. The current study also revealed that co-expression of immune activation and coagulation markers correlates positively with VL and negatively with CD4 counts, respectively. Furthermore, co-expression of immune activation and coagulation markers correlated positively with intermediate and non-classical monocyte subsets. By contrast, a negative correlation with classical monocytes was found. These data support the concept that the monocyte shift is related to higher immune activation and coagulation during HIV infection. Furthermore, correlations showed a positive relationship between activated Treg cells and increased inflammation and coagulation during HIV infection. TF was also positively correlated to CRP, further supporting a relationship between inflammation and coagulation in HIV-positive persons.

HIV-infected patients display a two- to tenfold higher risk of developing thromboembolic events and a two-fold higher risk of an acute myocardial infarction (Cerrato et al., 2015; Triant et al., 2012). The link between inflammation and coagulation during HIV infection is supported by previous work, e.g. the SMART Study highlighted the role of inflammation and coagulation (IL-6 and D-dimer) in the prediction of all-cause

mortality and CVD events (Kuller et al., 2008). Levi et al. (2003 and 2004) also reported that TF is usually expressed on mononuclear cells. However, the disturbance of vascular integrity and a relatively high levels of pro-inflammatory cytokines (IL-6) activate TF on mononuclear cells in circulation, which in turn can activate coagulation pathways (Funderburg and Lederman. 2014) (Figure 5.2). The current thesis is in agreement with such notions and demonstrates a link between inflammation and coagulation by the co-expression of CD38 and CD142 on CD8 and CD4 T lymphocytes during HIV infection. There is also a significant correlation between coagulation markers and atherogenic inflammatory monocyte subsets ($CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{++}$), with the latter thought to play a role in coagulation pathways during HIV infection.

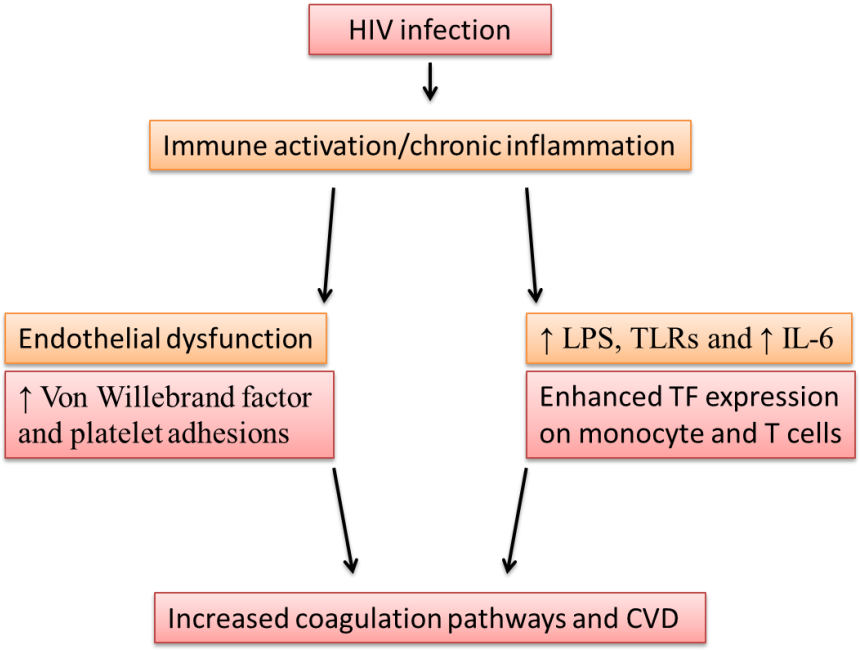

Figure 5-2: The link between inflammation and coagulation. Here the increase of the extrinsic coagulation pathway (TF activation) is highly linked to greater LPS and IL-6 levels that are in turn affected by increased immune activation during chronic HIV infection (reproduced from Levi et al., 2004).

5.3 UPREGULATION OF INTERMEDIATE AND NON-CLASSICAL MONOCYTE SUBSETS IN HIV-POSITIVE GROUPS WITH A RELATIVELY LOW CD4 COUNT

Our data showed an upregulation of both intermediate monocyte and non-classical monocyte subsets during HIV infection. In parallel, there was a decrease in the classical monocyte subset in the HIV-positive group, particularly for treated patients. These data are in agreement with others that also detected such an expansion of monocyte subsets with HIV infection (Funderburg et al., 2012).

Our monocyte subset profile also shows a positive correlation with Treg cell activation markers (SATB-1 and GARP). This relationship may help explain pro- and anti-inflammatory roles for SATB-1 and GARP, respectively, and may indicate an imbalance in cytokine regulation during HIV infection.

The proportion of non-classical monocytes correlated positively with immune activation and coagulation markers, indicating a link with inflammation and coagulation with HIV infection. Earlier in the thesis (Chapter 2) it was highlighted that non-classical monocytes express relatively high levels of the fractalkine receptor (CX3CL1). The latter is usually expressed on activated endothelial cells as a transmembrane-anchored adhesion receptor, thus attracting and arresting monocytes from the circulation into the atherosclerotic plaque (Ghattas et al., 2013). In addition, Funderburg et al. (2010) reported that non-classical monocytes express higher TF levels during HIV infection, further supporting the important role of non-classical monocytes in terms of promoting coagulation pathways in HIV-treated patients.

Our findings also show that intermediate monocytes correlated positively with coagulation markers, suggesting a link with intravascular coagulation. It also correlated positively with activated Treg cell markers such as SATB-1. This may reflect a relationship between intermediate monocytes and pro-inflammatory cytokines, where higher SATB-1 levels are linked to an increased pro-inflammatory role of T helper cells. In support, Rogacev et al. (2012) reported that intermediate monocytes may be an independent predictor of CVD events and that this could be linked to its ability to secrete inflammatory cytokines. Although there is disagreement in the literature regarding the exact role of each monocyte subset, it is beginning to emerge that both intermediate and non-classical monocytes may be highly atherogenic. In support, others found that such subsets are linked to inflammation and coagulation markers in persons saddled with chronic HIV infection (and receiving cART) (Stansfield et al., 2015; Rogacev et al., 2012; Yang et al., 2014).

5.4 INCREASED FOXP3 EXPRESSION IN TREG CELLS TOGETHER WITH UPREGULATION OF PRO-INFLAMMATORY (SATB-1) AND ANTI-INFLAMMATORY MARKERS (GARP)

5.4.1 Upregulation of $CD4^+FOXP3^+$ in HIV-infected Individuals

The present research study showed an expansion of $FOXP3^+$ Treg cells as a percentage of the total $CD4^+$ T cells with chronic HIV infection. This correlated negatively with CD4 count and positively with immune activation. The enhanced Treg frequency may be an indication of higher immune activation due to increased Treg activity and function (Chevalier and Weiss. 2013). However, there are conflicting findings regarding Treg cells in this context due to the lack of a clear surface-identifying marker and additional studies are required to identify more specific markers. Most studies link Treg expansion to increased FOXP3 induction under antigenic persistence, while HIV infection has direct effects on Treg expansion from $CD4^+CD25^-$ cells (T helper) in the presence of TGF-β (Amarnath et al., 2007). Moreover, Suchard et al. (2010) concluded that enhanced FOXP3 frequency in $CD4^+$ T cells can help explain elevated FOXP3 expression with greater lifespan of these cells or increased cell death of $FOXP3^-$ $CD4^+$ cells (AICD). The other suggestion made is that the progression from HIV to AIDS leads to an increase in the number of circulating $CD4^+CD25^+$ Tregs as a proportion of CD4 T cells, with a corresponding decrease in function (Février et al., 2011).Our study showed increased FOXP3 expression on $CD8^+$ T cells in the HIV-positive group with immune deficiency

(CD4 < 200), meaning that a small population of $CD8^+CD25^+FOXP3^+$ T cells are emerging as an important subset Treg cells with clinical significance (Churlaud et al., 2015). Mayer et al. (2011) demonstrated that $CD8^+FOXP3^+$ T cells share developmental and phenotypic features with classical $CD4^+FOXP3^+$ regulatory T cells but lack potent suppressive activity.

5.4.2 Upregulation of GARP in HIV-infected Groups and Correlation with Disease Progression

The current study showed increased GARP expression on $CD25^{++}$ $CD4^+$ cells in HIV-infected individuals with a relatively low CD4 count. This expansion correlated negatively with CD4 counts, and positively with immune activation markers and VL. The correlation between greater GARP activation and markers of disease progression may be explained by increased suppressive activity (viral-induced activation of the suppressor population). This would result in the inhibition of CD4+CD25- cells (T helper) leading to the inhibition of the HIV-specific T cell response and the development of a chronic model of HIV infection (Paiardini and Muller-Trutwin. 2013; Keynan et al., 2008). Thus GARP activation together with the persistence of HIV is associated with increased immune activation and inflammation, with downstream effects including upregulation of monocyte subsets and coagulation pathways.

5.4.3 Upregulation of GARP on both $CD4^+CD25^{++}$ and $CD4^+CD25^-$ Cells

Findings from the current study revealed an upregulation of GARP on CD4+CD25++ and CD4+CD25– cells during chronic HIV infection for the low CD4 count group. Such an increase is linked to disease progression markers e.g. VL, CD4 counts and immune activation. Moreover, GARP upregulation during HIV chronic infection correlated positively with an increase of both intermediate and non-classical monocyte subsets. This increase in GARP expression with chronic infection is further supported by Miller et al. (2013 and 2014) who demonstrated that FIV directly activated $CD4^+CD25^+$ regulatory T cells *in vitro* together with higher GARP expression on the surface of activated Treg cells. Such activation of Treg cells plays a role in converting $CD4^+CD25^-$ T helper cells into phenotypic and functional regulatory T cells. Furthermore, Miller et al. (2014) explains the role of the activated GARP–TGF-β complex in converting $CD4^+CD25^-$ T helper cells into Treg cells. This helps to explain the depletion of $CD4^+$ T cells during the chronic stages. For the current study, there was a positive correlation between GARP expression and VL and this underscores GARP's involvement under chronic conditions where it inhibited T effector cell function against the HIV infection.

The transcriptional factor, FOXP3 (suppressor function) interacts with target genes that promote suppression activity, e.g. GARP to be expressed on $CD4^+CD25^{++}$ Treg cells (Probst-Kepper and Buer. 2010). GARP is linked to naturally occurring activated Treg cells ($CD4^+CD25^{++}$) and is not expressed on induced Tregs in the presence of TGF-β (Wang et al., 2009). During the late stages of HIV infection FOXP3 expansion from the total $CD4^+$ cell population manifests together with increased GARP expression on $CD25^{++}$ and ectopic expression on $CD4^+CD25^-$ cells. This may explain the role of GARP in the induction of FOXP3 during chronic HIV infection.

What is the reason for GARP not being expressed on Tregs? Wang et al. (2009) demonstrated that GARP expression on activated Tregs correlated with their suppressive capacity. However, GARP was not induced on T cells activated in the presence of TGF-β. Here the T cells expressed relatively high FOXP3 levels and lacked suppressive function. This may indicate that increased FOXP3 induced in the presence of TGF-β during HIV infection may not be involved in suppression pathways (Wang et al., 2009) as such cells do not express GARP on their surface. By contrast, activated natural Treg cells ($CD4^{+}CD25^{++}FOXP3^{-}$) and ectopic Treg cells ($CD4^{+}CD25^{-}FOXP3^{-}$) express GARP during HIV infection (Elkord et al., 2015). This especially occurs during the chronic stages when such cells are involved in suppression pathways. Further studies are required to assess whether GARP upregulation during HIV infection is directly involved in CD4 depletion and if it contributes to immune deficiency during the latter stages of infection (Miller et al., 2014).

5.4.4 Increased SATB-1 Expression on CD25++ T Cells with low CD4 Counts and Correlation to Disease Progression

The upregulation of SATB-1 correlated negatively with the CD4 counts and positively with immune activation and coagulation markers. Our data revealed a significant increase of SATB-1 on $CD4^{+}CD25^{++}$ T cells in the HIV-treated group and also those with a relatively low CD4 count. This indicates a shift towards T effector cells together with the inhibition of Treg cell function. Relatively low levels of SATB-1 were observed in the control and the HIV-positive groups (CD4 count > 500 cells/μL) that indicate the normal suppressive function of Treg cells in this instance. By contrast, there is an upregulation of SATB-1 in Treg cells ($CD25^{++}FOXP3^{+}$) in the relatively low CD4 count group afflicted by chronic HIV infection; this correlated positively with immune activation markers and coagulation markers. High SATB-1 levels in Treg cells also correlated with intermediate and non-classical monocyte subsets. Moreover, increased SATB-1 levels in Treg cells is associated with the upregulation of T effector cytokines that may indicate the inability of Treg cells to suppress the activation of effector T cells. This may also show that there is skewing of the regulatory function towards the effector program and increasing pro-inflammatory pathways (Beyer et al., 2011; Thabet et al., 2012). The latter may help explain the correlation between SATB-1 and the relatively high levels of intermediate and non-classical monocyte subsets during chronic infection. It may also shed light on the positive correlation of SATB-1 with immune activation and coagulation markers expressed on CD8 T cells. There is also substantial upregulation of SATB-1 on $CD25^{-}$ $CD4^{+}$ T helper cells in the HIV-positive groups together with significant increases in the naïve group. Such an increase may indicate the pro-inflammatory role of T helper cells in early HIV infection (naïve).

There is some controversy regarding the increase of GARP and SATB-1 expression on Treg cells during chronic HIV infection and upregulation of the former in Tregs involved in the induction of adaptive regulatory T cells (Hahn et al., 2013). Such upregulation can act as a form of immunomodulation by inhibition of effector function in naive T cells and by FOXP3 induction. Conversely, the upregulation of SATB-1 in Treg cells can indicate a weakened suppressive function and an increase of pro-inflammatory properties. Whether the plasticity of Treg cells plays a role in this case and if there is an imbalance between

Treg and T helper cells due to the immunodeficiency state is not known at present. However, this may reflect an imbalance between anti- and pro-inflammatory cytokines during chronic HIV-infection. Further studies are required to gain additional insights into such intriguing questions.

5.5 INCREASING CRP IN CHRONIC HIV PATIENTS

CRP is an important marker for inflammation assessment and is linked to CVD in chronic HIV infection (Baker and Duprez. 2010). The results of the present study showed a significant increase in CRP levels in chronic HIV-positive patients, with the highest levels found in the HIV-positive group with a relatively low CD4 count. These findings are in agreement with other studies where increased CRP levels were associated with HIV disease progression, independently of CD4 lymphocyte counts and HIV RNA levels. This in turn may have consequences for CVD in HIV-infected persons (Lau et al., 2006). CRP elevation is an indicator of an inflammatory condition (as a consequence of infection) and increased levels are linked to pro-inflammatory cytokines such as IL-1 and IL-6. Here even relatively small changes in CRP concentrations can lead to increased CVD risk and hence lower levels in HIV-positive persons would predict a longer survival chance (Osman et al., 2006; Lau et al., 2006).

5.6 HIV INFECTION-ASSOCIATED DECREASE IN LIPID PROFILES

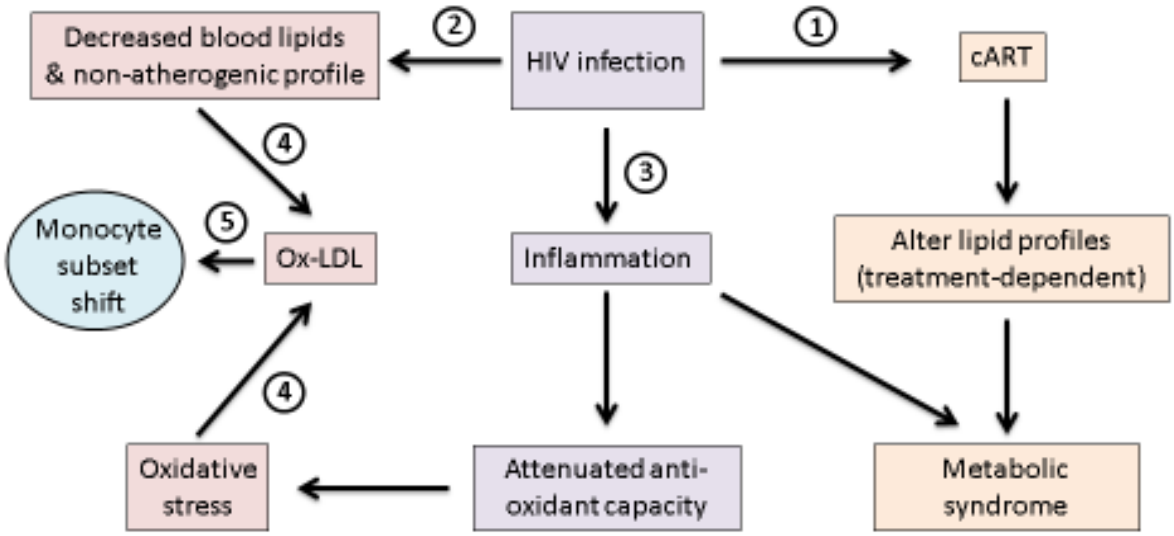

Figure 5-3: Lipid metabolism in HIV infection and the role of cART and inflammation. (1) HIV infection and cART may alter the lipid profile and eventually result in the onset of the metabolic syndrome. (2) The decreased lipid profile in cART naïve patients can contribute to damaging downstream effects; (3) Inflammation-induced oxidative stress can result in detrimental effects in circulation and the eventual onset of the metabolic syndrome. (4) Higher oxidative stress and altered lipid profile contribute to increased OX-LDL formation. (5) OX-LDL may contribute to the monocyte subset shift and increase pro-inflammatory cytokine production.

This study revealed a decrease in lipid blood metabolite levels in early HIV-infected (naïve) individuals, particularly triglyceride and total cholesterol levels (Souza et al., 2013)(Riddler et al., 2003). In addition, there was a significant decrease in circulating LDL levels in the HIV-positive group treated with second line therapy. Such alterations may be linked to HIV itself and/or increased inflammation and immune activation. After cART initiation the lipid profile undergoes further changes in accordance with the particular treatment regimen applied. For example, after treatment initiation there was an increase in HDL-cholesterol levels - this was particularly linked to the NNRTi group. These findings match other published studies (Souza et al., 2013; Riddler et al., 2003) where they showed that lipid profile values were substantially decreased in naïve persons and then slightly increased following cART (first line treatment) with such changes dependent on the type of treatment applied. cART can also lead to the metabolic syndrome during HIV infection and is associated with several risk factors, including insulin resistance, low HDL-cholesterol levels, hypertriglyceridemia and postprandial hyperlipidemia (Wijk and Cabezas. 2012)

The early inflammation and immune activation associated with HIV infection may enhance lipid peroxidation which is associated with decreased antioxidant capacity and a lower CD4 count in untreated asymptomatic HIV-infected individuals (Wanjiku et al., 2013). A persistent inflammatory environment during HIV infection together with relatively high levels of inflammatory cytokines (maintained by activation of oxidases released from macrophages and neutrophils) may promote pro-inflammatory alterations of the lipid profile (Gori et al., 2016; Lederman et al., 2013). This in turn can lead to increased formation of OX-LDL which further contributes to monocyte activation (Zidar et al., 2015) (Figure 5.3).

Decreased LDL levels during early HIV infection is accompanied by increased OX-LDL which alters the inflammatory profiles of blood monocytes and increases TF expression on monocytes (Zidar et al., 2015). Studies have revealed that HIV-infected patients display an oxidative imbalance early-on during the disease (Wanjiku et al., 2013). Here serum and tissue anti-oxidant levels were relatively low and peroxidation products elevated. Such dysregulation may be the source of immune activation during HIV infection. However, after the initiation of cART the lipid profile changes depending on specific treatment regimens. For example, PIs can affect the lipid profile by interference with intracellular regulation of lipid metabolism, increased VLDL production, and impaired catabolism of free fatty acids and increased liver triglyceride synthesis. Genetic background can also affect lipid abnormalities associated with cART (Estrada and Portilla. 2011)

The evaluation of lipid subclasses during HIV infection is important in order to identify pro-inflammatory and atherogenic molecules. The findings of this thesis revealed a significant increase in the non-atherogenic type of intermediate density lipoprotein type 3 (IDL-C) in cART-naïve and in HIV-treated (second line) patients. This parameter correlated positively with immune activation in treated patients. For the HDL subclasses there was an increase in the large HDL species for all HIV-positive groups (cART-naïve and cART-treated). Souza et al. (2013) showed the presence of small and large HDL in HIV-infected individuals at high risk for CVD onset. However, in this study there is significant decrease in small HDL in HIV-treated

(first and second line treatments) persons. Small HDL is the most protective lipoprotein in terms of atheroprotective effects and anti-inflammatory activities through inhibition of OX-LDL uptake and anti-thrombotic function. In support, some found that small HDL is inversely associated with IL-6 levels (Baker et al., 2010). Krychtiuk et al. (2014) demonstrated that small HDL levels correlated with circulating pro-inflammatory non-classical monocytes and showed an inverse relationship to circulating classical monocytes, independently from other lipid parameters. Thus lower small HDL availability would blunt its atheroprotective role. This phenomenon together with increased inflammatory monocytes may contribute to future CVD onset.

The analyses also revealed a positive correlation between atherogenic IDL-A and immune activation and immune activation + coagulation markers in the naïve group. This suggests an important role for immune activation and coagulation during early HIV-infection (naïve). The correlation in the naïve group also showed a positive relationship between non-atherogenic (I-HDL) and both immune activation and classic monocytes, while I-HDL correlated negatively with intermediate monocytes. Thus there is significant correlation between lipid subclasses and immune activation and coagulation markers in especially naïve individuals, suggesting a role for such metabolic modulators in this group. The atherogenic IDL-B also showed a positive correlation with Treg cells in HIV-treated patients. What is the value of such subclass lipid profiles? Atherogenic lipoprotein profiles are indeed very useful in the assessment of CVD risk and onset, especially as studies showed that 75% of patients with acute coronary syndrome or myocardial infarction displayed normal cholesterol, LDL-C and HDL-C plasma levels) (Haque et al., 2016). It can therefore be concluded that the traditional lipid profile in the current study's cohort is not significant for CVD risk, but that instead the atherogenic lipoprotein subclasses offer greater value to assess CVD events in HIV-infected individuals.

5.7 EVALUATION OF FMD WITH HIV INFECTION

The findings of this thesis showed a relatively small (insignificant) improvement in FMD in HIV-positive groups (7.3 ± 4%) versus controls (4.7 ± 3%). Further comparisons of FMD demonstrated that a relatively high FMD percentage characterized the HIV-positive group with a higher CD4 count (> 500 cells/µL). By contrast, others showed a decrease with HIV infection (indicating endothelial dysfunction) that improved with cART (Torriani et al., 2008; Solage et al., 2006). Moreover, Stein et al (2006) linked FMD changes in HIV-infection to abnormal lipid metabolism and insulin resistance (effects of PI) and proposed that this would not occur in the presence of a normal lipid profile. Together this means increased CVD risk with HIV-infection occurs together with greater inflammation and coagulation, and that this is usually accompanied by changes in endothelial function (decreased FMD percentage). However, this was not the case in our study and we propose that this may occur due to: a) limited samples sizes and b) the alternative lipid profile observed with HIV infection. Further studies with a greater number of subject recruits should help clarify such questions.

5.8 STUDY LIMITATIONS

The current study has three limiting factors. Firstly, a relatively small sample size was investigated that impacted on statistical significance in some instances. Although the sample size was relatively small, a considerable amount of time and resources were spent to launch the study. We also found that subject recruitment was difficult at times as patients did not always attend the clinic due to work commitments, lack of transport and/or inclement weather conditions in the Worcester area. As this is an ongoing study, samples from additional subject recruits can be further investigated and this should further strengthen existing findings. Secondly, the current research project was a cross-sectional one and a longitudinal study would be essential to confirm the interesting data thus far generated. Finally, further research would also be required to delineate mechanisms underlying immune activation, coagulation, monocyte subclass shifts and altered lipid profile changes observed and how exactly these are contributing to CVD onset in HIV-infected individuals.

5.9 CONCLUDING REMARKS

This study has demonstrated greater immune activation in cytotoxic T cells with relatively lower CD4 counts in HIV-infected persons from the Worcester region in South Africa. In parallel, there was evidence for increased coagulation in such cells. These findings therefore support the link between inflammation and coagulation markers during HIV infection and emphasize its importance in persons with immune dysregulation. The current study also identified a unique coagulation marker expressed on CD4 and CD8 T cells that is expressed relatively early on in HIV-infected individuals. In addition, there is activation of Treg cell markers together with upregulation of particular monocyte subsets that are linked to immune activation and coagulation. Together such perturbations increase the risk for atherosclerosis and CVD onset, especially in HIV-positive individuals with some degree of immune dysregulation. This therefore alerts to the nature of future clinical management of HIV-positive patients and makes a case for earlier therapeutic interventions to decrease chronic inflammation and immune activation. Longer-term benefits of such a strategy would be decreased coagulation and lowered CVD risk in HIV-infected individuals treated with cART. In addition, another group of HIV-infected patients, i.e. with poor immune recovery (failure to boost CD4 counts despite treatment) is also at high risk for inflammation and coagulation. Thus, it is recommended that clinicians should:

a) Be mindful of immune activation and coagulation with HIV infection, even at relatively early stages of disease progression;

b) request additional evaluations to be completed on blood samples collected from such individuals, e.g. immune activation and coagulation markers, and detailed lipid profiles; and

c) Actively manage such identified high-risk patients with appropriate pharmacotherapy and lifestyle modifications, e.g. changing smoking habits and improving dietary habits.

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ADDENDUM – A: FLOW CYTOMETRY RESULT AND OPTIMIZATION

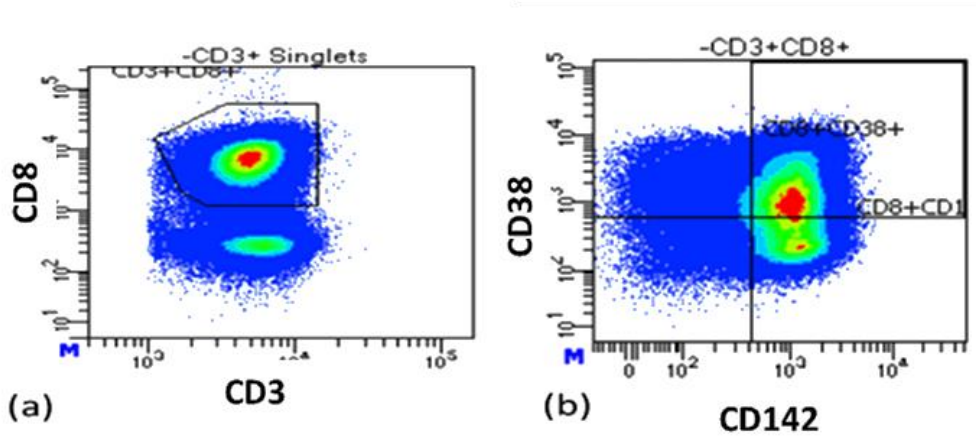

Figure 1: Diagrams Show CD8 T cell co- expression of CD38 and CD142 : a) APC-H7 mouse anti-human (CD3)versus BV412 mouse anti-human CD8; b) Co-expression of APC anti-human CD142 and PE anti CD38

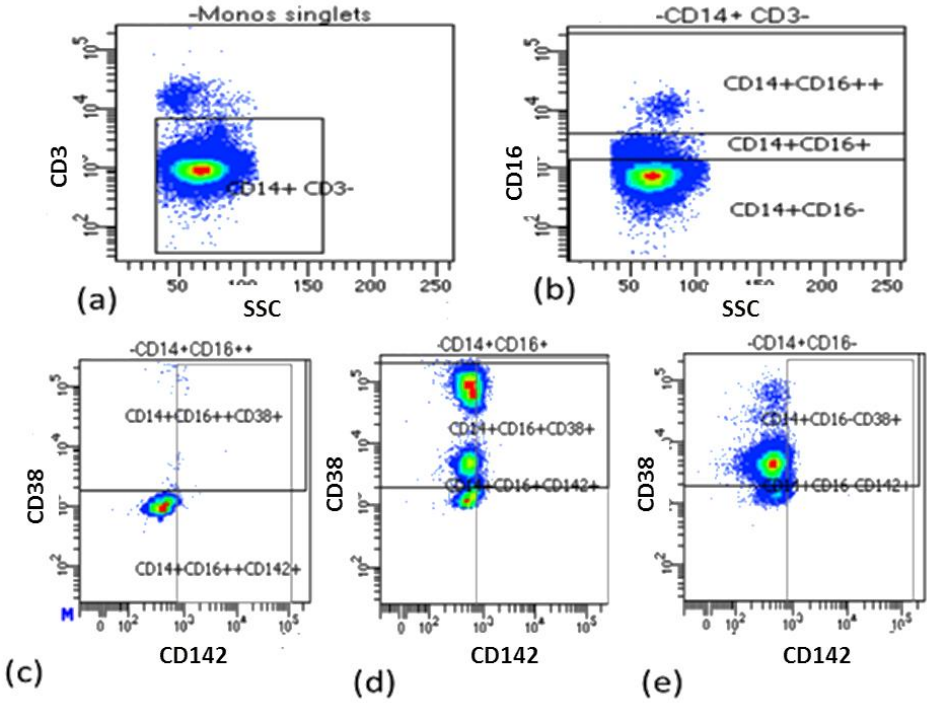

Figure 2: The monocytes subsets and the expression of CD38 and CD 142 on each subset : a) Gate for $CD14^{+}$ and $CD3^{-}$ (SSC) versus CD3 APC-CY7 to exclude any CD3 positive from monocyte subsets; b) CD16 PE-CY7 versus SSC to differentiate monocyte subsets (classical, intermediate and non-classical monocyte); c) Non-classical monocyte and co-expression of CD142 and CD38; d) Intermediate monocyte and co-expression of

CD142 and CD38; e) Classical monocyte and co-expression of CD142 and CD38

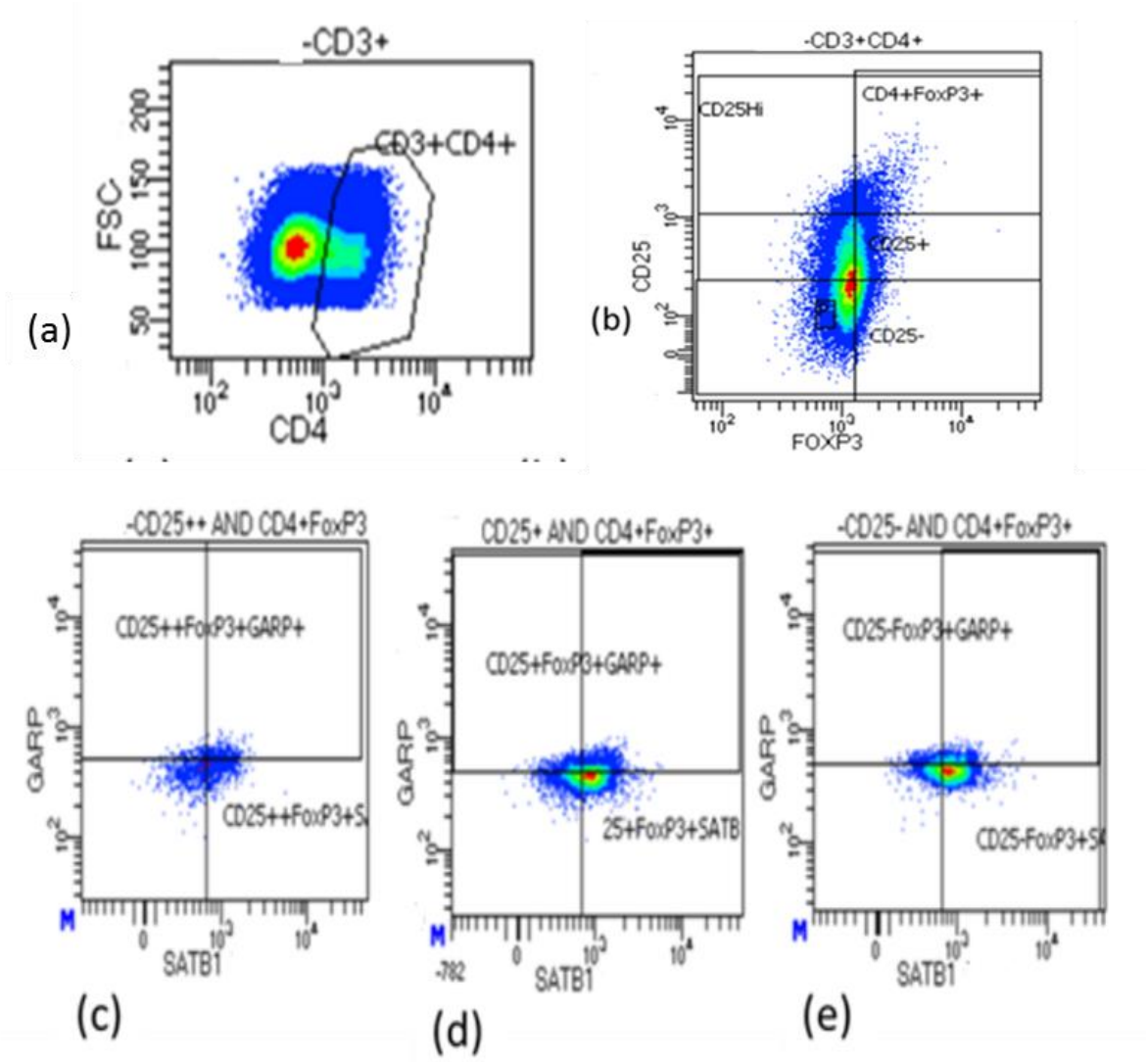

Figure 3: Treg (CD25++FOXP3+) and expression of SATB-1 and GARP. (a) CD4 FITC versus FSC (b) CD25 PE-CY7 versus FOXP3 and 3 different subsets($CD25^{++}FOXP3^{+}$,$CD25^{+}FOXP3^{+}$ and $CD25^{-}FOXP3^{+}$) (c) Co-expression of SATB-1 and GARP in $CD25^{++}FOXP3^{+}$ (d) Co-expression of SATB-1 and GARP in $CD25^{+}$ FOXP3+ (e) Co-expression of SATB-1 and GARP in $CD25^{-}FOXP3$

Optimization data Fluorescence minus one (FMO)

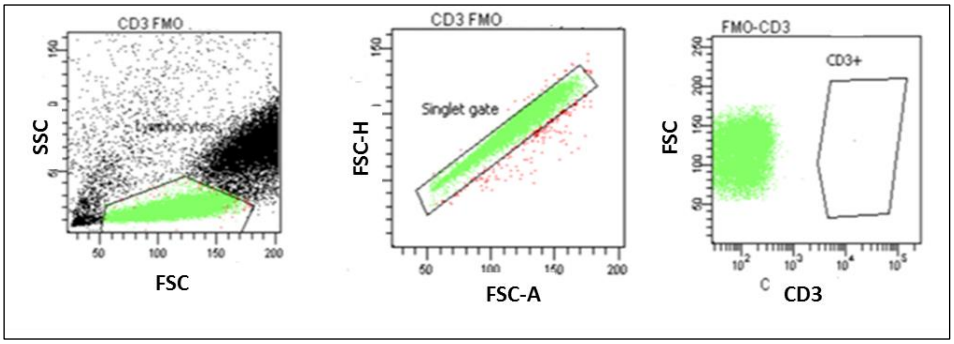

Figure 4: Fluorescence minus CD3

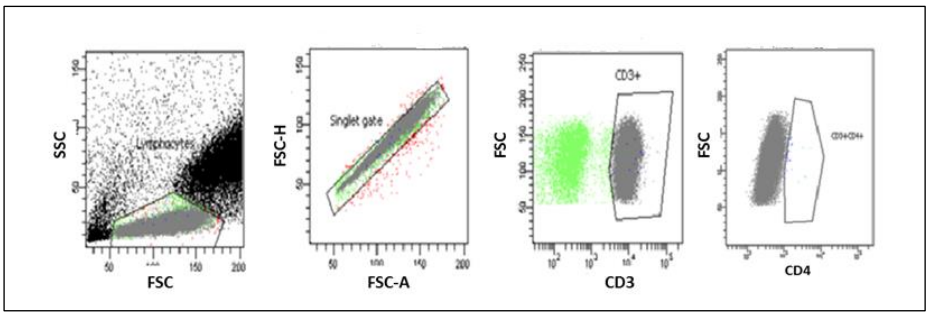

Figure 5: Fluorescence minus CD4

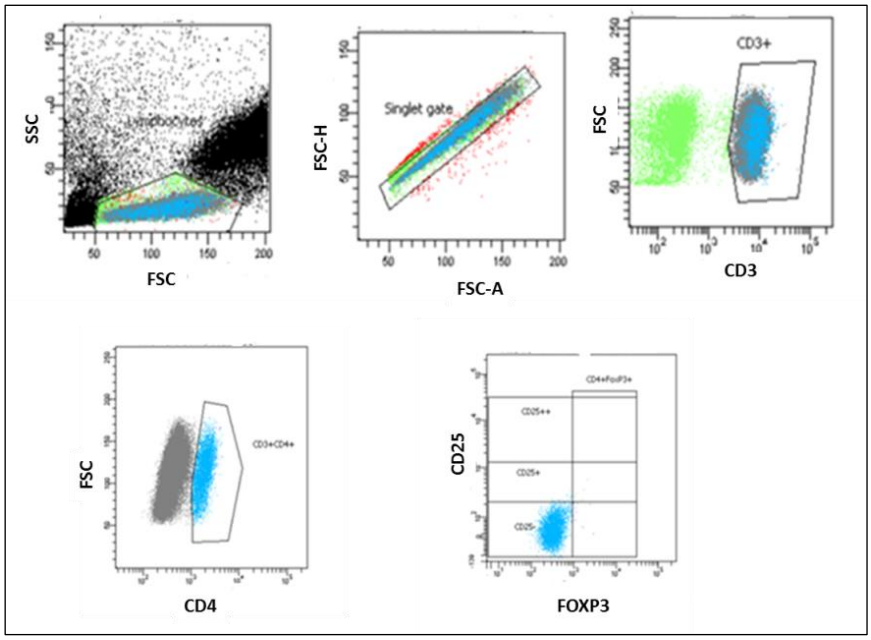

Figure 6: Fluorescence minus FOXP3

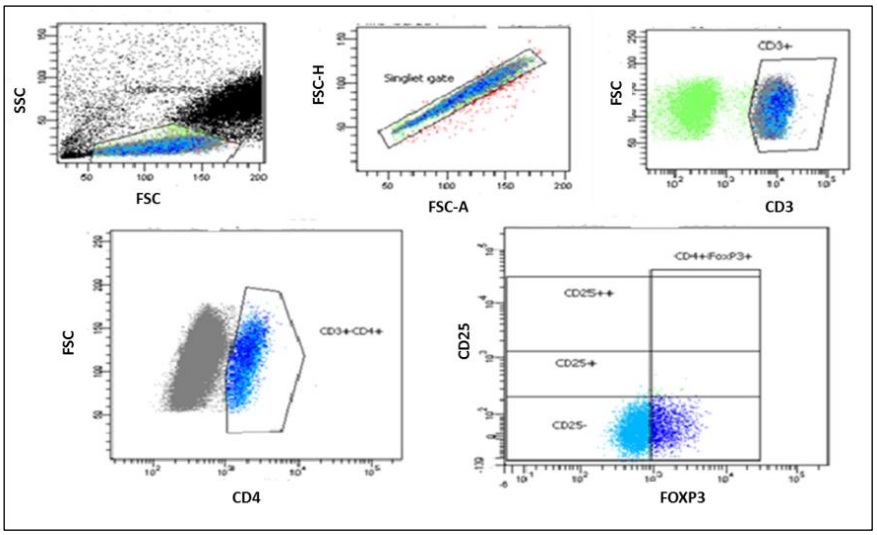

Figure 7: Fluorescence minus CD25

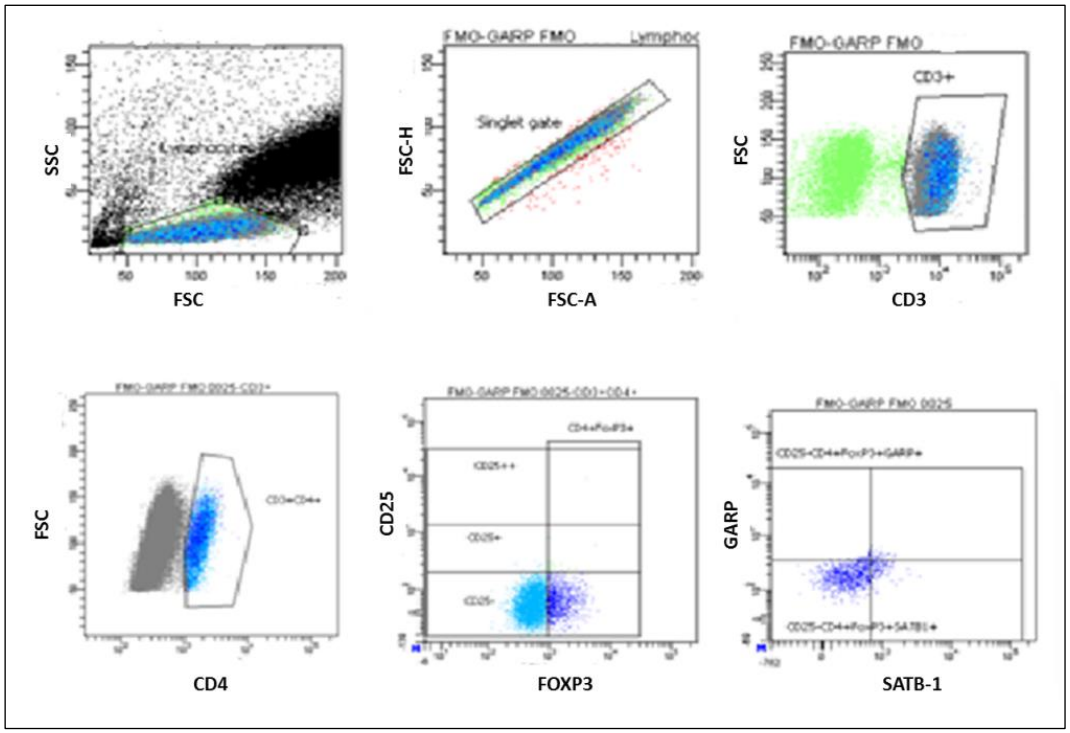

Figure 8: Fluorescence minus GARP

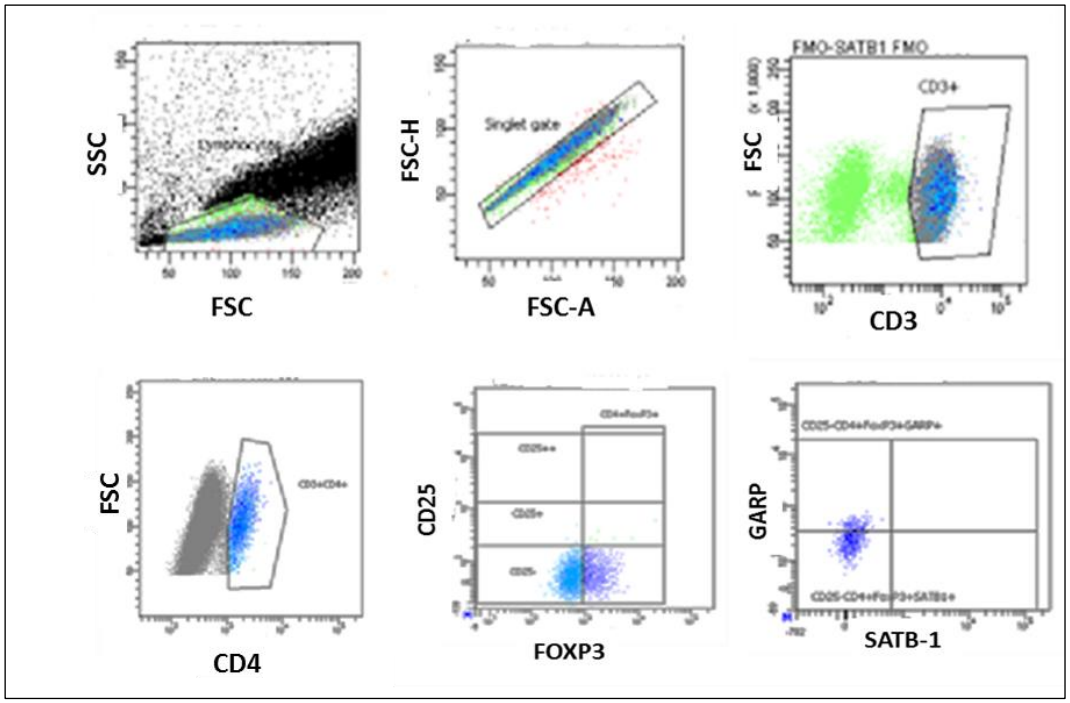

Figure 9: Fluorescence minus SATB-1

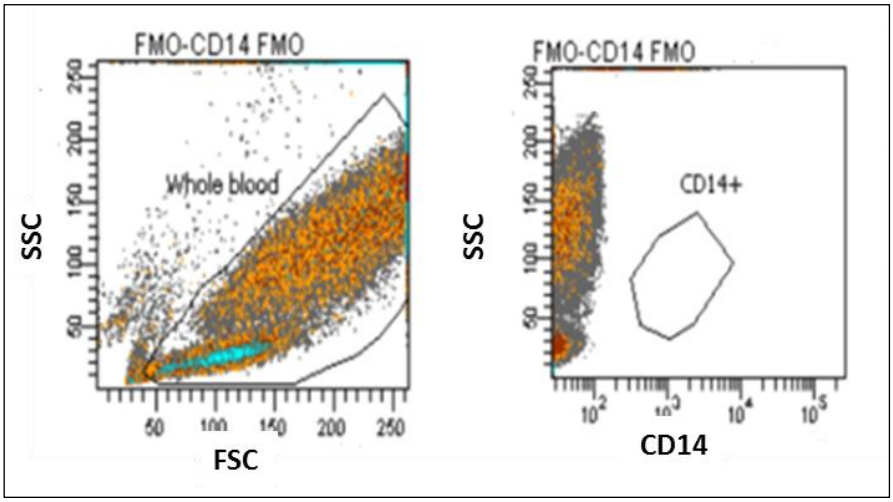

Figure 10: Fluorescence minus CD14

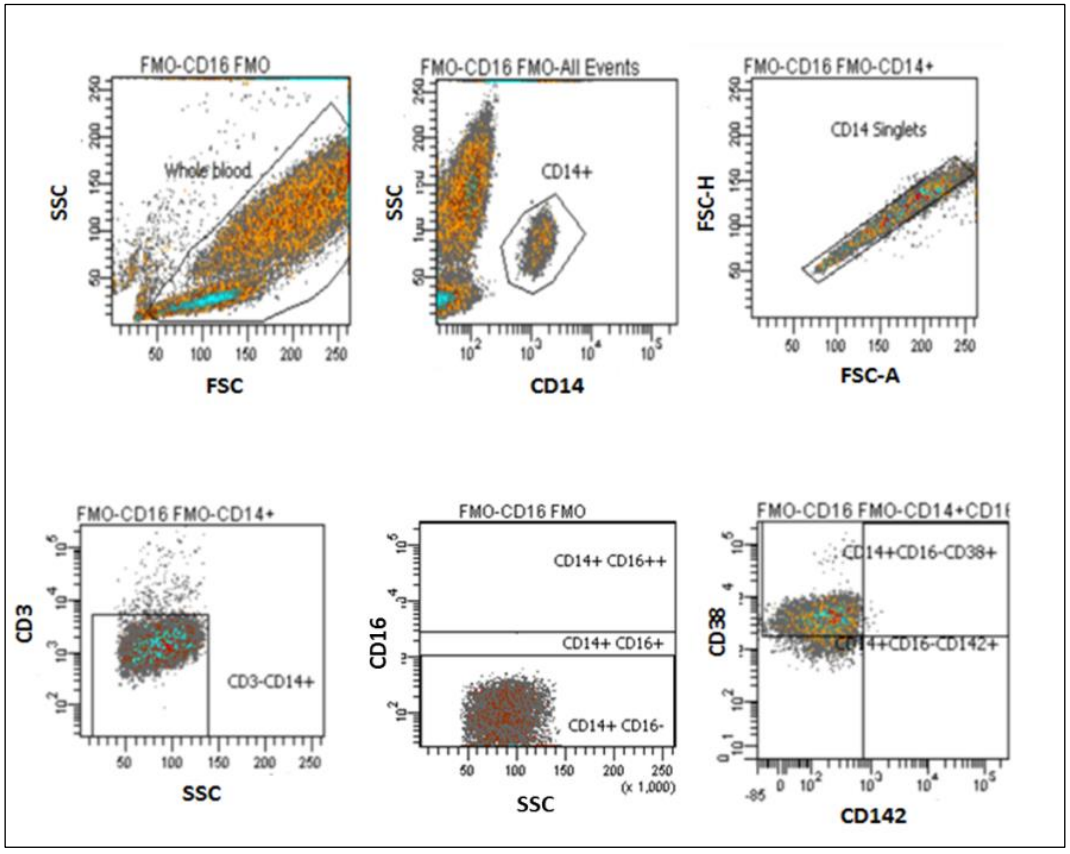

Figure 11 Fluorescence minus CD16

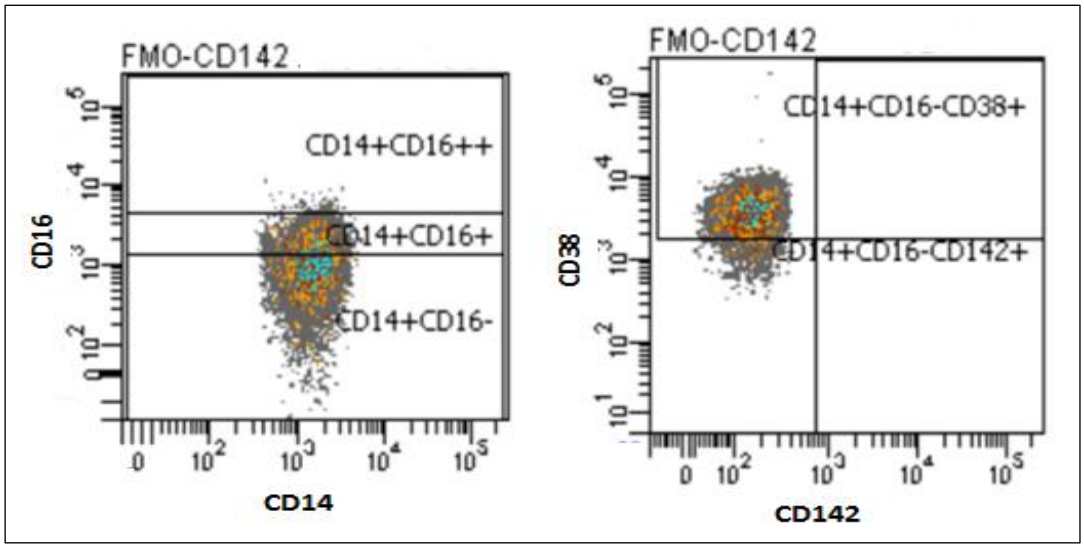

Figure 12: Fluorescence minus CD142 (coagulation factor expression on monocytes)

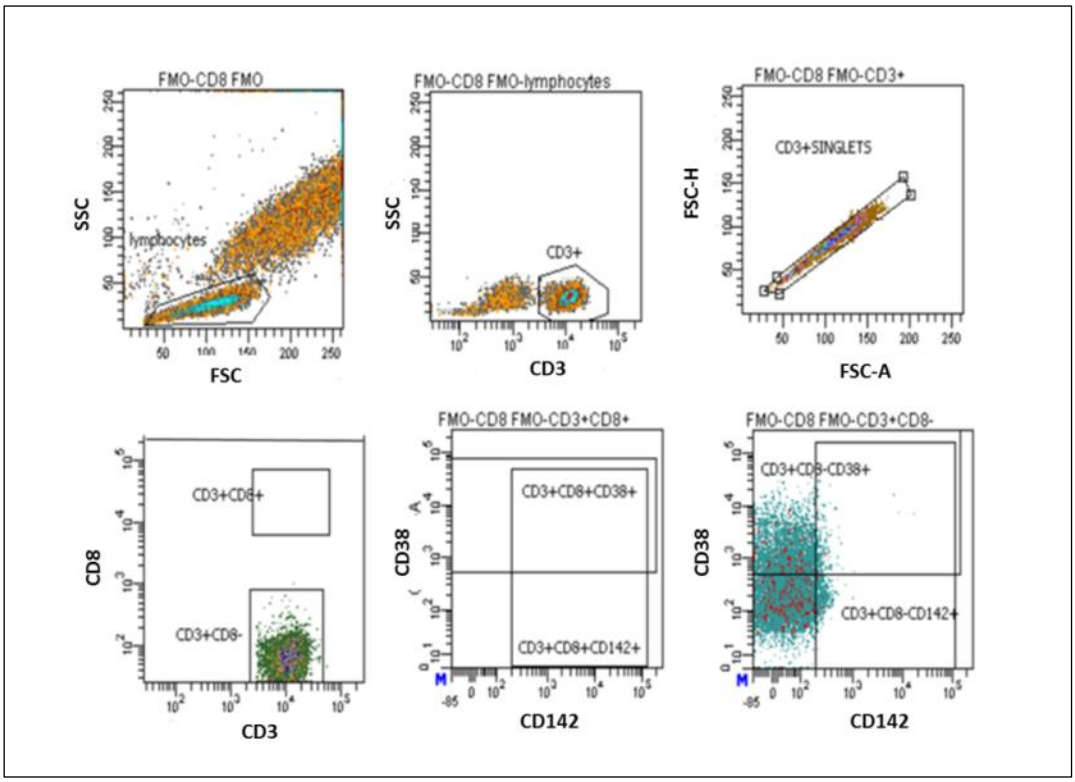

Figure 13: Fluorescence minus CD8 T cell

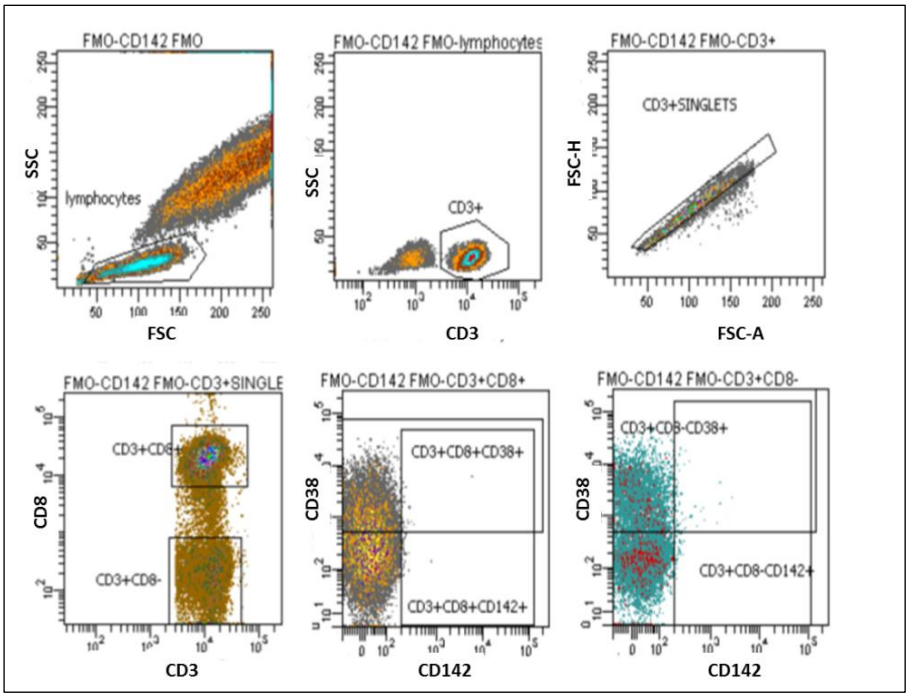

Figure 14: Fluorescence minus CD142 (coagulation factor expressed on T cells)

Antibody titration

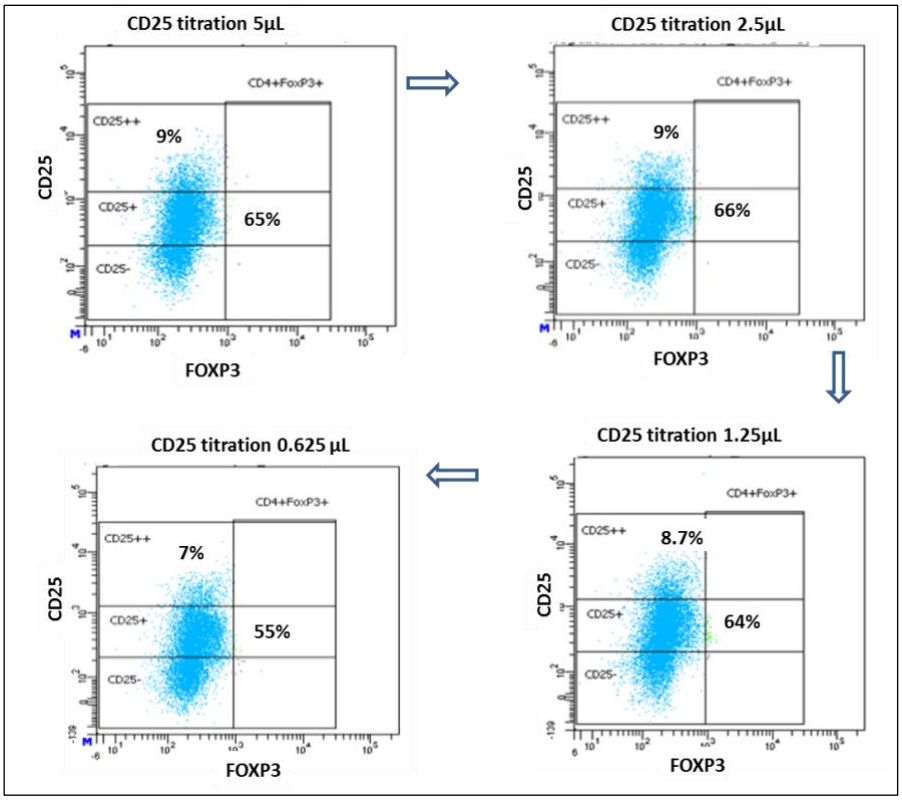

Figure 15: Titration of CD25 with different antibody concentration: The titration shows a bright result (intense signal) with a relatively low antibody concentration. (2.5µL)

ADDENDUM – B: Data for FMD

Table 5-1: Data for FMD (baseline diameter, maximum diameter and percentage of FMD)

	Baseline diameter (mm)	Maximum diameter (mm)	FMD%
CWH2H008	3.82	3.91	2.52
CWH2H0012	4.72	4.85	2.86
CWH2H0013	4.26	4.37	2.79
CWH2H0016	3.57	3.87	8.38
CWH2H0020	3.21	3.51	9.47
CWH2H0022	3.28	3.63	10.61
CWH2H0023	3.17	3.41	7.74
CWH2H0030	3.27	3.85	17.89
CWH2H0031	3.59	3.85	7.37
CWH2H0033	3.33	3.68	10.61
CWH2H0039	3.59	3.69	2.61
CWH2H0040	3.32	3.66	10.19
CWH2H0041	3.11	3.17	2.01
CWH2H0043	5.25	5.27	0.55
CWH2H0046	3.25	3.45	6.16
CWH2H0047	3.82	3.98	4.11
CWH2H0051	5.36	5.47	2.09
CWH2H0053	3.31	3.59	8.32
CWH2H0054	3.27	3.54	8.30
CWH2H0055	2.98	3.29	10.34
CWH2H0056	2.39	2.58	8.19
CWH2H0057	3.58	3.73	4.07
CWH2H0058	3.22	3.28	1.97
CWH2H0059	3.27	3.49	6.91
CWH2H0065	2.25	2.54	12.65
CWH2H0071	4.17	4.43	6.25
CWH2H0075	3.05	3.35	9.59
CWH2H0076	3.06	3.33	8.81
CWH2H0077	3.53	3.89	10.10
CWH2H0078	2.74	2.92	6.60
CWH2H0079	4.52	4.73	4.53
CWH2H0080	4.51	4.56	1.27
CWH2H0081	4.06	4.12	1.56
CWH2H0083	3.69	3.93	6.54
CWH2H0085	3.90	4.07	4.20
CWH2H0086	3.15	3.37	6.78
CWH2H0088	3.15	3.35	6.54
CWH2H0092	3.64	4.03	10.69
CWH2H0093	2.03	2.25	10.61
CWH2H0095	3.58	3.78	5.59
CWH2H0096	3.96	4.12	3.92
CWH2H0097	2.86	3.36	17.42
CWH2H0098	4.13	4.32	4.55
CWH2H0099	3.08	3.45	12.08
CWH2H0100	4.89	5.02	1.72
CWH2H0101	4.58	4.88	6.68
CWH2H0102	2.20	2.41	9.52
CWH2H0103	3.47	3.77	8.70
CWH2H0104	3.68	3.61	-2.03
CWH2H0105	4.42	4.64	4.96
CWH2H0106	3.46	3.52	1.65
CWH2H0107	4.00	4.40	10.12
CWH2H0109	3.19	3.54	11.17
CWH2H0111	3.54	3.83	8.20
CWH2H0113	3.99	4.39	10.19

ADDENDUM – C: Health Research Ethics Committee approval

Health Research Ethics Committee approval

Western Cape Government
Health

STRATEGY & HEALTH SUPPORT
Health.Research@westerncape.gov.za
tel: +27 21 483 6857; fax: +27 21 483 9895
5th Floor, Norton Rose House, 8 Riebeek Street, Cape Town, 8001
www.capegateway.gov.za

REFERENCE: RP 090 /2013
ENQUIRIES: Ms Charlene Roderick

Private Bag X1
Matieland
7602

For attention: **Prof. MF Essop, Dr Theo Nell, Dr E Calitz, Dr. C Gunst, Dr C Klussmann, Dr. E Teer, Dr F Abaid, Dr. M Lombard, Dr. R Essop**

Re: A cross-sectional survey of the metabolic dysfunction and onset of cardiac diseases in HIV patients on HAART treatment within the Winelands region

Thank you for submitting your proposal to undertake the above-mentioned study. We are pleased to inform you that the department has granted you approval for your research.
Please contact to assist you with any further enquiries in accessing the following sites:

Cape Winelands	**Ms S Neethling**	**Contact No. 023 348 8111**
Paarl Hospital	**Dr B Kruger**	**Contact No. 021 872 1711**
Worcester Hospital	**Dr W Marais**	**Contact No.023 348 1101**

Kindly ensure that the following are adhered to:

1. Arrangements can be made with managers, providing that normal activities at requested facilities are not interrupted.
2. Researchers, in accessing provincial health facilities, are expressing consent to provide the department with an electronic copy of the final report within six months of completion of research. This can be submitted to the provincial Research Co-ordinator (Health.Research@westerncape.gov.za).
3. The reference number above should be quoted in all future correspondence.

We look forward to hearing from you.

Yours sincerely

DR NT Nafedi
DIRECTOR: HEALTH IMPACT ASSESSMENT
DATE: 30/10/2013
CC **DR L PHILLIPS** **DIRECTOR: CAPE WINELANDS**

Page 1 of 1

ADDENDUM – D: Letter from the Health Research Ethic Committee

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

Ethics Letter

05-Aug-2015

Ethics Reference #: N12/12/086
Clinical Trial Reference #:
Title: Winelands HAART to HEART study (Prevalence)

Dear Prof Mogammad Essop,

At a meeting of HREC1 on 29 July 2015 the following progress report was approved:

Progress Report dated 8 July 2015

The approval of this project is extended for a further year.

Approval date: 29 July 2015

Expiry date: 29 July 2016

If you have any queries or need further assistance, please contact the HREC Office 0219399657.

Sincerely,

REC Coordinator
Franklin Weber
Health Research Ethics Committee 1

ADDENDUM – E: Consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

WINELANDS HAART to HEART STUDY

REFERENCE NUMBER: N12/12/086

PRINCIPAL INVESTIGATOR: Prof MF Essop

ADDRESS:

Department of Physiological Sciences

Mike de Vries Building

Room 2005a

Stellenbosch University

CONTACT NUMBER: 021 8083146

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- *This study will only be done in the Stellenbosch district and surrounding areas. We will need approximately 300 Patients. We are trying to gather information on laboratory tests, body composition and patient questionnaires profiles of chronic HIV patients on ARV treatment (1st and 2nd line). By getting this information we would be able to assess the attenuation of metabolic function and contractail dysfunction caused by ARV treatment.*
- *As Africa adopts a more westernised lifestyle there is a higher incidence of HIV infection, diabetes and heart diseases.*

Your blood will be taken by a registered medical nurse. It will then be sent away to Pathcare (Stellenbosch) where metabolic-associated parameters will be measured. Other biochemical tests that will be done by Pathcare include C-reactive protein measurements. The remainder of your blood samples will be used to obtain genetic material (RNA).

The blood presser assessment, evaluation of cardiac function (ECG, echocardiography and carotid intima media thickness), full anthropometric evaluation and life style questionnaire will be done at the clinic

Why have you been invited to participate?

- *We are trying to gather information on laboratory tests, body composition and patient questionnaires profiles of chronic HIV patients on ARV treatment (1st and 2nd line). By donating blood to our study you will be helping us to determine these profiles and how we can relate them to the current diagnostic tests to investigate cardio-metabolic effects of ARV treatment. By getting this information we would be able to assess the attenuation of metabolic function and contractail dysfunction caused by ARV treatment. With your help we hope to identify the suitable therapeutic interventions that will blunt metabolic dysfunction, oxidative stress and enhance mitochondrial function under conditions where PIs are chronically administered.*

What will your responsibilities be?

- *As we need to examine the selected patients and then take the blood for laboratory tests and lifestyle questionnaire regarding your line of ARV treatment. A registered anthropometrist, Dr Theo Nel, will also perform anthropometric measurements that include waist circumference, waist: hip ratio, height and mass.*

Will you benefit from taking part in this research?

- *Although there may not be any direct benefits to me/the participant by participating at this stage, future generations may benefit if the researchers succeed in finding out more about how ARV treatment can lead to metabolic dysfunction and cardiac disease. If you choose to know the results of your blood tests we will make these available. However, you would have to discuss this information with your personal doctor, at your own cost, in order to assess your medical status with ARV treatment (metabolic and cardiac status).*

Are there in risks involved in your taking part in this research?

There are no more than minimal medical or physiological risks associated with this study.

- *I/the participant may feel some pain associated with having blood drawn from a vein and may experience some discomfort, bruising and/or slight bleeding at the site.*

If you do not agree to take part, what alternatives do you have?

- *It is your decision to participate or not and nothing will be done from the researchers' part to in any way to persuade you to take part.*

Who will have access to your medical records?

- *Only the principal researcher (Prof Faadiel Essop and the following Doctoral student: Dr. Eman Teer) will have access to your data and records. All information will be treated with respect and utmost confidentiality. Under no circumstances will your name or any form of identification be used in any publication, poster, lecture or thesis that results from this study. Professor Faadiel Essop and Dr. Eman Teer will be the only authorised personnel who will have access to your results.*

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

- *Not applicable here.*

Will you be paid to take part in this study and are there any costs involved?

- *No, you will not be paid to take part in the study. There will be no costs involved for you, if you do take part. Travelling costs in order to get to the Department of Physiological Sciences will not be covered by this study and you will need to cover this cost yourself.*

Would you like to know the results of your blood tests?

- Please indicate by marking the correct box with an X
- YES ☐
NO ☐

Is there anything else that you should know or do?

- You can contact Professor MF Essop on 021 808 3146 if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled **(Investigation of cardio-metabolic effects of ARV treatment in the winelands region).**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....……. On (*date*) ……...……...

....

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…..…….. on (*date*) ……...……...

....

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. Using the language medium of Tswana/English.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……..……..

....

Signature of interpreter **Signature of witnes**

ADDENDUM – F: Data Sheet

Project title: Cape Winelands HAART 2 HEART

Ethics approval nr: N12/12/086

IDENTIFICATION CODE: CWH2H_____				
Date of interview:				
Interviewer:				
Health District Site:	Worcester	TC Newman	Mbekweni	Stellenbosch
Age:				
Blood pressure (Sys/Dia):				
Heart rate (beats/min):				
HIV classification	Negative	Naive	1st line	2nd line
WHO stage:	1	2	3	4
Time since HIV diagnosis (months):				
HAART start date:				
Time on HAART:				
Current HAART regimen:				
Time on current HAART:				
Switch from regimen:				
Adherence:	YES	NO		
Viral load:				
Previous co-infections:				
Current co-infections:				
Drug history:				
Family history CVD:				
Smoking/snuff:				
Quantity per day:				
Frequency per week:				

ADDENDUM – G: Demographic questioner

Project title: Cape Winelands HAART to HEART study

Ethics approval nr: N12/12/086

IDENTIFICATION CODE: CWH2H_____							
Date of interview:							
Interviewer:							
Health District Site:	Worcester	TC Newman	Mbekweni	Stellenbosch			
Household (Brick house, shack other dwelling)							
Total in household	**Male**				**Female**		
Children 0-6 yrs							
Children 7-12 yrs							
Children 13-18 yrs							
Adults 18-30							
Adults 31-45							
Adults 46-60							
Adults 61+							
LANGUAGE AND ACCULTURATION							
Home language of respondent	Afrikaans	Xhosa	Zulu	English	Sotho	Other	
Household head speaks	Home language only		Home language + Afr/Eng		English/Afrikaans only		
Can you read and understand a newspaper in your home language easily, difficult or not at all?	Easily	1					
	With difficulty	2					
	Not at all	3					
Can you read and understand a newspaper in the English language easily, difficult or not at all?	Easily	1					
	With difficulty	2					
	Not at all	3					
NEAREST CLINIC/HOSPITAL							
Name of clinic/hospital	**Walk (minutes)**				**Mode of transport Walk=1; Taxi=2; Own car=3; bicycle=4**		

ADDENDUM – H: Normal range of lipid profile

PARAMETER	REFERENCE RANGE	UNIT
∗TC	Desirable < 5.2 Borderline 5.2–6.2 High > 6.2	mmol/L
∗TG	Desirable 1.7–2.2 High > 2.2	mmol/L
∗HDL	Desirable > 1.2 Low < 1	mmol/L
∗LDL	Desirable < 2.6 Borderline 2.6–3.3 High > 3.3	mmol/L
CRP	Normal < 10	mg/L