Monocytes in Chronic HIV-1 infection: Changes in Phenotypic Marker Expression and their Relationship with Immune Activation

by

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Declaration

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Abstract

HIV-infection is characterized by depletion of $CD4^+$ T-cells from the gut-associated lymphoid tissue (GALT) which causes irreparable gastrointestinal tract damage and subsequent microbial translocation of bacterial products such as lipopolysaccharide (LPS), a component of Gram-negative bacteria, into systemic circulation. HIV infection also affects the functions and relative population sizes of various immune cells, such as monocytes. Monocytes are important innate immune cells as they are often the first cells recruited to sites of infection and inflammation. They then either promote inflammatory processes; elicit adaptive immune responses, through their antigen presenting ability; aid in pathogen and debris clearance or aid in damage repair.

This cross-sectional study investigated functional changes to monocytes and monocyte subsets (CD14+CD16- and CD14+CD16+) in HIV+, treatment naïve individuals and healthy uninfected controls, using whole blood assays and isolated monocytes. A number of chemokine receptors associated with function and homing, and specific gut-homing receptors, were investigated. Monocyte activation, apoptotic potential and intracellular monocyte cytokine production were also investigated. All markers were evaluated using multi-parameter flow cytometry. Monocyte responsiveness to *in vitro* LPS stimulation and expression of the afore-mentioned chemokine receptors to viral load, $CD4^+$ count and CD38/8 T-cell expression was also assessed.

During HIV-infection monocytes appeared primed to exit systemic circulation and migrate towards the gut, as seen through elevated CD62-L ($p < 0.005$) and CCR7 ($p < 0.005$), whereas the CD14+CD16+ subset was increased ($p = 0.0461$) and exhibited a higher activation status through increased CD69 expression ($p < 0.005$) compared to the CD14+CD16- subset. An interesting observation was the significantly increased IL-10 production by the CD14+CD16+ subset ($p < 0.005$). An elevated CCR5 expression in total monocytes ($p < 0.005$) was also seen. After LPS stimulation, the HIV+ group displayed unique and significant percentage increases in the total monocyte population.

The findings of the current study suggest that monocyte functionality may be retained during HIV-infection and that CD14+CD16+ monocytes play a vital role during HIV-infection evidenced by their preferential expansion and priming for GALT migration. The production of IL-10 by this subset further highlights their importance and emphasizes the need for future studies on the role of these cells in chronic stable HIV-1 infection and whilst disease progresses.

Opsomming

MIV-infeksie word gekenmerk deur die uitputting van $CD4^+$ T-selle, veral uit die derm-verwante limfweefsel (GALT). Dit veroorsaak onherstelbare skade aan die spysverteringskanaal en die daaropvolgende mikrobiese translokasie van bakteriële produkte soos LPS, ‘n komponent van Gram-negatiewe bakterieë, wat gaan binne sistemiese sirkulasie. MIV-infeksie beinvloed die funksies en relatiewe bevolkingsgrootte van verskeie immuun selle, insluitend monosiete. Monosiete is belangrike ingebore immuun selle en is dikwels die eerste selle wat gewerf word na areas van infeksie en inflammasie. Monosiete kan inflammatoriese prosesse bevorder of aanpabare immuunstelsel reaksies ontlok deur middel van hul antigeen aanbiedings vermoë of help met patogeen en puin klaring en skade herstel.

In hierdie deursnee-studie het ons veranderinge aan monosiete (CD14+CD16+ en CD14+CD16-) ondersoek in MIV+ behandelde naïef individue en gesonde onbesmette kontroles, deur die gebruik van hele bloed toetse en geïsoleerde monosiete. 'n Aantal chemokine reseptore, wat verband hou met homing en funksie was ondersoek in toevoeging tot spesifieke derm-homing reseptore. Monosiet aktivering, apoptese potensiaal en intrasellulêre monosiet sitokien produksie was ook ondersoek. Alle merkers is ondersoek deur multi-parameter vloeisitometrie. Die beoordeel reaksies van monosiete na *in vitro* LPS stimulasie en die uitdrukking van die merkers met merkers van algemene immuun aktivering en MIV-siekte patogenese was ook ondersoek.

CD14+CD16+ monosiete was gedurende MIV-infeksie verhoog (p-waarde = 0.0461). Daar was 'n hoër algehele monosiet uitdrukking van verskeie chemokine merkers soos CD69 (p-waarde < 0.005) uitdrukking; CD62-L (p-waarde < 0.005), en CCR7 (p-waarde < 0.005) uitdrukking in die CD14+CD16+ subgroep. Daar was ook ‘n toename in IL-10 produksie, veral in die CD14+CD16+ subgroep (p-waarde < 0.005). Hoewel baie funksionele merker reaksies dieselfde was, het die MIV+ groep ‘n unieke en beduidende persentasie verhooging in die totale monosiet bevolking getoon.

Ons algehele bevindinge dui op 'n voorkeur uitbreiding van CD14+CD16+ monosiete tydens MIV-infeksie. Die CD14+CD16+ monosiet subgroep blyk ook bevoordeel word met betrekking tot voorbereiding vir migrasie na limfknope en die GALT. Die toename in geaktiveer de CD14+CD16+ monosiete op siekte webwerwe is waarskynlik 'n groot bydraende faktor tot aanhoudende immuun aktivering wat op sy beurt virale replikasie bevorder. Hierdie resultate

beklemtoon die behoefte om die rol van hierdie selle en in veral die CD14+CD16+ subgroep, in kroniese stabiele MIV-1 infeksie verder te studeer en terwyl siekte bevorder.

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List of Abbreviations

HIV	Human immunodeficiency virus
NK	Natural killer cells
DC	Dendrtic cells
APC	Antigen presenting cells
CD	Cluster differential
GALT	Gut associate lymphoid tissue
AIDS	Acquired immunodeficiency syndrome
VIP	Vaso-intestinal peptide
VPAC	Vaso-intestinal peptide receptor
ART	Anti-retroviral therapy
ARV	Anti-retroviral
UNAIDS	Joint United Nations Programme on HIV/AIDS
RNA	Ribonucleic acid
ORF	Open reading frame
CMV	Cytomegalovirus
PPRs	Pattern recognition receptors
PAMPs	Pathogen-Associated Molecular Patterns
TLRs	Toll-like receptors
NOD	Nucleotide-binding oligomerisation-domain protein
NLRs	Nod-like receptors
RLRs	RIG-like receptors
CLRs	C-type Lectin Receptors
ROS	Reactive oxygen species
NOS	Reactive nitrogen species
TGF-β	Transforming growth factor-beta
CTL	Cytotoxic T lymphocytes
IL	Interleukin
TNF-α	Tumour necrosis factor-alpha
TNF	Tumour necrosis factor

IFN-γ	Interferon-gamma
LPS	Lipopolysaccharide
SIV	Simian immunodeficiency virus
AGM	African green monkeys
SMs	Sootey mangabeys
RM	Rhesus macaques
GIT	Gastrointestinal tract
mCD14	Membrane associated CD14
mDC	Myeloid dendritic cells
cDC	Conventional dendritic cells
M-CSF	Macrophage-colony stimulating factor
CCR	Chemokine receptor
CCL	Chemokine ligand
MCP-1	Monocyte-chemotactic protein-1
MHC	Major histocompatibility complex
GM-CSF	Granulocyte-macrophage colony stimulating factor
TNFR1/2	Tumour necrosis factor receptor 1/1
TRAIL	Tumour necrosis factor apoptosis-inducing ligand
TRAIL-R1	Tumour necrosis factor apoptosis-inducing ligand-receptor 1
SANAS	South African National Accreditation System
FITC	Fluorescein isothiocyanate
APC	Allophycocyanin
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
APC-H7	Allophycocyanin-Hilite®7
SSC	Side scatter
FSC-H	Forward scatter height
FSC-A	Forward scatter area
TCM	Tissue culture media
7AAD	7-Aminoactinomycin
MFI	Mean fluorescence intensity
FMO	Fluorescence minus one

PBS	Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
SOP	Standard operating prodecure
NHLS	National health laboratory services
BD	Becton Dickson
BM	Bone marrow

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Chapter 1: Introduction

Since its emergence in 1983, Human Immunodeficiency virus-1 (HIV-1) infection has become a serious worldwide problem. Much has been learned about the pathogenesis of the disease in the years since initial discovery, but with new knowledge has come the challenge of further questions. An area of particular importance has been in trying to discern the exact role the immune system plays in HIV-1 infection. In the absence of any individual ever being cured naturally, researchers have been unable to define any clear immune correlates of protection. Understanding is hampered by pleiotropic effects of various components of the immune system, leading to conflicting reports of protective vs. damaging roles. A particularly fertile area of research is the role of the innate immune system, its constituent cells and their mediators in the pathogenesis of HIV-1 infection. The innate immune system includes various cell types such as monocytes/macrophages, natural killer cells (NK), mast cells, granulocytes, and dendritic cells (DCs). Monocytes are one of the most important cells of the immune system as they are often the first cells that encounter invading pathogens as resident tissue macrophages, and they are often also the first cells to respond to danger signals provided by pathogens. Macrophages are tissue/organ associated cells and whilst monocytes are often precursors to macrophages, not all tissue macrophages originate from blood monocytes, with some macrophages now known to originate from embryotic somatic cells (Guilliams *et al*., 2013), see **Figure 1-1**.

Monocytes usually circulate systemically, after being released from the bone marrow, until they are recruited to sites of infection and in inflammatory situations, where they act upon invading pathogens. Monocytes are also the precursors of macrophages involved in inflammation resolution and promotion of tissue repair. Once migration into various tissues has occurred, monocytes differentiate into macrophages including specialised organ- or tissue-associated populations. These include, Kupffer cells in the liver, microglial cells in the brain and also the M1 (pro-inflammatory) or M2 (tissue repair) macrophage populations representing infection site recruits, which are involved in initiation of immune responses, and later in their resolution (Le Douce *et al*., 2010; Cassol *et al*., 2010; Laforge *et al*., 2011; Wu 2011; Mir *et al*., 2012). As early recruits to sites of inflammation and infection, monocytes play an important role as antigen presenting cells (APCs), inflammatory mediators and as phagocytes. However, they are also vital in the resolution of inflammation and in tissue repair; linked to the latter is their implication as

important role players in fibrotic conditions. Fibrosis is the aberrant deposition of collagen, which in moderation represents a normal repair mechanism, but in pathological conditions is problematic. The role monocytes play during immune responses, as phagocytes and APCs, has been extensively studied and well-characterised. The impact of HIV-1 infection on these cells has also been investigated. Monocytes may be directly infected with HIV-1; however their major role in this disease would appear to be in the induction (and continuation) of inflammation, the promotion and stimulation of acquired immunity and its role players, and also in mediation in lymphoid tissue fibrosis. However, due to the variability across the field in study populations, diseases status, infecting virus and baseline immunity, much conflicting and confusing data have been generated. More research is needed to clarify the impact HIV-1 has on monocytes (both directly and indirectly) and the consequence for the host's immune system and its ability to deal with the infection.

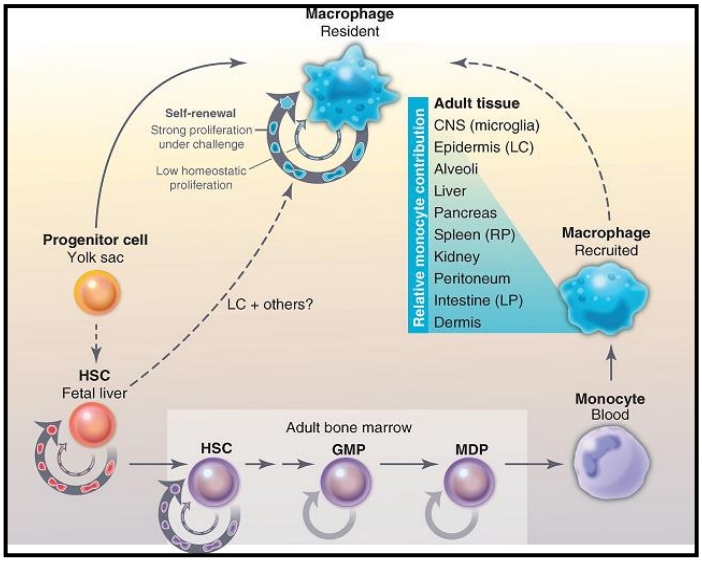

Figure 1- 1: Diagram showing monocyte and macrophage origins. Monocytes originate from the bone marrow and become released into circulation, whereas macrophages are either monocyte or somatic in origin (Sieweke & Allen 2013)

Significant changes have been reported in monocytes during HIV-1 infection, such as expansion of particular subsets, impaired functional ability and resistance to programmed cell death. Macrophages, together with the main target cells of HIV-1, $CD4^+$ T-cells are the first to encounter the virus and are also the two cells primarily responsible for viral replication and dissemination. A large number of $CD4^+$ T-cells (particularly memory $CD4^+$ T cells expressing CCR5) reside in the gut associated lymphoid tissue (GALT) and generally in infection these cells

are targeted by the virus, resulting in massive depletion of these T-cells. In parallel to this depletion, damage to the gut barrier occurs as a result of the pro-inflammatory milieu. Subsequent microbial translocation of bacterial products, such as lipopolysaccharide (LPS), into the systemic circulation then occurs. Microbial translocation itself leads to immune activation, both localised and systemic. Recruitment of monocytes from the bloodstream into the GALT and other lymphoid tissues then occurs resulting in on-going promotion of inflammation and immune activation ultimately leading to immune exhaustion. Monocytes thus play an important role during both immune activation persistence and repair in the lymph nodes and gut (Laforge *et al*., 2011; Wu, 2011; Mir *et al*., 2012).

Aberrant immune activation poses a new challenge in the fight against HIV-1 infection. The inability of the immune system to clear HIV-1 infection despite the advancement of anti-retroviral therapy highlights the importance of immune activation in disease progression to acquired immunodeficiency syndrome (AIDS). This phenomenon has opened a new area of research into selective modulation or dampening of inflammation and immune activation. Several potential immuno-regulatory molecules and drugs have been proposed as intervention agents. A particularly promising mediator is vasoactive intestinal peptide (VIP), a neuropeptide that functions as a neurotransmitter and neuromodulator that when engaging with its receptors, vasoactive intestinal peptide receptor 1 (VPAC-1) and vasoactive intestinal peptide receptor 2 (VPAC-2), has been seen to promote anti-inflammatory effects (Delgado *et al*., 2004). Although only recently investigated in the HIV-1 field, this mediator has been well studied in other inflammatory disorders and represents a unique and potentially powerful selective mediator which may both increase resistance to HIV-1 infection in monocytes/macrophages and dampen aberrant pro-inflammatory activity in chronic infection.

Given the important role monocytes play in the immune system and particularly in HIV-1 pathogenesis it is vital their role be further investigated. Most especially the role they play in linked processes of immune activation and immune exhaustion. A number of studies have been performed in Africa have reported conflicting results regarding immune activation caused by LPS in HIV-1 infection. The conflicting nature of current literature regarding the characterisation, phenotype and functioning of monocytes in different disease settings further highlight the importance of conducting studies on this innate cell subset. Therefore the current study set out to

determine if HIV-infection affects the function of monocytes. If monocytes are affected, how are they affected? Lastly, could monocyte be used as a therapeutic intervention?

The aim of this research project was to investigate the phenotype and function of monocytes in South African individuals with a stable CD4 count (> 300cells/µl) and who are asymptomatic. Furthermore, this study set out to elucidate the role of these cells in HIV-1 infection in relation to markers of homing (gut and lymph node), activation, inflammation and apoptosis. The novel aspects of this study were the simultaneous evaluation of putative gut and lymph node homing markers and assessment of their expression in relation to standard markers of HIV disease (CD4 count and viral load) as well as a general marker of immune activation status (CD38 on $CD8^+$ T cells). The expression of the VIP receptor VPAC-2 on monocytes was also assessed with the aim of defining whether this modulatory pathway could be exploited in the monocyte cell population in this disease.

This thesis will discuss the research in the following manner: **Chapter 2** will be a review of current monocyte literature in relation to HIV-1 infection and will also outline the motivation, hypothesis, aims and objectives of the study. **Chapter 3** will describe the materials and methods used to perform the research. The results obtained will be presented in **Chapter 4** and discussed in **Chapter 5**. **Chapter 6** will provide a concise conclusion to the work.

Chapter 2: Literature review

The primary focus of this literature review is monocytes and their relation to HIV-1 pathogenesis. A brief introduction to HIV-1 epidemiology and virology is given, followed by the processes involved in inflammation, and HIV-1 pathogenesis. The chapter will address monocytes in detail, their role in HIV-1 pathogenesis and an overview of selected key monocyte markers which have been studied in more detail in this research project.

2.1. HIV-1

2.1.1. History

HIV was first reported as the cause of acquired immunodeficiency syndrome (AIDS) in 1983 (Barré-Sinoussi *et al*., 1983). There are two major sub-species of HIV namely; HIV-1 and HIV-2. HIV-1 is responsible for the current global AIDS pandemic and has successfully spread worldwide (Sasse & Van Beckhoven, 2012). HIV-2 on the other hand is not commonly found outside of Africa and was first isolated in West Africa in 1986 (Clavel *et al*., 1986).

2.1.2. Epidemiology

At the end of 2013 the Joint United Nations Programme on HIV/AIDS (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013), released a report estimating that 35.3 million people were living with HIV in 2012, an increase from previous years due to more people receiving anti-retroviral therapy (ART) (**Figure 2-1**). This is most likely as a result of successful roll-out of ARV therapies in many areas. Sub-Saharan Africa continues to be disproportionately affected with 70% of all new global infections occurring in this region in 2012. Surveys in several sub-Saharan African countries have shown that there is a decrease in condom use and/or an increase in the number of sexual partners, which highlights the fact that societal attitudes impose a major restriction to successful programmes aimed at eradicating this disease. Despite this, the annual number of new HIV infections amongst adults in sub-Saharan Africa has reduced by 34% from 2001 to 2012 (**Figure 2-2**). Globally there has been a 33% decline in the number of new infections from 3.4 million in 2001 to 2.3 million in 2012 (**Figure 2-1**). The number of AIDS

deaths has also been declining from 2.3 million AIDS deaths in 2005 to 1.6 million in 2012 (**Figure 2-1**) (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013).

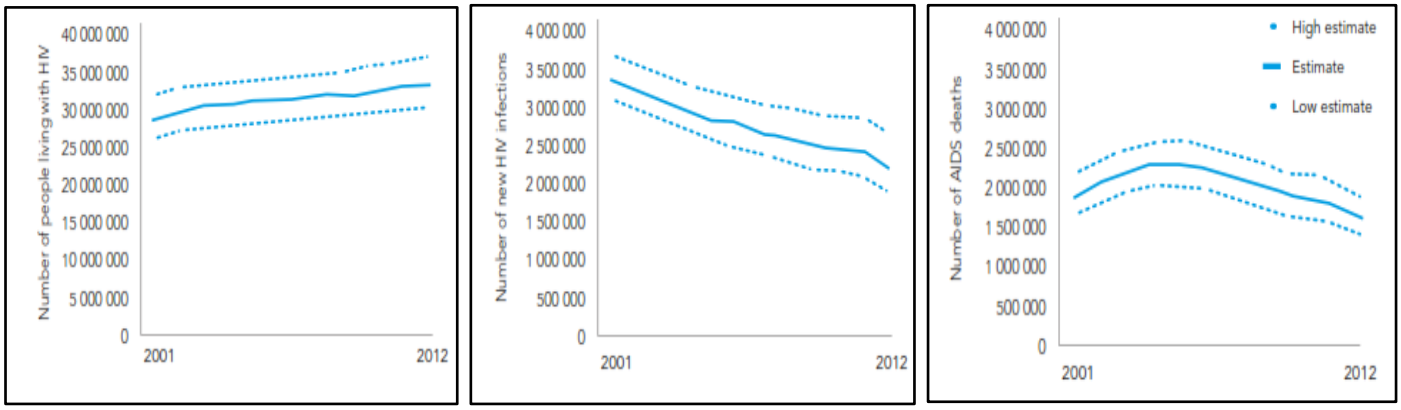

Figure 2- 1: Graphs depicting HIV epidemiology globally and within Sub-Saharan Africa. The graph on the far left number shows the number of people living with HIV globally from 2001-2012. The graph in the middle shows the number of newly infected people, globally from 2001-2012 and the graph on the right shows the number of AIDS deaths in sub-Saharan Africa from 2001-2012. The amount of people living with HIV globally has increased in 2012 with an estimated 35.3 million people are living with HIV, whilst the number of .newly infected adults declined by 33% in 2012.There has also been a decline of AIDS deaths from 2005 to 2012(Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013).

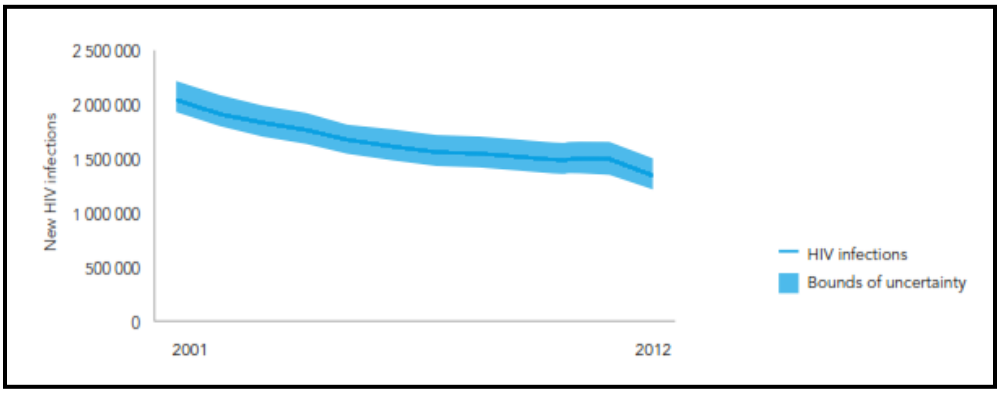

Figure 2- 2: The number of new HIV infections amongst adults in Sub-Saharan Africa from 2001-2012. The annual number of newly infected adults has decreased by 34% since 2001 (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013).

Notable reductions in HIV prevalence has been achieved amongst young people (15-24 years) across diverse countries of sub-Saharan Africa. HIV prevalence amongst young women and men has declined by 42% from 2001 to 2012. However, the prevalence is still more than double amongst young women than in young men throughout sub-Saharan Africa (**Figure 2-3**).

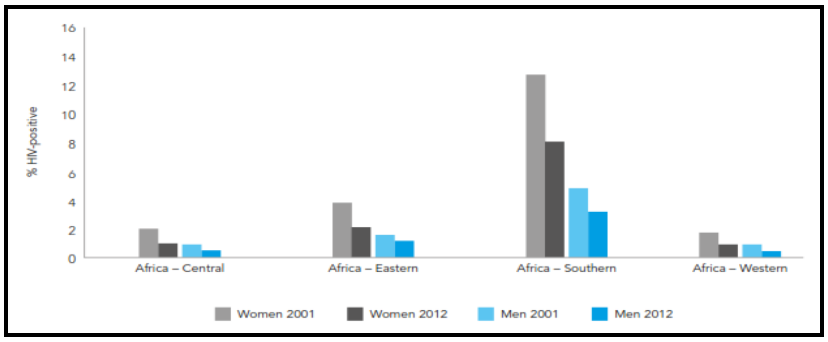

Figure 2- 3: The prevalence of HIV infection amongst young people from different parts of Africa from 2001-2012. The prevalence of HIV infection amongst young people from different parts of Africa from 2001-2012. There was a decrease in the prevalence of HIV amongst young men and women of Southern Africa from 2001-2012. However, the prevalence amongst young women of Southern Africa remains higher than in young men of Southern Africa despite the decrease seen since 2001 (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013).

Despite an improvement in both globally and particularly in sub-Saharan Africa, HIV-1 infection and AIDS remains a major health concern. ARTs are effective in most cases but universal access and compliance remain logistical problems. Attitudes to sex (e.g. use of condom, multiple partners) remains an area of concern. In the absence of an effective vaccine or cure, global efforts at optimising therapy remain a primary focus area.

2.2. HIV-1 structure

HIV belongs to the Lentivirus group of the family Retroviridae. It is an enveloped RNA virus of spherical shape and particle size of 100-120nm in diameter. The HIV-1 genome is made up of two identical 9.2 kb single stranded RNA molecules enclosed in a cone-shaped nucleocapsid, surrounded by a lipid bilayer membrane (Frankel & Young, 1998).

Encoded in the genome are nine open reading frames (ORFs) (**Figure 2-4**). Three of the ORFs encode for a structural proteins; Gag, Env and Pol. The *gag* gene encodes four glycoproteins, viz. matrix (**p17**), capsid (**p24**), nucleocapsid (**p9**), and **p**6; Env is cleaved to produce **gp120** and **gp41**. These are all structural proteins of which the virion core and outer membrane envelope consist. The Pol proteins, viz. viral protease (**p10**), RNA polymerase (**p15**), reverse transcriptase (**p50**) and integrase (**p31**) play a vital enzymatic role promoting the processes of transcription and integration. *Tat* and Rev are regulatory proteins that play critical roles in gene regulation. *Tat* is essential for HIV-1 replication by lengthening the phase if HIV-1 transcription, whilst Rev plays a role in the transition from early to late phase of HIV gene expression. The viral genome also

encodes four accessory proteins, Vif, Vpr, Vpu and Nef (the latter also sometimes included as a regulatory protein) that represent important virulence factors *in vivo* (Frankel & Young 1998).

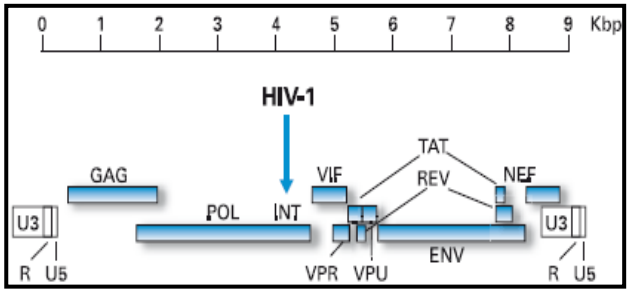

Figure 2- 4: Genomic structure of HIV-1. The figure shows the genes gag, pol and env and the accessory genes *vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*. Kbp represents kilo-base pair (Frankel & Young 1998).

2.3. HIV-1 replication and transmission

HIV infection is usually characterised by three disease stages (**Figure 2-5**); the Acute Phase where initial viral replication occurs unhindered by specific immune pressure. This phase includes the process of initiation of inflammatory responses and stimulation of innate immune cells. During this time the initiation and amplification of acquired immunity also occurs. Cell-mediated and humoral immunity develops later in this stage creating selective pressure that results in a decrease in viral replication. This decline in viral replication results in the establishment of a relatively stable viral load, defined as the viral load set point. The establishment of the viral set point is accompanied by stabilisation of the CD4 count, and both these phenomena generally represent the Chronic Phase (i.e. a transition from acute to chronic infection).During the Chronic Phase infected individuals are usually asymptomatic, however, this phase is also characterised by continued unresolved inflammation and immune activation, which may be caused by antigenemia.

Antigenemia may arise as a result of HIV itself, but also to other chronic viral infections (e.g. Cytomegalovirus (CMV)) and to increased gut content antigens entering the circulation due to translocation. This clinically latent phase may last many years and eventually leads to the destruction of the host's immune system as a result of chronic immune activation eventually leading to immune exhaustion. This culminates in the onset of AIDS, the appearance of opportunistic infections in the host and ultimately the death of many un-treated patients

(Coleman & Wu, 2009; Kedzierska *et al*., 2003). The implementation of combination ART (cART) has however led to a decrease in the number of AIDS deaths, allowing HIV-infected individuals to live much longer. The use of ART drugs leads to a reduction in plasma viral load to undetectable levels. Infected individuals will subsequently be less infectious and this could most likely decrease the transmission of HIV via sexual contact. With the use of ART the chronic phase of HIV infection is prolonged and the onset of AIDS delayed (Centers for Disease Control and Prevention, 2009).

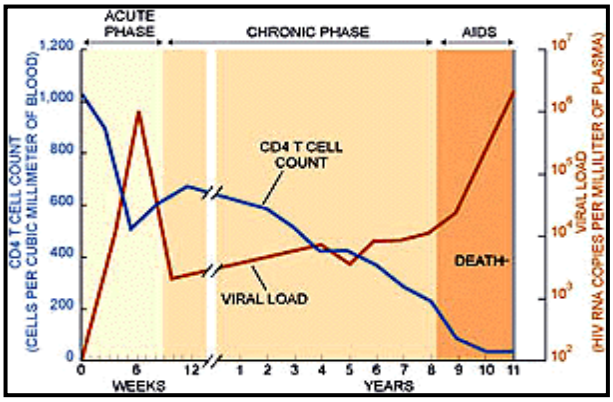

Figure 2- 5: Diagram illustrating the stages of HIV infection, including the length of each stage as well as the emergence of viral load and its effect on CD4$^+$ T-cell count (William Chris Woodward 1999).

There are three major routes of transmission, namely:

- Horizontal (sexual contact, both hetero- and homosexual)
- Vertical (mother-to-child)
- Intravenous drug users

Irrespective of the route of HIV infection and the first cells which are infected, viral replication converges on GALT in only a few days (Cohen, 2011). This is subsequent to detection of infection in localized draining lymph nodes (usually of the genital tract). Once the GALT is infected, a massive destruction of memory CD4$^+$ T cells occurs. This event marks a major turning point in the pathogenesis process, with the scales turning toward the virus's advantage. Normally, the immune system ensures that infection is contained and eradicated before irreversible damage to the host occurs. However, this does not occur during HIV infection.

The pathogenesis of HIV-1 infection is an extremely complex process involving multiple role players. For the purposes of this thesis, the primary focus will be on inflammation, now an accepted driver of disease pathogenesis, and host immune response, particularly innate responses and the role of monocytes in HIV-1 disease progression.

2.4. The host immune system

2.4.1. An overview of the inflammatory response

The acute inflammatory response is initiated by infection or tissue injury and consists of the coordinated delivery of blood components, such as plasma proteins and leukocytes to the site of infection or tissue injury. This response has been best characterised for microbial infections where the response is triggered by a family of Pattern-Recognition Receptors (PPRs), which recognise Pathogen-Associated Molecular Patterns (PAMPs). Toll-like Receptors (TLRs) and NOD (nucleotide-binding oligomerisation-domain protein)-like receptors (NLRs) are two families of PPRs that represent the major receptors of the innate immune system. TLRs recognize extracellular or endosomal antigens as they are membrane bound receptors. NLRs recognize intra-cytoplasmic antigens as they are located intra-cytoplasmically. TLRs and NLRs are two important groups of PRRs that serve to initiate inflammatory responses through the recognition of various PAMPs (Lehner, 2002).. There are also other PPRs including RIG-Like Receptors (RLRs) and C-type Lectin Receptors (CLRs). The initial recognition of infection is facilitated through tissue-resident macrophages, mast cells and dendritic cells. Following antigen recognition via PRRs, innate cells are induced to secrete various inflammatory mediators such as, chemokines, cytokines, vasoamines, eicosanoids and other products of proteolytic cascades. The central and most important role of these mediators is to initiate an inflammatory response that creates a local pro-inflammatory microenvironment. This involves the translocation of plasma proteins and leukocytes from the blood vessels to the extra-vascular tissues at the site of infection through post-capillary venules (**Figure 2-6**). The activated endothelium of blood vessels allows for selective extravasation of leukocytes whilst preventing the exit of erythrocytes from the blood stream. This selective ability is achieved through the inducible ligation of endothelial-cell selectins with intergrins and chemokine receptors present on leukocytes (Medzhitov, 2008; Qu *et al*., 2014).

Upon arrival at the infected tissue site, neutrophils become activated. This occurs through either direct contact with the pathogen or through the effects of secreted cytokines by resident tissue cells. Neutrophils then make an attempt to eradicate the invading agents either through phagocytosis or through the secretion of the contents of their granules. These granules contain reactive oxygen species (ROS) and reactive nitrogen species (NOS), proteinase 3, cathepsin G and elastase. These extremely powerful effectors are not able to discriminate between microbial and host targets which leads to inevitable damage to surrounding host tissues (Medzhitov, 2008; Qu *et al*., 2014).

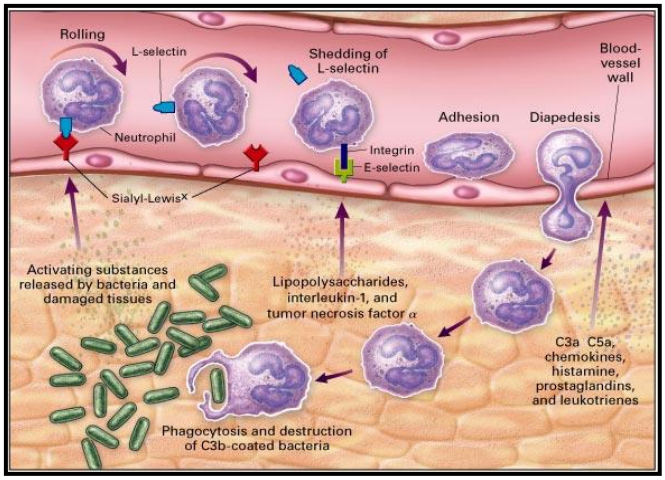

Figure 2- 6: Graphical representation of the inflammatory response showing activation of neutrophil via bacterial products, recognition of neutrophils through the tetrasaccharide carbohydrate Sialyl-LewisX and the involvement of integrins and selectins in the diapesdis of neutrophils through the blood vessel wall as well as the subsequent phagocytosis and destruction of bacteria (Medzhitov, 2008).

If an inflammatory response is successful, elimination of the infectious agent will be achieved followed by termination of the inflammatory process and then a repair phase that is mediated mostly by resident tissue macrophages and other recruited monocytes/macrophages. One very important step in the resolution of the inflammatory response is the switch from the expression of pro-inflammatory prostaglandins, to anti-inflammatory lipoxins. Lipoxins are involved in inhibiting the recruitment of neutrophils and instead encourage the recruitment of monocytes to sites of inflammation. Here the monocytes are responsible for clearing of dead cells and facilitating tissue remodelling. Other non-cellular mediators include lipid mediators (resolvins & protectins), and cytokines e.g. Transforming Growth Factor-β (TGF- β) secreted by macrophages. These lipid mediators and cytokines also play an essential role in the initiation of tissue repair

following an inflammatory response via the stimulation of cell replication and deposition of collagen by fibroblasts (Medzhitov, 2008; Qu *et al*., 2014).

If, however, the initiated inflammatory response is unable to clear the infectious agent the inflammatory response persists and develops novel characteristics. Neutrophils initially present at the site of infection are replaced with T-cells and monocytes that differentiate into macrophages. If the activity of both macrophages and T-cells are inadequate to eradicate the antigen, the inflammation becomes chronic and a chronic inflammatory state is established. This occurs in the lymph nodes and other lymphoid tissues (especially the GALT) during HIV-1 infection. The features of this type of inflammatory response are highly dependent on the effector class of T-cells that are present, and in HIV infection during most stages of disease it is a pro-inflammatory Th1-type milieu (Medzhitov, 2008; Qu *et al*., 2014).

The mechanisms involved in infection-induced inflammation are understood far better than any other inflammatory process. However, it has been said that even though infection-induced inflammation is important in clearing an infection, it may be unique for particular conditions (infectious agent, infectious dose, host status etc.). Therefore, it is essential to fully understand the mechanisms involved in infection-induced inflammation and how these mechanisms can be applied to other inflammatory processes (Medzhitov, 2008).

In HIV infection the initiation of inflammation is important in the early acute stage as an attempt to control the virus and eradicate it. The virally-induced inflammation is also important for the initiation of effective acquired immunity. Innate cell-derived cytokines prime a pro-inflammatory Th1 antiviral immunity. The initial inflammation becomes problematic when the pathogen remains un-cleared. There is no resolution of inflammation, rather continued promotion of damaging pro-inflammatory immune responses (including helper and CTL activity, production of cytokines such as IL-6, tumour necrosis factor-alpha (TNF-α) and IFN-γ and hyper-stimulation of B cells. Chronic inflammation, particularly in lymphoid tissues, leads to chronic immune activation, hyper-responsiveness and ultimately exhaustion. Coincidental with immune collapse is the development of fibrosis in lymphoid tissue due to initiation aberrant repair mechanisms. This accumulation of multiple drivers towards immune collapse culminates in death of the host. The initial trigger is the natural initiation of normal innate immunity by the incoming virus (Medzhitov, 2008).

2.4.2. Innate immune system

The immune system comprises of innate and adaptive arms. Innate immune responses are said to be older and are evolutionarily more conserved. Innate immunity is a first line defence system intimately linked to inflammation that includes phagocytes, both mononuclear (monocytes and dendritic cells) and polymorphonuclear (granulocytes); cytolytic cells, primarily NK cells, other innate lymphocytes, and all cellular associated cytokines (**Figure 2-7**). The purpose of the innate immune system is to eradicate invading pathogens without causing extensive host damage. The type of response can vary depending on which immune cells initiate the response and which predominate, as well as which cytokines are produced. Monocytes play an essential role in the early response of the innate immune system (Sasse & Van Beckhoven, 2012).

The innate immune system is active during the acute phase of infection and precedes the development of specific adaptive immunity. Innate immune cells first function as effector cells in the innate response but later may act to dampen or enhance adaptive immune responses (Lehner, 2002). The pro-inflammatory mediators in the inflammatory response are mostly produced by the innate immune cells that are stimulated through PPRs that are specific to bacterial products (Brenchley & Douek, 2012)..

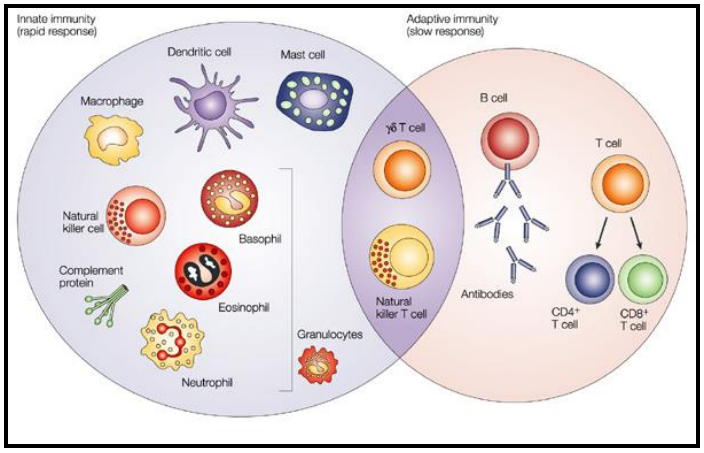

Figure 2- 7: Immune cells that comprise the innate and adaptive immune system (Dranoff, 2004).

2.4.3. Role of the Gut-Associated Lymphoid Tissue (GALT) in HIV-1 pathogenesis

Microbial translocation, whether it is microorganisms or microorganism components, from the gut lumen of the gastrointestinal tract (GIT) into the systemic circulation may have dramatic consequences, including promoting immune activation. A possible cause for this phenomenon is the extremely high levels of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), that are produced in the GALT in response to gut leakage. Despite the fact that the release of these cytokines initiates a favourable immune response, the over production of these cytokines leads to an enhanced systemic inflammatory response. In some cases this is more damaging than the infection itself. If microbial products such as Lipopolysaccharide (LPS), an outer-membrane component of Gram-negative bacteria, move across the mucosal and epithelial barriers a large amount of specialized resident macrophages prevent the bacterial products from translocating into the systemic circulation (Brenchley & Douek, 2012).

HIV-1 infection that usually stems from mucosal exposure results in epithelial and resident innate immune cell stimulation and production of pro-inflammatory cytokines. Dendritic cells aid in translocation of viral particles to the draining lymph nodes. Once within the lymphoid tissue, the virus has optimal exposure to its primary target, $CD4^+$ T-cells. Following initial viral expansion in the lymph nodes, the virus gains access to the GALT, a $CD4^+$ T-cell store, this results in the characteristic rapid depletion of these cells. $CD4^+$ T-cell depletion affects primarily the lamina propria of the gut and to a lesser degree the Peyer's patches. This phenomenon is also seen in rhesus macaques (RM) infected with simian immunodeficiency virus (SIV) and the natural hosts of SIV, African green monkeys (AGM) and Sootey Mangabeys (SMs) (Cohen, 2011). $CD4^+$ T-cells present in the gut are said to have a "memory' phenotype and express CCR5, the main co-receptor for M-Tropic HIV strains. It has been suggested that half of the $CD4^+$ T-cells found in the body are present in the GALT (Stellbrink *et al*., 2010). The environment of the GALT is optimal for viral replication. Within a few days most of the cells present in the GALT are infected and they die due to cytopathic effects of the virus infection or due to bystander cell death. Bystander death occurs as a result of cellular activation and activation of death receptor pathways (Cohen, 2011).

Weakening of gut barrier integrity also occurs as tight junctions are breached during the $CD4^+$ T-cell depletion, this facilitates the entry of microbial products into the body, such as LPS, from the

gut lumen into the tissues of the host (**Figure 2-8**) (Cohen, 2011; Wasserman *et al*., 2011). This leads to the activation of both the innate and adaptive immune responses (Cohen, 2011). During HIV infection chronic systemic immune activation persists, various causes have been suggested. Many of these causes are multifactorial, including: the constant increased levels of type I and type II interferons, innate and adaptive immune responses to HIV-1 infection, bacterial products that translocate from the damaged or "leaky" gut, effects of the HIV-1 virions or viral proteins, co-infections with non-HIV pathogens (e.g. CMV), and the dysregulation of cytokine and chemokine production. This persistent immune activation is also evident in the later stages of infection as the host progresses towards AIDS (Kamat *et al*., 2012).

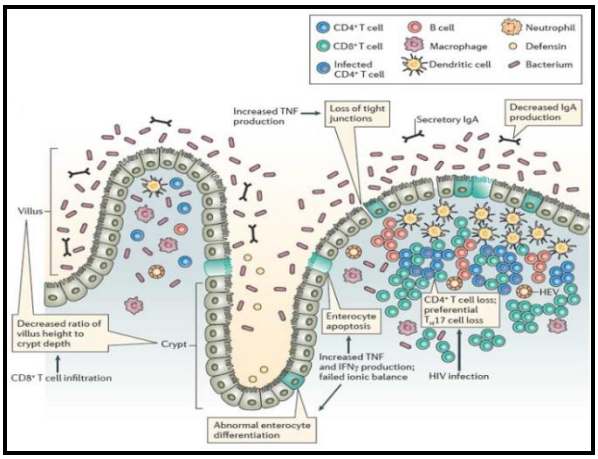

Figure 2- 8: "Leaky gut" observed during HIV infection. The height of the villi decrease leading to increased crypt depth, which has been associated with $CD8^+$ T-cell infiltration. Abnormal enterocyte differentiation and apoptosis is seen as well as the loss of the tight junctions (Sandler & Douek, 2012).

Interestingly there is no increase in the levels of LPS translocation and low levels of immune activation are seen in AGMs and SMs infected with SIV, where non-pathogenic disease is observed. Furthermore, it has been shown that there is an absence of microbial translocation in SIV's natural hosts (Douek, 2007). It has therefore been suggested that the $CD4^+$ T-cell depletion on its own cannot account for the translocation of LPS seen in chronic HIV-1 infection and that on-going immune activation is rather linked to GIT damage and microbial translocation (Brenchley *et al*., 2006; Hofer *et al*., 2010; Estes *et al*., 2010; Vassallo *et al*., 2012; Hunt, 2012). This further highlights the pivotal role of LPS translocation and the role immune activation plays in the pathogenesis of SIV infection and ultimately its application in understanding HIV-1 infection.

2.4.4. Role of Lipopolysaccharide in HIV pathogenesis

LPS is an important bacterial component that can enter systemic circulation due to GIT damage and translocation. Due to the importance of LPS as a stimulus for monocytes, this will be discussed in more detail.

The concept of microbial translocation is seen in many disease settings. These include graft versus host disease, inflammatory bowel disease and surgery of the gut. Microbial translocation has been correlated with systemic immune activation in several of these conditions. Since 2007 it has been acknowledged that those infected with HIV-1 may have a significant increase in gut permeability. Immunological and structural defects act together in HIV-1 infection in causing microbial translocation (**Figure 2-9**).

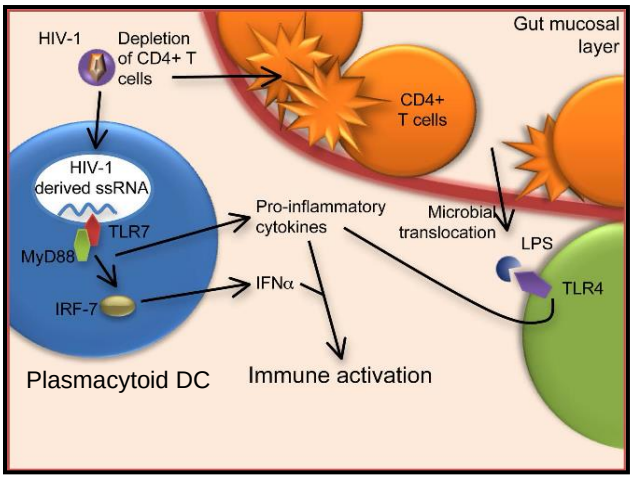

Figure 2- 9: HIV-1 infection leading to CD4$^+$ T-cell depletion and consequent damage to gut barrier and "leaky gut" syndrome. Microbial translocation follows, resulting in stimulation of TLR4, present on monocytes, through elevated levels of LPS leading to release of pro-inflammatory cytokines that can cause immune activation. Additionally stimulation by HIV-1- encoded TLR7 ligands on plasmacytoid DC can also lead to immune activation through pro-inflammatory cytokines (Chang & Altfeld, 2009).

LPS is a well-known immune stimulator. This immune stimulation is linked to the binding of LPS to the CD14/TLR-4 complex. CD14 is the primary LPS receptor and exists in both cell membrane (mCD14) bound and soluble (sCD14) forms. mCD14 is expressed on cells of the monocyte lineage and sCD14 is thought to represent mCD14 which has been cleaved in response to either monocyte activation or differentiation. SIV infection in SMs has been connected to low levels of immune activation, even in the presence of high viral loads. Microbial translocation that occurs early in HIV infection has been implicated as a cause for the systemic immune activation

seen during chronic infection (Douek, 2007). It is suggested that the mucosal damage that occurs in the GIT of SIV-infected RMs and HIV-1 infected individuals may lead to high levels of LPS being released. This could hinder the ability of the host's defence mechanisms and therefore permits the generation of persistent immune activation (Vassallo *et al*., 2012). The degree of mucosal damage seen in pathogenic SIV infection has been linked to the extent of the microbial translocation, which in turn drives the production of inflammatory cytokines from resident macrophages and recruited monocytes (Vassallo *et al*., 2012) Innate immune cells and specifically monocytes, from HIV-1 infected individuals, have been shown to be refractory to LPS stimulation *ex vivo*; it was then suggested these monocytes had already been stimulated with LPS *in vivo* (Brenchley *et al*., 2006).

This is evidence that LPS is a contributor to systemic activation and that this concept may be applied to diseases like HIV-1 infection, where the microbial translocation is seen. Therefore, the level of microbial translocation seen in HIV-1 infected individuals may be used to establish the rate of progression to AIDS (Douek, 2007). Due to its importance as a monocyte stimulus, LPS stimulation of whole blood or purified monocytes can be used to test monocyte function. This procedure has been applied in the current study to assess functional responsiveness of monocytes in HIV-infected and uninfected individuals.

2.4.5. Immune activation and its role in HIV pathogenesis

As mentioned previously, a key feature of both pathogenic SIV and HIV-1 infection is the persistent immune activation that leads to, pro-inflammatory cytokine production, uninhibited viral replication in activated T-cells and ultimately immune exhaustion. Continuous immune activation in HIV-1/SIV infection is characterized by polyclonal B-cell activation, an increased T-cell production as well as elevated levels of activated T-cells. Also seen is augmented production of pro-inflammatory cytokines (Estes *et al*., 2010; Hunt, 2012). Immune activation and inflammation observed during HIV-1 infection are mediated directly and indirectly by HIV-1 itself. Direct immune activation is characterized by HIV-1 specific $CD8^+$ T-cell cytolytic responses, a decrease in $CD4^+$ T-cells responses to the HIV-1 infection as a result of $CD4^+$ T-cell depletion as well as activation of HIV-1-specific and non-specific antibody producing B-cells, NK cells and monocytes. Indirect immune activation, on the other hand, can occur due reactivation of other latent viruses such as CMV or Herpes viruses (Douek, 2007; Lehner, 2002; Wasserman *et al*., 2011; Brenchley & Douek, 2012; Kamat *et al*., 2012; Mir *et al*., 2012).

Immune activation has both negative and positive effects during HIV-1 infection by promoting aspects of immunity to control the virus but also systematically causing damage. Immune activation has also been suggested as a better measure of disease progression than viral load or $CD4^+$ T-cell count (Haynes, 2006; Douek, 2007; Cassol *et al*., 2010; Kwon *et al*., 2012).

The excessive nature of the immune activation observed in HIV-1 infection causes it to be a major driver of HIV-1 replication and progression to AIDS. In a study performed by Brenchley & Douek (2012), elevated levels of circulating LPS were a contributing factor to the abnormal immune activation seen in those infected with HIV-1, as a result of microbial translocation. Consequently it can be stated that in developed countries, microbial translocation and immune activation may play a significant role in HIV-1 pathogenesis but it still remains, to some extent, unclear what role they play in HIV-1 infection in Africa. A study performed in Uganda, showed no association was found between circulating LPS and progression to AIDS (Redd *et al*., 2009). However, another 2009 study conducted in Kenya on female sex workers, identified a significant association between HIV-1 infection and plasma LPS (Lester *et al*., 2009). The authors further stated the inflammatory responses to LPS were extremely variable between the individuals. This would suggest differences in the host immunity and levels of background immune activation are able to modify the influence of microbial translocation on HIV-1 disease. These contrasting studies highlight the necessity to elucidate the relationship between the pathogenesis of HIV-1 infection and immune activation through microbial translocation (Brenchley & Douek, 2012). It further highlights the need to understand the role the immune system plays in HIV-1 infection and its progression to AIDS, especially in the African setting.

Various studies have recently linked surrogate markers of the persistent inflammatory state to clinical outcomes; this confirms persistent immune activation as a therapeutic target worth exploring. Widespread immune activation has been acknowledged as an important feature of AIDS pathogenesis. According to Hunt (2012), the polyclonal T-cell activation that is seen in untreated HIV-1 infection should be attributed to the generalized immune activation seen and not an outcome of the expansion of HIV-specific $CD8^+$ T-cells. During HIV-1 infection in addition to immune activation, higher frequencies of immune cell proliferation, activation and apoptosis have been observed (Mir *et al*., 2012).

2.5. Monocytes

2.5.1. Origin

Cells of the myeloid lineage include monocytes, macrophages, myeloid or conventional dendritic cells (mDC/cDC), granulocytes and mast cells. These cells play an extremely important role in the innate immune system as they are the first line of defence against invading pathogens. These cells also play an important role in HIV-1 infection and transmission (Le Douce *et al*., 2010; Cassol *et al*., 2010; Laforge *et al*., 2011; Wu, 2011; Mir *et al*., 2012). Cells of this lineage, particularly monocytes/macrophages are important to study as they may be targeted by HIV-1 *in vivo* and are also the main responders to LPS thus serving as the link between GIT translocation and immune activation in HIV-1 infection.

Monocytes are generated in the bone marrow from haematopoietic stem cells. They are released into the blood stream and circulate for up to three days before migrating to specific tissues, relocating to the spleen and entering the stored monocyte pool or being eliminated through natural homeostatic apoptosis. Whilst circulating in the blood stream, monocytes are able to detect a large number of pathogens via their broad range of PRRs. Monocytes make up 3-8% of blood leukocytes and serve as precursor cells to macrophages and in some cases mDCs. Macrophages and DCs are important APCs and play a central role in HIV pathogenesis. Monocytes are able to respond in the blood to circulating antigen, this characteristic may be important in HIV as increased levels of LPS (and other GIT products) in the blood is well described (Le Douce *et al*., 2010; Cassol *et al*., 2010; Laforge *et al*., 2011; Wu 2011; Mir *et al*., 2012).

In a recent study by Sassè *et al*. (2012), it was shown that the spleen is a reservoir for a large amount of monocytes. The authors provide clear evidence that there are un-differentiated monocytes inhabiting the spleen and that the monocytes outnumber those that are in circulation. This observation identified the spleen as a site of storage and deployment of splenic monocytes which performs as a reserve the body uses to control inflammatory responses. The current working hypothesis is that monocytes freely circulate in the blood stream and they patrol vessels and irreversibly differentiate into macrophages and mDCs.

2.5.2. Properties

Monocytes are characterized by a large degree of plasticity, widespread tissue distribution and the ability to respond to a large variety of environmental stimuli, such as microbial products and host cytokines. Whilst in circulation monocytes are exposed to cytokines such as macrophage-colony stimulating factor (M-CSF) which alter their phenotype and functional properties. Therefore monocytes can be classified into subsets based on their cellular surface expression of functional receptors. It has not been clarified whether the monocyte subsets signify cells that are already predisposed to differentiate into polarized M1 or M2 macrophages or DCs. It has been suggested that during a non-disease state blood monocytes are most likely predisposed toward M2 phenotype, which is aligned with tissue repair, due to their stimulation by elevated levels of M-CSF that is present in plasma (Cassol *et al*., 2010; Laforge *et al*., 2011).

Monocytes are phagocytic cells that are able to recognize and eradicate pathogens in a rapid fashion, with no need for antigen sensitization. This is achieved via a network of innate immune receptors that are expressed on their cellular surface which leads to either phagocytosis, killing of pathogens, the production of cytokines as well as the production of reactive oxygen and nitrogen species (Le Douce *et al*., 2010; Wu, 2011; Ackerman & Dugast *et al*., 2012; Gama *et al*., 2012; Mir *et al*., 2012).

2.5.3. Function and activation

Monocytes primarily function to initiate innate immune responses against a large range of pathogens. They make use of phagocytosis, intracellular killing (phagocytic burst response), and the secretion of cytokines, chemokines and other mediators to eradicate microbes (Le Douce *et al*., 2010; Mir *et al*., 2012). Monocytes have the ability to assimilate phagocytosed antigen and then present them in the lymphoid tissues. This then results in the prompt induction of adaptive immunity and the initiation of long-lived memory responses (Ackerman & Dugast *et al*., 2012; Gama *et al*., 2012). The differentiation that blood monocytes undergo in tissues is dependent on signals received from the local microenvironment (Le Douce *et al*., 2010; Mir *et al*., 2012).

2.6. Monocyte subsets

Several schemes of monocyte classification exist but monocytes are usually divided into two major subsets. This division is based on surface protein marker expression, but extends to cytokine production and the gene expression profiles (Mir *et al*., 2012). Monocytes can be either termed "classical", "intermediate" or "non-classical" depending on their varied expression levels of CD14 and CD16, however, there is much debate surrounding the delineation of monocyte subsets. For the purposes of the current study monocyte subsets have been defined using the expression of CD16 on monocytes as CD14+CD16- or CD14+CD16+.

2.6.1. CD14+CD16- monocyte subset

CD14+CD16- monocytes are the largest subset, accounting for >80% of blood monocytes in disease free individuals. They express high levels of CD14, almost no CD16 and are reported to have a pro-inflammatory phenotype. CD14+CD16- monocytes express elevated levels of chemokine receptor 2 (CCR2), the receptor for chemotactic ligand 2 (CCL2), also known as Monocyte Chemotactic Protein-1 (MCP-1). This receptor is involved in directing the migration of these monocytes towards sites of inflammation. CCL2 is expressed by cells in response to activation either by, pro-inflammatory cytokines, or stimulation of innate immune receptors by a range of microbial molecules (Shi & Pamer, 2011). At these sites of infection there is a high concentration of CCL2 present, which is the ligand for CCR2 (Sasse & Van Beckhoven, 2012).

L-selectin (CD62-L), which is best described as being involved in homing of lymphocytes to lymph nodes, is also expressed at high levels in CD14+CD16- monocytes. MCP-1/CCR2 facilitates the migration of $CD14^{+}CD16^{-}CCR2^{+}CD62\text{-}L^{+}$ monocytes into lymph nodes during inflammation (Ancuta *et al*., 2003; Hearps *et al*., 2012). However, CD14+CD16- monocytes may possess anti-inflammatory properties via their ability to produce high levels of IL-10.

2.6.2. CD14+CD16+ monocyte subset

CD14+CD16+ monocytes also have a pro-inflammatory phenotype; however, they preferentially express the fractalkine receptor (CX3CR1) instead of CCR2, and using CX3CR1, CD14+CD16+ monocytes migrate into tissues in response to CX3CL1 (Bocchino *et al*., 2001; Ancuta *et al*., 2003; Bergamaschi & Pancino, 2010; Iijima *et al*., 2011; Zawada *et al*., 2011; Mir *et al*., 2012).

CD14+CD16+ monocytes account for 5-10% of the total circulating monocyte population. CD14+CD16+ monocytes display characteristics of tissue macrophages and therefore may represent a higher level of differentiation as compared to the CD14+CD16- subset (Bergamaschi & Pancino, 2010). This monocyte subset is more susceptible to HIV-1 infection due to the high expression levels of CD4 and CCR5 receptors. Unlike $CD4^+$ T-cells, CD14+CD16+ monocytes are expanded up to 40% during HIV-1 infection and have the ability to migrate to tissues where they are able to form long-lived viral reservoirs. Various studies have shown that monocytic functions such as phagocytosis, chemotaxis and cytokine secretion both *in vivo* and *in vitro* are changed during HIV-1 infection (Locher *et al*., 1994; Kedzierska & Crowe, 2002; Vlad *et al*., 2003; Bergamaschi & Pancino, 2010; Cassol *et al*., 2010; Le Douce *et al*., 2010; Ackerman & Dugast *et al*., 2012; Gama *et al*., 2012; Hunt, 2012; Hearps *et al*., 2012; Mir *et al*., 2012; Sasse & Van Beckhoven, 2012; Martin *et al*., 2013).Furthermore, it has been shown that *in vivo* and *in vitro* CD14+CD16+ monocytes are an important source of the pro-inflammatory cytokine TNF-α (Cassol *et al*., 2010; Hearps *et al*., 2012).

CD14+CD16+ monocytes secrete elevated levels of TNF-α and lower levels of IL-10, a regulatory cytokine primarily involved in dampening immune responses, compared to CD14+CD16- monocytes. IL-10 was first identified and described as an important facilitator of T-cell exhaustion and a critical determining factor of viral persistence in the lymphocytic choriomeningitis virus mouse model (Sasse & Van Beckhoven, 2012). CD14+CD16+ monocytes also have been reported to possess diminished phagocytic capacity whilst possessing a higher antigen presenting capacity. However, others have reported conflicting findings, with increased phagocytic capacity as compared to CD14+CD16- monocytes. CD62-L is expressed in very low levels in CD14+CD16+ monocytes. This suggests that CD14+CD16+ monocytes are not preferentially recruited to the lymph nodes during inflammation (Ancuta *et al*., 2003; Hearps *et al*., 2012).

A study done in mice by Gautier *et al*. (2009) showed the behaviour of the CD14+CD16+ monocyte subset is context dependent. The authors showed that infiltration of the CD14+CD16+ monocyte subset into sites of tissue injury occurred in the late stages of inflammation and CD14+CD16+ monocytes are thought to play a role in tissue repair. However, in bacterial infections their recruitment is earlier. In myocardial infarction and skeletal injury studies,

CD14+CD16- monocytes are critical for setting up the resolution of inflammation by cleaning cellular debris and CD14+CD16+ monocytes are seen to mediate tissue repair.

2.7. HIV Infection and monocyte impairment

2.7.1. Monocyte population changes and functional impairment as a consequence of HIV-1 infection

Alterations in circulating monocyte subsets are seen in HIV-1 and pathogenic SIV infection, including the above-mentioned and well described expansion of the CD14+CD16+ monocyte subset. The expansion of the CD14+CD16+ subset is known to correlate with plasma HIV-1 viral load; this is also observed in SIV-infected RMs, where it has also been shown to correlate with SIV viral load; whilst peripheral blood $CD4^+$ T-cell count correlates inversely with CD14+CD16+ monocytes. It has also been seen that HIV-1 and pathogenic SIV infection lead to changes in the trafficking and the turnover of myeloid cells. SIV-infected RMs have shown increased monocyte turnover and this is said to be a better indicator of disease progression than CD4 count or viral load. In addition, this turnover probably reflects the increased recruitment from the bone marrow in response to elevated macrophage turnover that occurs in both peripheral and lymphoid tissues (Cassol *et al*., 2010; Mir *et al*., 2012).

A study performed which involved three groups of HIV-infected individuals, namely; (1) asymptomatic untreated patients with $CD4^+$-cell counts above 500cells/µl, (2) asymptomatic and symptomatic non-AIDS patients with $CD4^+$-cell counts below 500cells/µl, and (3) AIDS patients, found that the monocyte count in group 1 was elevated when compared to groups 2 and 3. An overall more activated status of monocytes in HIV-1 infected individuals was also seen (Elbim *et al*., 1999).

Monocytes play a very important role in the innate immune system through their antigen presentation function. Defects in this antigen presentation are a major issue during HIV-1 infection, and its systemic impairment happens at every stage of the plasma viremia. Moreover, defects in major histocompatibility complex (MHC) class II expression, formation of class II antigen complexes and antigen uptake have been observed in chronically infected monocytes during HIV-1 infection. The impaired antigen presentation could be related to severe immune dysfunction in HIV-1 infected individuals. The means by which this process occurs is not fully

clarified but it has been seen that there is reduced capacity of HIV-1 infected monocytes to stimulate or present antigen to $CD4^+$ T-cells. These defects may contribute to the immunosuppression seen during AIDS. Another reason that can account for this impaired stimulation of T-cells is the production of TNF-α induced by gp-120, which has been linked to defective antigen presentation capacity of monocytes in AIDS individuals (Sasse & Van Beckhoven, 2012).

Monocytes and macrophages are highly secretory cells and play a role in the altered chemokine and cytokine secretion patterns observed during HIV-1 infection. Production of several cytokines such as IL-10 and TNF-α, are affected during HIV-1 infection. TNF-α is known to up-regulate HIV-1 expression whilst other cytokines like Interferon-α have been shown to be potent inhibitors of HIV-1 replication. In addition to causing dysfunctional cytokine production, HIV-1 infection is also known to disrupt the normal synthesis of cytokines. It is important to note that in addition to direct infection, HIV protein released from infected cells may also affect monocyte and macrophage functioning in a bystander effect process (Sasse & Van Beckhoven, 2012).

Monocytes together with lung alveolar macrophages isolated from HIV-1 infected individuals showed reduced phagocytic activity, diminished phagosome-lysosome fusion and decreased intracellular killing of opportunistic pathogens. Monocytes that were isolated from patients suffering from AIDS also displayed impaired migratory responses; this phenotype has been linked to a down-regulation of receptors for chemotactic ligands (Cassol *et al*., 2010; Laforge *et al*., 2011). However, in *in vitro* studies conducted on circulating monocytes, it was noted that these cells lack any considerable impairment to important functions such as antigen presentation and differentiation into macrophages and myeloid DCs (Lederman, 2004).

2.7.2. Deficient intracellular killing

Monocytes infected with HIV-1 appear to display an impaired ability to eradicate opportunistic pathogens via intracellular killing. The fusion of phagosome and lysosome is pivotal for the killing of ingested pathogens. However, this fusion is considerably reduced in HIV-1 infection of peripheral blood monocytes and monocyte-derived macrophages (MDMs) which correlates with disease progression (Kedzierska & Crowe, 2002).

2.7.3. Impaired chemotaxis

HIV-1 infection is responsible for the deficient chemotactic ability observed in monocytes. Monocytic cells from AIDS patients have been shown to display defective migratory responses to chemotactic stimuli. This impaired chemotaxis is associated with the down-regulation of CCR2 surface expression (Kedzierska & Crowe, 2002).

2.7.4. Impaired phagocytosis

According to Kedzierska and Crowe (2002) there is no correlation between inhibition of phagocytosis, seen during HIV-1 infection, and viral load or $CD4^+$ T-cell count. This suggests that the defective phagocytosis observed in HIV-1 infection is not as a result of either of those measures of disease progression. It has been suggested that restoration of this impaired phagocytosis can be achieved by therapy with immuno-modulators such as granulocyte-macrophage colony stimulation factor (GM-CSF). GM-CSF is said to enhance the ability of monocytes and MDMs to phagocytose almost to the levels of un-infected cells.

2.7.5. Altered cytokine and chemokine production profile

A key feature of HIV-1 infection is immune activation and altered cytokine production various immune cell types. Monocytes play a major role in the altered cytokine and chemokine profile seen during HIV-1 infection. Cells of the monocyte lineage are associated with increased production of inflammatory cytokines. The elevated levels of inflammatory cytokine production are believed to activate HIV-1 replication and sustain active HIV-1 expression (Kedzierska & Crowe, 2002).

In the following sections a selection of cytokines and chemokines and their receptors which have been studied in this current study will be discussed.

TNF-α and IL-10

Increased levels of the pro-inflammatory cytokines TNF-α and GM-CSF seen during HIV-1 infection could result in the shift of virus-infected monocytes towards differentiation into macrophages or DCs (Bennasser *et al*., 2002; Iijima *et al*., 2011; Hou *et al*., 2012; Mir *et al*., 2012). It was found that HIV-infected humanized-mice had an elevated level of plasma TNF-α, which resulted in disruption of tight junctions and apoptosis of intestinal epithelia (Hofer et al.

2010). This is further supported by the observed apoptosis of intestinal epithelia which contributes to barrier dysfunction and therefore facilitates microbial translocation (Guillemard *et al*., 2004).

In chronically HIV-1 infected individuals, monocytes have been shown to produce more TNF-α upon re-stimulation with LPS. Monocytes from HIV-1 infected individuals also display impaired phagocytosis of Mycobacterium and there is also a decrease in LPS-mediated enhancement of phagocytosis when compared to the enhancement of monocyte phagocytosis in un-infected individuals (Bocchino *et al*., 2001). *Ex vivo*, monocytes of HIV-1 infected patients have shown a diminished pro-inflammatory cytokine response when stimulated with LPS; this has been speculated to be due to *in vivo* pre-stimulation (Hofer *et al*., 2010).

Monocytes from viremic patients that had been stimulated with LPS showed an over-production of TNF-α (Sauce *et al*., 2013).TNF-α production has been linked to at least two consquences. Firstly, the stimulation of HIV-1 replication in chronically infected cells and the second is linked to neurological disorders. The HIV-1 protein *Tat* has also been implicated in acting extra-cellularly to cause the production of TNF-α by peripheral blood monocytes. The Tat-generated production of TNF-α seems to contribute towards disease progression as a pathogenic factor through its pro-inflammatory characteristics (Bennasser *et al*., 2002). According to Cassol *et al*. (2010), the levels of TNF-α is already maximally expressed by HIV-1 infected individuals during the late stages of HIV-1 infection. The high level of viral replication that is observed during the late stages of chronic HIV-1 infection is expected to drive continual LPS responses and persistent immune activation; together these factors drive the progression of HIV-1 infection to AIDS. In contrast it has been found that in whole blood assays no significant difference in TNF-α production between HIV-1 infected and un-infected individuals were seen. Whole blood from SIV-infected SMs which had been stimulated by LPS was shown to secrete lower levels of TNF-α than uninfected SMs, suggesting that there is a down-regulation of TNF-α in SIV-infected SMs. Furthermore, when whole blood samples from SMs, RMs and human volunteers were stimulated with LPS it was found that in control SMs TNF-α expression was 20%, whereas SIV-infected SMs showed an expression of 7%. SIV-infected RMs and HIV-1 infected human volunteers did not display this reduced expression of TNF-α after LPS stimulation. Together this means that SIV-infected SMs experience a decrease in TNF-α production upon LPS stimulation but not SIV-infected RMs or HIV-1 infected humans (Cassol *et al*., 2010; Mir *et al*., 2012).

The down-regulation of TNF-α production due to LPS stimulation that is seen in SIV-infected SMs could be an early step in preventing high levels of immune activation. Through maintaining low levels of aberrant immune activation the disease progression to AIDS is hindered in non-pathogenic infection. Since there is rapid regulation of monocyte responses to LPS in SIV-infected SMs, it suggests that there needs to be stringent regulation of monocyte responsiveness. In addition, other monocyte responses must then also be regulated to prevent shift of immune activation towards disease progression (Mir *et al*., 2012).

The decrease of TNF-α secretion seen in SIV-infected SMs is not in any way related to a diminished capacity of monocytes to phagocytose infectious agents. Therefore, the innate effector mechanisms that play a role in clearing opportunistic infections are preserved. Simultaneously the ability to promote immune activation is dampened by SIV infection in SMs. The regulation of TNF-α is very important as it's one of the earliest and most concentrated pro-inflammatory cytokines produced in response to LPS. Furthermore, TNF-α is a major role player in cell survival and cell death depending on which receptor it binds (TNFR1 or TNFR2). The decline of monocyte responsiveness in SMs to LPS is not likely due to direct infection with SIV infection since monocytes are usually infected at very low levels during HIV infection in humans and SIV infection in RMs. TNF-α secretion in response to LPS can add to the increased $CD8^+$ T cell activation in SMs and highlights the importance of regulating monocyte LPS responses during non-pathogenic SIV infection. Natural hosts also alleviate the development of immune activation by increasing IL-10 production coupled with lower levels of T-cell activation and apoptosis during the acute and chronic phases of the disease. Monocytes have been shown to preserve their response to LPS in HIV-1 infection which implies that monocyte-derived pro-inflammatory cytokines play a role in facilitating an immune activation environment. The importance of uninhibited monocyte TNF-α production as being detrimental to clearing the disease is highlighted during pathogenic HIV-1 infection (Iijima *et al*., 2011; Mir *et al*., 2012).

Recent data has provided evidence that there are increased levels of IL-10 and CD14 in AIDS patients (Sasse & Van Beckhoven, 2012). Soluble CD14 is the cleaved version of the cellular marker and increased plasma levels of CD14 have been correlated with levels of inflammation. The level of IL-10 has also interestingly been shown to correlate with levels of CD14, suggesting that IL-10 may be a potential driving force for the elevated levels of CD14 seen during systemic infection. IL-10 plays many roles in HIV-1 pathogenesis. In chronic infection it is elevated and

these levels closely correlate with viral load. Studies involving the blocking of IL-10 signalling resulted in the restoration of both $CD4^+$ and $CD8^+$ T-cell proliferation as well as cytokine production. This suggests that IL-10 could possibly play a role in HIV-1 mediated immune exhaustion in that the cytokine induces hypo-responsiveness. Studies have shown that IL-10's anti-inflammatory effects could be protective during HIV-1 driven chronic immune activation. HIV-1 infected individuals who have genetic polymorphisms in their IL-10 gene, that are associated with elevated levels of IL-10 production, have shown slowed progression to AIDS, decreased decline of $CD4^+$ T-cell count and a longer lifespan. Collectively, this means IL-10 may have divergent roles in HIV-1 associated immune dysfunction and disease progression (Mir *et al*., 2012; Sasse & Van Beckhoven, 2012).

Production of IL-10 has been described in numerous cell types such as B cells, T- cells, NK cells as well as monocytes. However, monocytes *ex vivo* have been shown to be the primary producers of IL-10 in HIV-1 infected, untreated individuals. Studies have reported that monocyte subsets are vital in the regulation of T-cell responses in progressive HIV-1 infection and that increased IL-10 levels are characteristic of chronic HIV-1 disease. IL-10 is considered to specifically affect T-cell function through its effects on monocytes. This proposes the idea that T-regulatory (Tregs) cells may be stimulated by monocyte-derived IL-10. This would lead to a decreased ability of monocyte function as APCs through down-regulation of major histocompatibility complex class II (HLA-DR) and CD80/86 as well as a diminished cytokine production capacity – both via Treg activity. This interplay of Tregs and monocytes and the resulting up-regulation of IL-10 may lead to disease progression through limitation of host immune responses, impairment of pathogen clearance and its role in controlling inflammation. This interplay may also have the beneficial effect of restricting immune-mediated damage to the host (Kwon *et al*., 2012).

IL-10 is capable of acting in an autocrine or paracrine manner and its role is to prevent the production of TNF-α and other pro-inflammatory cytokines (Mir *et al*., 2012). IL-10 is able to up-regulate CCR1, CCR2 and CCR5 expression in human monocytes; its effect on CCR3, which is expressed in low levels, has been shown to be selective. IL-10 has also been shown to promote the active replication of HIV in monocytes; this has been linked with an increase in viral entry. An up-regulation of CCR5 contributes to IL-10 augmentation of HIV-1 replication. It has been suggested that the increase in CCR5 on the cellular surface of monocytes as well as other chemokine receptors by IL-10 may facilitate the accumulation of free circulating virions and their

eventual cellular entry. In spite of this, it should also be noted that IL-10 exercise various functions during the course of HIV-1 infection for instance hindering HIV-1 replication (Sozzani *et al*., 1998; Kwon *et al*., 2012).

IL-10's role in immune regulation is complex and it seems that this pleiotropic cytokine is responsible for the balance between the immune responses that are vital for pathogen clearance as well as excessive inflammation that results in collateral damage

CCR2 and CX3CR1

In SIV-infected RMs monocytes belonging to the CD14+CD16- subset displayed limited CCR2 expression. When compared to $CCR2^{+}$ CD14+CD16- monocytes the $CCR2^{low}$ monocyte subset showed impaired expression of various cytokine including TNF-α and IL-10. The $CCR2^{low}$ subset also exhibited diminished capacity for chemotaxis, and phagocytosis. A comparable subset was found in HIV-1 infected individuals with the same characteristics as the $CCR2^{low}$ monocyte subset RMs. It has been proposed that these cells developed in response to the virus-mediated immune activation and that the characteristics that the $CCR2^{low}$ monocytes exhibit may be accelerate disease progression by hindering the development of the antiviral cytotoxic response mediated by T-cells (Iijima *et al*., 2011; Mir *et al*., 2012).

Changes in monocyte phenotype in HIV-1 infection may impact early immune responses which could in the long run affect virus pathogenesis. Loss of surface CCR2 receptor expression may have an impact on cell trafficking as well as efficient responses during infection and inflammation. Monocytes in the CD14+CD16- subset that display a $CD4^{+}CD16^{-}CCR2^{low/negative}$ phenotype showing diminished expression of CD14 as well as a low CCR5 surface expression may be more resistant to HIV-1 infection (Gama *et al*., 2012). It is also known that CCR2 is required for the migration of monocytes from the bone marrow and that it may also be used by monocytes to diffuse into inflamed tissues (Iijima *et al*., 2011). CD14+CD16+ monocytes do not use CCR2 to migrate into tissues but CX3CR1 as they represent an advanced stage of differentiation closer to resident tissue macrophages (Kedzierska & Crowe, 2002).

CX3CR1, like all chemokine receptors, is a G-protein-coupled receptor. Its ligand is fractalkine that is a unique chemokine that has various functions including being a chemo attractant and an adhesion molecule. Fractalkine plays a central role in the recruitment of monocytes of the CD14+CD16+ subset. It has also been suggested that CD14+CD16+ monocytes are favoured for

recruitment to sites that express fractalkine and that they add to vascular and tissue injury seen during pathological conditions where their numbers are elevated (Ancuta *et al*., 2003). CX3CR1 has been implicated as a contributor to disease progression in HIV-1 infection. It was shown that progression to AIDS is either delayed or rapid and that the progression is influenced by specific mutations in the fractalkine receptor gene. The two polymorphisms detected, V249I and T280M have been shown to cause a slow progression or a fast progression to AIDS respectively, confirming that CX3CR1 plays a role in disease progression. When compared to HIV negative individuals HIV-1 infected patients have a higher percentage of cells expressing fractalkine in the T cell zones of their lymph nodes. Furthermore, there is no expression of fractalkine in the gut's Peyer's patches in healthy individuals, it is however, expressed by neurons and endothelial cells of the blood vessels (Becker, 2007).

Studies done on fractalkine expression in tissues of HIV-1 infected individuals show that there is an up-regulation of fractalkine in the lymph nodes and in the plasma cells of the GALT. The absence of fractalkine found in the GIT of healthy individuals suggests that it does not contribute to the homing of immune cells to an un-inflamed GIT. Fractalkine molecules on blood endothelia have been observed to interact with CX3CR1 on monocytes and this interaction initiated the capture, firm adhesion and activation of circulating monocytes. This has elucidated a novel mechanism by which leukocytes can be recruited to sites of infection. It has also been seen that pro-inflammatory cytokines released upon LPS secretion such as TNF-α also play a role in fractalkine synthesis (Becker, 2007).

2.7.6. Susceptibility to HIV-1 infection

In addition to resting $CD4^+$ T-cells, blood monocytes *in vivo* are known to be a major long-lived compartment of HIV-1 despite the low frequency of HIV-1 infection of these cells (Le Douce *et al*., 2010; Wu, 2011; Mir *et al*., 2012). HIV-1 exhibits tropism for both monocytes and macrophages, even though HIV-1 infection of monocytes and/or macrophages occurs at a low frequency. Subsequent to infection numerous direct and indirect effects on the innate immune system are induced. These effects include changes in immune recognition of pathogens, cytokine signalling, phagocytosis and also life span of monocytes, all of which directly affect the regulation and implementation of effective innate immune responses (Collini *et al*., 2010). *Ex vivo* studies have shown that peripheral monocytes are fairly resistant to productive infection

before differentiation into tissue macrophages. However, about 0.001-1% of monocytes are known to harbour HIV-1 during the course of infection (Le Douce *et al*., 2010; Mir *et al*., 2012).

Ancuta *et al*. (2008), suggested that monocytes may become susceptible to HIV-1 infection after being activated in the bone marrow or whilst circulating in the blood of HIV-1 infected individuals, as a result of the inflammatory environment and immune activation. HIV-1 infected monocytes are known to be recruited to the GIT where they later differentiate into macrophages and may serve as viral reservoirs (Elbim *et al*., 1999). However, it has been noted that blood monocytes are rarely infected with HIV-1, which may down-play their role as reservoir cells (Collini *et al*., 2010).

It has been shown that there is a direct association between the levels of CCR5 and the rate of monocyte differentiation into macrophages. The level of CCR5 expression is also related to monocyte resistance to infection. In addition, it has also been observed that the levels of CCR5 correlate with the ability of maturing monocytes to be in infected with HIV-1 (Sasse & Van Beckhoven, 2012). One of the interesting clinical consequences of immune activation is described by Munsaka *et al*. (2009), who showed LPS stimulation of monocytes in HIV-1 negative individuals resulted in an enhanced surface expression of CCR5 from approximately 21% to 99% of cells. This elevated expression may increase the risk of infection in non-infected monocytes.

2.8. Monocytes and Apoptosis

Monocytes are fairly resistant to apoptosis following HIV-1 infection and may thus be a participant in viral persistence in a similar way to latently infected resting memory $CD4^+$ T-cells, despite the low infectivity rate of monocytes. MDMs that are infected with HIV-1 have an increase in anti-apoptotic proteins, Bcl-2 and Bcl-xl and a decrease in pro-apoptotic proteins Bax and Bad through a mechanism that is TNF-dependent (Collini *et al*., 2010).

Bcl-2 expression is up-regulated by R5-tropic (CCR5 dependant HIV-1 viral entry) HIV-1 or exogenous *Tat* in MDMs. The gp120 activated M-CSF regulated pathway results in a down-regulation of tumour necrosis factor-alpha-related apoptosis inducing ligand (TRAIL) receptor TRAIL-R1 (DR4) and in monocytes from seropositive HIV-1 individuals it has been observed to display an increased resistance to Fas ligand- (FasL, CD95L) induced death. Irrespective of these

anti-apoptotic measures monocytes and macrophages may still undergo apoptosis (Collini *et al*., 2010).

Increased rates of macrophage apoptosis in mesenteric lymph nodes is linked with progression to AIDS. It has still not been determined however, if this is a consequence or the driver of monocyte turnover, and whether there is a direct contribution of HIV-1 to the observed apoptosis. In HIV-1 infected individuals it has been observed that there is an increase in spontaneous monocyte apoptosis when compared to HIV negative controls. As a result of the vital role that apoptosis plays in immunity, the regulation of monocyte life span is of particular relevance in both physiological and pathological processes in HIV disease (Busca *et al*., 2009).

Although infection of monocytes appears to confer apoptotic resistance, monocytes may be made more susceptible due to bystander effects. The pro-inflammatory milieu may up-regulate death receptor expression which allows for activated T-cells expressing death ligands to target monocytes/macrophages for elimination.

2.8.1. The extrinsic and intrinsic pathways of apoptosis

Generally there are two pathways that eventually lead to apoptosis of cells and they are dependent on the origin of the signal (**Figure 2-10**). The extrinsic pathway begins with the binding of ligands to molecules of the death receptor family; the intrinsic pathway however, is instigated by damage to the mitochondrial membrane that results in the release of cytochrome c into the cytosol and the eventual caspase activation. Bcl-2 is an anti-apoptotic protein that is part of three classes of anti-apoptotic proteins that are able to intervene at different stages of the caspase activation. They counteract death receptor-induced signalling and therefore prevent apoptosis. Bcl-2 maintains the integrity of the mitochondrial membrane and prevents the release of cytochrome c.

During the process of maturation monocytes increase their resistance to spontaneous and induced apoptosis. This is a beneficial mechanism during times of immune responses to pathogens. This enhanced survival is critical in pathological conditions such as infections with intracellular viral and bacterial pathogens, inflammatory conditions as well as monocytic malignancies. When their survival is increased during these conditions it then ceases to be beneficial but rather becomes a factor in disease pathogenesis. Apoptosis plays a very important role in host immunity against HIV-1 infection.

These various roles include:

1) Elimination or reduction of viability of intracellular pathogens,
2) Prevention of microbe dissemination,
3) Presenting microbial antigens in apoptotic bodies to other APCs,
4) Prevention of persistent infection and reservoir formation.

There is evidence, however, that shows intracellular pathogens may escape apoptosis of infected monocytes through the up-regulation of a number of host anti-apoptotic genes that are able to disrupt both the extrinsic and intrinsic apoptotic pathways of these cells (Busca *et al*., 2009).

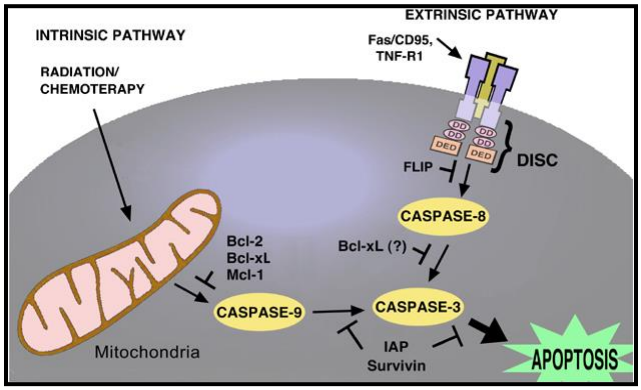

Figure 2- 10: Overview of the extrinsic and intrinsic pathway involved in apoptosis (Pai *et al*., 2006).

2.8.2. The role of anti-apoptotic and pro-apoptotic proteins in monocyte survival

Suppression of HIV-1 infection in monocytes plays a part in viral latency and persistence via numerous mechanisms. Monocytes, which remains undifferentiated *in vivo*, are resistant to HIV-1 infection post-entry, while MDMs and myeloid DCs are much more susceptible to productive HIV-1 infection. Resistance to post-entry infection in blood monocytes may be explained by differentiation-dependant cellular factors. The mechanisms used by HIV-1 infection, during monocyte differentiation into macrophages, however, still remains to be clarified. It has been suggested that HIV-1- infected monocytes are able to function as cellular reservoirs and that once they differentiate into macrophages they are able to encourage productive HIV-1 infection (Guillemard *et al*., 2004; Wu, 2011).

Exogenous recombinant HIV-1 *Tat* protein is known to enhance monocyte survival rates through the up-regulation of Bcl-2, an anti-apoptotic protein, which could be a way to establish viral persistence. With the use of an *in vitro* model of monocyte mediated death by TRAIL, it was seen that the *Tat* protein promotes the survival of monocytes in circumstances where they would usually undergo apoptosis and be removed. *Tat* is also known to increase the expression levels of IL-10 of monocytes *in vitro*. These interactions between HIV-1 virus and monocytes may augment the establishment of chronic HIV-1 infection and assist in disease progression (Coleman & Wu, 2009). Monocytes seem to be more susceptible to apoptosis following death receptor ligation compared to macrophages. These receptors include Fas (CD95), TRAIL-Receptor and TNFR1/2. The interaction between the death-receptors and their ligand result in the induction of death signalling. In mice with defective Fas/FasL genes an increase in monocytic cell counts was observed. During viral infections monocytes are known to be susceptible to FasL-induced death. In chronically infected SIV RMs there appears to be extensive turnover of peripheral monocytes that undergo apoptosis (Zheng *et al*., 2007; Laforge *et al*., 2011).

During the late stages of infection monocytes and macrophages have been shown to be a major source of virus in the lymphoid tissues of HIV-1 infected individuals. Studies have shown that infected monocytes/macrophages are able to induce apoptotic death in $CD4^+$ and $CD8^+$ T lymphocytes. It can therefore be said that viral persistence in monocytes/macrophages may play a major role in the dissemination and disease progression of HIV-1 infection. Resistance to virus-induced death is known to be a common feature of monocytes/macrophages in HIV-1 infection. A down-regulation of Bcl-2 was observed in blood monocytes of HIV-1 infected individuals during the asymptomatic phase, whilst there appeared to be a gradual increase in the expression of Bcl-2 during the late stages of infection. Levels of TNF-α are known to be highly elevated in HIV+ patients. It has been shown *in vitro* that HIV-1 infection induces the production of TNF-α which in turn induces Bcl-2 expression. The high activation status of monocytes that is observed in conditions such as stimulation with LPS is linked to a higher capacity for pro-inflammatory cytokine production, such as TNF-α. This high level of monocytic cellular activation in the inflammatory context of AIDS can contribute to establishment of a viral reservoir (Elbim *et al*., 1999; Guillemard *et al*., 2004; Le Douce *et al*., 2010).

2.9 VIP as a putative modulator of monocyte activation in HIV

Exploring novel therapeutic targets that are able to diminish monocyte/macrophage responsiveness would be a good step forward in trying to diminish immune activation in a selective manner. One proposed therapy would is of neuropeptide, VIP, which has been shown to exhibit immuno-modulatory effects. Upon release from sensory neurons and T-lymphocytes at sites of lymphoid tissue it binds to its receptors VPAC-1 and VPAC-2, which are present in immune cells such as lymphocytes and monocytes (Delgado *et al*., 2004). VPAC-1 has been shown to mediate productive HIV infection, whilst VPAC-2 could be involved with inhibition of viral replication. When bound to $CD4^+$ T-cells via VPAC-1/2 VIP acts to limit apoptosis and cell activation, VIP may also decrease responsiveness of monocytes/macrophages via VPAC-1/2, which have been shown to be expressed on these cells (Lara-Marquez *et al*., 2001). In this way through its receptors, VPAC-1/2, VIP may be used as a therapeutic approach to diminish the hyper-responsiveness of immune cells (including monocytes) during HIV infection and alleviate the burden placed upon the immune system due to persistent immune activation.

Persistent immune activation that is present during chronic HIV-1 infection provides an environment that aids in viral replication and facilitates disease progression. Cells belonging to myeloid lineage such as monocytes, macrophages and DCs are the primary targets of LPS-induced activation and are important regulators of both inflammatory and anti-inflammatory responses. Therefore it is important to understand the relationship between immune activation, monocyte function and HIV-1 disease (Cassol *et al*., 2010).

2.10 Research question, motivation, aim and objectives of the study

The prevalence of HIV-1 infections in Sub-Saharan Africa makes up the majority of global HIV infections (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013). Although several studies characterising the role of monocytes have been performed in the United States of America, Europe and some African countries that characterise the role of monocytes, very few have been performed in South Africa. Furthermore studies performed in Africa, mentioned earlier, have reported conflicting results regarding immune activation caused by LPS in HIV-1 infection. Therefore it's very important that studies be undertaken in South Africa so that the function of monocytes in HIV-1 pathogenesis can be clarified and their role be contextualised in

the South African setting. Unique features of this setting include higher levels of background infections, pre-existing pro-inflammatory immune status, infection with subtype C, and heterosexual transmission (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013).

The hypothesis of this study was that HIV-1 infection induces changes in the monocyte cell population that affects their phenotype and functional responsiveness to LPS stimulation. The current study set out to determine the following research questions:

1. Does HIV-infection affect the function of monocytes?
2. If monocytes are affected, how are they affected?
3. Lastly, could monocytes be used as a therapeutic intervention?

Therefore, the aim of this study was to characterise the phenotype and function of monocytes in stable, untreated chronic HIV-1 infection in South Africa. An additional aim was to determine how markers of migration, homing, activation and apoptosis relate to standard markers of disease progression (CD4 count and viral load) and to generalised immune activation (CD38/8 T-cell expression).

To achieve this, the following objectives were set:

- To assess changes in CD14+CD16- and CD14+CD16+ monocyte subsets
- To assess monocyte phenotype in HIV+ and HIV- individuals, with particular focus on
    - Activation markers (HLA-DR & CD69)
    - Migration-associated (CCR1, CCR2, CX3CR, CD62-L) and gut-associated (CCR7, CCR9) chemokine receptors
    - Expression of HIV co-receptor, CCR5
- To evaluate the apoptotic potential of monocytes during HIV-infection
- To evaluate responsiveness of monocytes to TLR4 stimulation with LPS, and assess changes to marker expression in monocyte subsets
- To relate novel marker expression patterns to general immune activation status

Chapter 3: Materials & Methods

3.1 Study design

This was a cross-sectional observational study with functional experimental components. The main objective of the study was to determine the phenotypic expression of chemokine homing receptors (CCR1, CCR2, CX3CR1, CCR9, and CCR7), other homing and activation markers plus the anti-apoptotic protein Bcl-2 in relation to immune activation. Whole blood assays were used to assess the phenotypic marker expression, cytokine expression as well as functional integrity of monocytes (following LPS stimulation) in chronic HIV-1 infection. Three (3) Flow cytometry panels (Panel 1, 2 and 3) were used for this purpose. Another objective was to determine the apoptotic sensitivity/potential of monocytes during chronic HIV-1 infection. Isolated monocytes and Flow cytometry Panel 4 were used for this purpose.

3.2 Study participants

Study participants were recruited from Emavudleni clinic in Crossroads and from local clinics in the Durbanville and Kayamandi areas in Cape Town. Ethical approval was granted by the Stellenbosch University Ethics Committee (N07/09/197). Study participants were recruited by a research nurse. All participants had a CD4 count above 300 cells/µl, either at the time of the blood draw or at a prior visit, of maximally three weeks before the blood draw. Donors had no history of TB and HIV+ donors were not on anti-retroviral therapy/treatment naïve (determined using information from patient folders). Participants were also above 18 years of age. All donors were briefed on the study and signed consent forms in their preferred language. Study participants were split into two groups: HIV negative (n=26) and HIV positive (n=40), see **Table 4-1** for summary of study participant demographics. HIV status was confirmed by standard serological testing. CD4 count and viral load

3.3 Venipuncture

Whole blood was collected by a research nurse using 10ml sodium-heparin Vacutainer tubes (BD Biosciences, San Jose, California). Approximately 20 ml was acquired from each donor. The research nurse transported the blood samples to the Division of Medical Virology within two

hours of collection. CD4 count and viral load tests (HIV+ only) were performed, for research purposes only, using blood collected in 5ml EDTA Vacutainer® tubes (BD Biosciences, San Jose, California).

3.4 CD4 Count

TruCOUNT tubes (BD Biosciences, San Jose, California) and the Becton Dickson (BD) MultiTEST^{TM} CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC Reagent (BD Biosciences, San Jose, California) were used to perform CD4 counts. A TruCOUNT tube for each patient was labelled with an identification number. 20µl of MultiTEST CD3-FITC/ CD8-PE/ CD45-PerCP/ CD4-APC reagent was added to the TruCOUNT tube just above the steel retainer. Following this, 50µl of EDTA blood was added to the TruCOUNT tube and mixed gently. The blood and reagent was incubated in the tube for 15 minutes at room temperature, in the dark. Then, 450µl of 1X Fluorescent Activated Cell Sorter (FACS) lysing solution (BD Biosciences, San Jose, California) was added to the tube and vortexed to mix. The tube was incubated for a further 15 minutes at room temperature in the dark, after which the cells were analysed on a Becton Dickinson FACSCalibur four-colour flow cytometer with MultiSET^{TM} software (BD Biosciences, San Jose, California). In addition to CD4 count; $CD8^+$ count, $\%CD4^+$ and $\%CD8^+$ as well as $CD4^+CD8^+$ ratios were also determined. This method is the standard CD4 count standard operation procedure (SOP) used in the Division of Medical Virology, which is accredited by South African National Accreditation System (SANAS).

3.5 Viral Load

The viral load test is a HIV-1 RNA quantitative assay based on nucleic acid sequence-based (NASBA) technology in combination with the detection of real-time molecular beacons. The test has a detection range from 350 to 10^6 copies/ml.

2ml of blood was removed from EDTA tubes and placed in appropriately labelled 5ml BD Falcon Polystyrene Round-Bottom tube (BD Biosciences, San Jose, California). The tube was then centrifuged at 375 X gravity (g) for 5 minutes at 20°C. Approximately 1ml of plasma was decanted into a 1.7ml micro-centrifuge tube (Axygen, Union City USA). Samples were sent for routine viral load testing in the Division of Medical Virology. The NucliSens EasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands) was used to perform the viral load

assay. This protocol is the viral load SOP used in NHLS laboratories located in the Division of Medical Virology and is also SANAS approved.

3.6 CD38 on $CD8^+$ T-cell expression

The expression of CD38 on $CD8^+$ T-cells is a measure of immune activation (Liu *et al*. 1997). A routine and standardized CD38 and PD-1 staining protocol was used for this purpose. 100µl of blood was removed and placed in appropriately labelled 5ml BD Falcon Polystyrene Round-Bottom tube (BD Biosciences, San Jose, California). An antibody cocktail containing 10µl CD3-Fluorescein isothiocyanate (FITC), 2.5µl CD38- Allophycocyanin (APC), 10µl CD8- Peridinin chlorophyll protein (PerCP) and 2.5µl PD-1- Phycoerythrin (PE) was added to the tube followed by a 30 minute incubation step. All antibody volumes were based on earlier titration procedures and currently in use in the standardized assay. Thereafter, 2ml of 10x BD Lysing™ solution (BD Biosciences, San Jose, California) was added to each tube and the tubes incubated for a further 10 minutes at room temperature. After the incubation period the tubes were centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was decanted using 3ml sterile Pastuer pipettes (Lasec, Ndabeni, Cape Town, South Africa), the cell pellet was resuspended and 2ml of BD CellWash™ (BD Biosciences, San Jose, California) was added to each tube. The tubes were centrifuged again at 375g for 5 minutes with the brake off at 18°C. The supernatant was decanted, the cell pellet was resuspended and 400 µL of 1x BD Fixative TM solution (BD Biosciences, San Jose, California) was added. The tubes were stored in a 4°C fridge until flow cytometry acquisition the following day. Flow cytometry was performed on a BD FACSCalibur 4-colour instrument with BD CellQuest analysis software (BD Biosciences, San Jose, California). Please see **Figure 3-1** for flow cytometry gating strategy for CD38 expression on $CD8^+$ T-cells. Lymphocytes were gated based on scatter (P1 = FSC vs. SSC), and CD3 FITC (P2). The expression of CD38 and PD-1 was determined in a CD8 PerCP vs. CD38 APC or PD-1 PE plot with quadrant statistics.

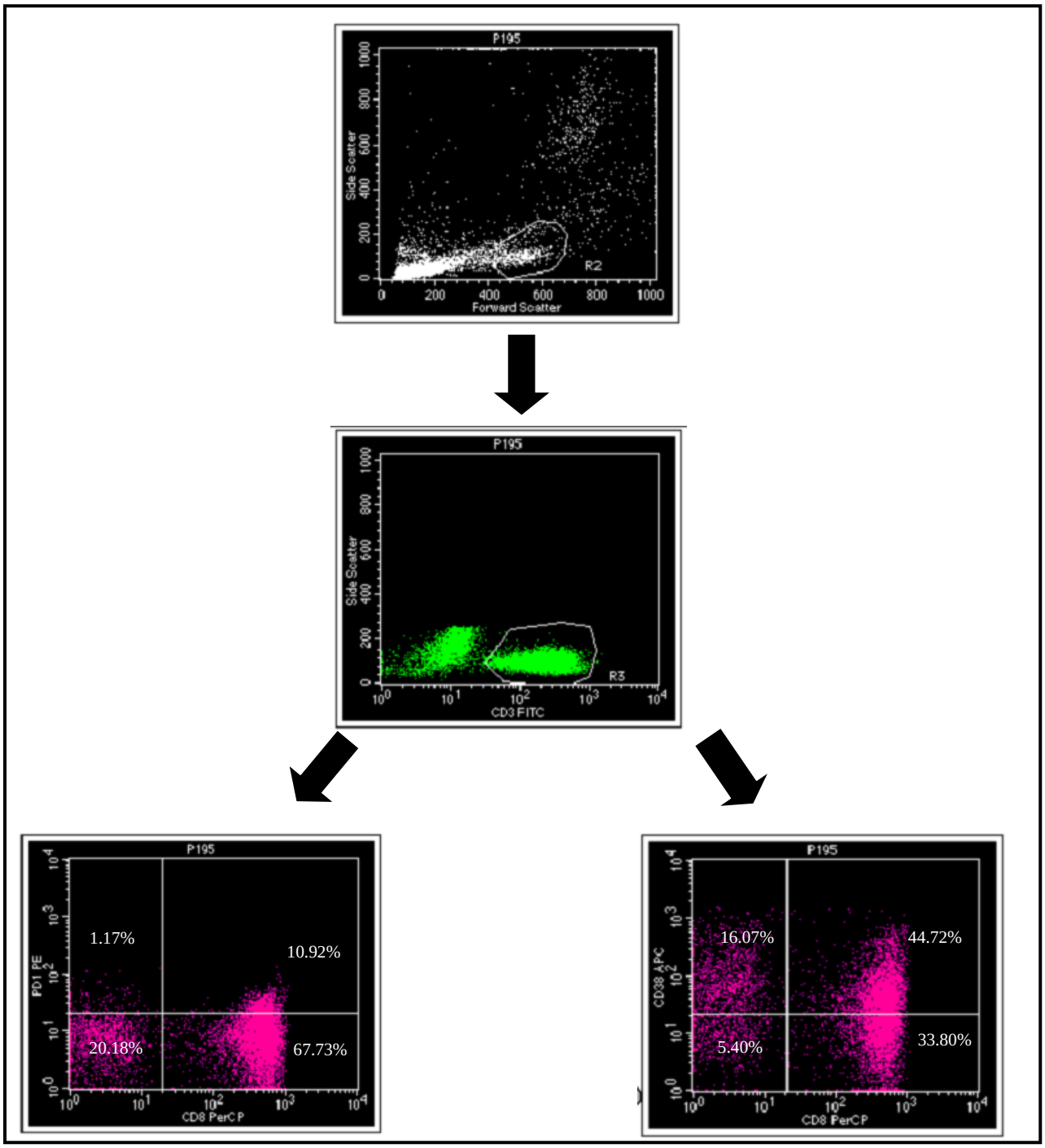

Figure 3- 1: Flow cytometry gating strategy used to acquire CD38 on $CD8^+$ T-cell expression, illustrated using a HIV+ sample (P195). Side Scatter Height (SSC-H) is plotted against Forward-Scatter (FSC-H), this is used to differentiate between lymphocytes (R2), monocytes and granulocytes. A dot plot showing SSC vs. CD3-FITC is used to define the $CD3^+$ T-cells (R3). Using R3, CD38-APC expression (right dot plot) and PD-1-PE expression (left dot plot) of $CD3^+CD8^+$ T-cells is determined.

3.7 Optimisation and quality control of multiparameter flow cytometry

Multiparameter/multicolour flow cytometry can generate a large amount of biological information from a single sample. It is often the only technique available to sufficiently identify or characterise complex cell populations. The field of flow cyomtery has rapidly evolved in recent years leading to increases in the number of parameters that can be analysed simultaneously. High performance instruments with additional laser and detector options and data computational power are now available. With the added complexity of parameter detection there is a need to ensure proper experiment setup for the generation of accurate and meaningful results (BD Biosciences, San Jose, California, 2009). Factors influencing proper experiment setup include: Compensation, Antibody Titrations and Fluorescence-Minus-One (FMO) procedures for the discrimination of positive vs. negative events. These are all discussed in the sections to follow.

3.7.1 Antibody Titrations

Antibody titrations are performed for mainly two reasons, to determine the optimal antibody volume that produces bright labelling of target cells and to save money by being able to use lower adjusted volumes per test. The optimal antibody volume used in each flow cytometry panel (see **Table 3-2**) was established by performing a titration of each antibody. A total of 21 antibodies were used in the study, the antibodies were divided into four different panels, namely (see **Table 3-1**):

- Chemokine (CCR5, CCR1, CCR7, CCR9, CCR2, CX3CR1)
- Activation and Apoptosis (CD69, TRAIL-R1, TNFR1, HLA-DR, CD62-L, CD95)
- Cytokines and Function 1 (CD116, Bcl-2, VPAC-2, IL-10, TNF-α)
- Apoptotic potential (Annexin V, 7AAD)

The highest volume of each antibody used during titration experiments was selected according to the manufacturer's instructions. However, it should be noted that choosing a higher volume than that recommended would have been ideal to observe a tapering off at the top of the curve. This

[1] In addition to fresh whole blood, isolated monocytes were also stained with antibodies from this panel as a quality control for the use of isolated monocytes in panel 4.

volume was then titrated down in 5 µl increments to 1.5 µl, e.g. the manufacturers recommended volume for CCR5-FITC is 20 µl; in the titration experiment the volumes tested were 20 µl, 15 µl, 10 µl, 5 µl, 2.5 µl and 1.5 µl. An unstained control was also used for each antibody. Blood from uninfected donors was used during the titration experiments. Tubes were processed and treated as a patient sample.

3.7.1.1 Cellular surface marker staining titrations

Titrations were carried out on both LPS-stimulated and unstimulated blood to determine if stimulation had an effect on the antibody volume needed and also to generate a positive expression profile for markers that are not expressed at any significant level. After completion of the titration experiments it was determined that stimulation did not required altered dilutions for optimal expression. The protocol presented below is for unstimulated blood. Refer to section **3.8** for LPS-stimulation protocol.

3.7.1.1.1 Whole blood antibody titrations - Panel 1, 2 & 3

Each tube was appropriately labelled with the antibody and the amount of antibody to be added during the staining protocol. 100µl of whole blood and 200µl tissue culture media (TCM) was added to each 5ml BD Falcon™ Polystyrene Round-Bottom tube in an antibody's titration series, with the exception of tubes which had intracellular antibodies added to them. Tubes which would have intracellular antibodies added, also had 0.8 µL GolgiPlug™ added (BD Biosciences, San Jose, California). The tubes were then incubated for 4 hours at 30°C at 5% CO2. Post-incubation, 120µl of a 2% EDTA (Sigma Aldrich, St Louis, Missouri, USA) solution was added to the tubes. After 10 minutes incubation at room temperature, the cells were stained with the appropriate volume of antibody and incubated in the dark for 30 minutes at room temperature. Thereafter, 2ml of 10x BD Lysing™ solution (BD Biosciences, San Jose, California) was added to each tube and the tubes incubated for a further 10 minutes at room temperature. After the incubation period, the tubes were centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was decanted using 3ml sterile Pasteur pipettes (Lasec, Ndabeni, Cape Town, South Africa), the cell pellet resuspended and 2ml of BD CellWash™ (BD Biosciences, San Jose, California) was added to each tube. The tubes were centrifuged again at 375g for 5 minutes with the brake off at 18°C. The supernatant was decanted, the pellet resuspended and 400 µL of 1x BD Fixative TM solution

(BD Biosciences, San Jose, California) was added. The tubes were stored in a 4°C fridge until flow cytometry acquisition the following day.

3.7.1.1.2 Isolated monocytes titrations - Antibody panels 3 and 4

Monocytes were isolated from whole blood in a two phase process, namely monocyte enrichment using the RosetteSep® Negative Selection kit with Human Monocyte Enrichment Cocktail (Catalogue #15028, STEMCELLTM Technologies, Vancouver, Canada) and EasySep® Human CD14 Selection Kit (Catalogue #18058, STEMCELLTM Technologies, Vancouver, Canada) for positive selection. All isolation experiments were performed in a tissue culture biosafety flow cabinet. For the RosetteSep® and EasySep® protocol detailed in the FMO section, see **Figure 3-3**. and **Figure 3-5**, respectively.

Antibody titrations were not performed for antibodies AnnexinV-FITC and 7AAD-PerCP from panel 4 as the recommended volume was 5 µl, which was applied directly in the current study. Antibody titrations were also not performed for the panel 3 antibodies used in the staining of isolated monocytes as the titrated values obtained during the whole blood antibody titrations were applied for staining isolated monocytes.

3.7.1.2 Intracellular marker antibody staining titrations –Panel 3 only

3.7.1.2.1 Whole blood

The staining protocol for cellular surface staining was followed for tubes from antibody panel 3, which is the only antibody panel that included intracellular antibodies (Bcl-2, IL-10 and TNF-α). The standard protocol was adhered to until the second centrifugation and supernatant decanting step. In the place of BD Fixative™ (BD Biosciences, San Jose, California) being added to the tubes, 100µL of BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, California) was added to the tubes and placed in a 4°C fridge to incubate for 20 minutes. After 20 minutes 300µl of 1x PermWash™ (BD Biosciences, San Jose, California) was added, the tubes were mixed gently and then centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was removed, the cell pellet resuspended and the intracellular antibody mix was added. The tubes were then incubated at 4°C for 30 minutes in the dark. After 30 minutes, 300µl of PermWash™ (BD Biosciences, San Jose, California) was added and the tubes centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was discarded, the pellet resuspended and 400µl of

BD Fixative™ (BD Biosciences, San Jose, California) was added and the tubes stored in a 4°C fridge until flow acquisition the next day.

3.7.1.2.2 Purified monocyte titrations

The staining protocol for cellular surface marker detection previously detailed was adhered to for isolated monocytes. The protocol was adhered to until the second centrifugation and supernatant decanting step. In the place of BD Fixative™ (BD Biosciences, San Jose, California) being added to the tubes, 100µL of BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, California) was added to the tubes and placed in a 4°C fridge to incubate for 20 minutes. After 20 minutes 300µl of 1x PermWash™ (BD Biosciences, San Jose, California) was added, the tubes were mixed gently and then centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was removed, the cell pellet resuspended and the intracellular antibody mix was added. The tubes were then incubated at 4°C for 30 minutes in the dark. After 30 minutes, 300µl of PermWash™ (BD Biosciences, San Jose, California) was added and the tubes centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was discarded, the cells resuspended and 400µl of BD Fixative™ (BD Biosciences, San Jose, California) was added and the tubes stored in a 4°C fridge until flow acquisition the next day.

3.7.1.3 Flow cytometry acquisition

Titration samples were acquired and analysed on a BD FACS Canto II flow cytometer with FACS Diva software (BD Biosciences, San Jose, California). Additional post-acquisition analysis was performed using the same FACS Diva software (BD Biosciences, San Jose, California). Five thousand (5000) gated events were collected per a tube, the cells were gated according to monocyte population (population 2, P2) was defined based on P1 and using Side Scatter (SSC) versus CD14-V500. P1 was based on single cell (singlet) population (population 1, P1) using Forward Scatter-Height (FSC-H) versus Forward Scatter-Area (FSC-A). Note: CD14 titration was performed with only singlet and FSC vs. SSC gating. To ensure all monocytes had been gated, a dot plot was created using SSC versus FSC-H for back-gate display. This population was designated population 3 (P3). Histograms for each antibody titration value were then created using P3. The negative population marker was set using the unstained control tube. The negative marker indicator was then copy- pasted onto every histogram. The titration tubes were then acquired using the flow cytometer and the fluorescent peaks of each antibody visualised on the

histograms. The histograms displayed P2 and P3 events. Peaks that were seen to the right of the negative marker were deemed as positive. Population statistics of the histograms were generated and these values exported into an excel spread sheet for each antibody.

Using these values a standard curve was created. In order to do this the staining index value for each titration value was determined by using the following formula:

$$\left(\frac{(MFI_{pos} - MFI_{neg})}{SD_{neg}} \right)$$

Equation 1: Formula used to calculate the staining index, which is used to generate a titration curve. MFI = mean fluorescence intensity, SD = standard deviation.

Using the standard curve see **Figure 3-2**, the antibody volume that produced a positive fluorescent peak closest to the fluorescent peak created by the manufacturer suggested volume was determined to be the optimal volume to use in the assay.

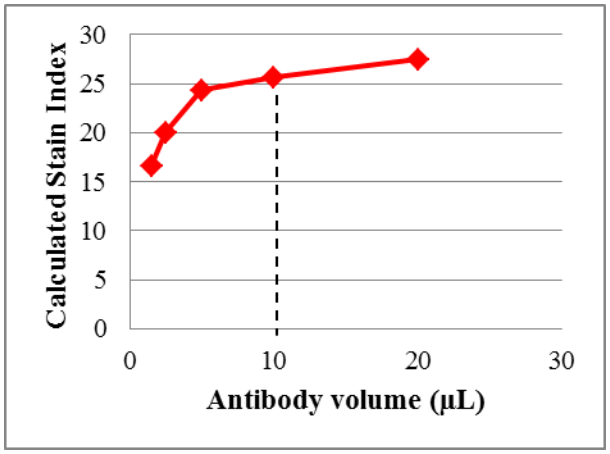

Figure 3- 2: Titration curve for CD116-FITC. Calculated stain index is plotted against the correlating antibody. The curve is then used to determine the lowest antibody volume required (in this case 10µl) to produce a positive signal similar to the signal produced by the manufacturer recommended volume (20µl).

3.7.2 Fluorescence-Minus-One (FMOs)

FMOs were performed in order to accurately determine positive vs. negative event discrimination for antibody markers that are novel or where there is a level of difficulty in determining whether a true positive signal is seen or t accuracy is questionable due to the strength of the fluorescent signal. In the cases of certain antibody markers a positive signal is seen at a low level and if

FMOs are not performed this low level of positivity can be overlooked or not seen at all. FMOs are also performed when a new batch of antibody is introduced in a panel. FMOs were performed as discussed below.

3.7.2.1 Whole blood- Antibody panels 1, 2 and 3

5ml BD Falcon™ Polystyrene Round-Bottom tubes (BD Biosciences, San Jose, California) were labelled with the name of each antibody. Control (uninfected donor) blood was during the FMO experiments. Following on from the antibody titrations, the optimal volume of each antibody was added to each tube except the antibody that the tube was labelled with. For example, for a tube labelled -CD95-APC, which is part of the activation and apoptosis panel, every antibody in that panel except CD95-APC was added to the tube. After the correct antibodies were added to the appropriate tubes, 200µl of fresh whole blood was added and the tubes were gently mixed and incubated for 30 minutes in the dark at room temperature. The staining protocol used during the antibody titration experiment, detailed earlier, was followed for cellular surface and intracellular markers, up until the step before the addition of cell fixative. At this step half of the supernatant was removed and 350µl of BD CellWash (BD Biosciences, San Jose, California) was added instead of cell fixative. A negative, unstained control was also included. The tubes were mixed well and then data were acquired on the BD FACS Canto II.

During flow cytometry acquisition, FSC-H versus FSC-A was used to determine single cells (singlets) (population 1, P1). Following this, SSC versus CD14-V500 was plotted to determine the monocyte population (population 2, P2). The control tube was acquired first followed by the tubes containing the antibody mixes. Histograms were then generated for each antibody including the unstained tube. A marker was set over the negative signal. This marker was copy and pasted onto the histograms of all the antibodies. As the tubes were acquired the negative control marker on each histogram was adjusted according to the strength of the negative signal. The final negative marker was used to determine a true positive fluorescent signal seen during implementation of the assay on patient's samples.

LPS stimulation was also performed to determine whether stimulation would have an effect on positive signal detection. The full staining protocol and flow cytometry acquisition was also followed. No difference was seen between LPS stimulation FMOs and unstimulated FMOs.

3.7.2.2 Isolated Monocytes – Antibody panels 3 & 4

FMOs were not performed on isolated monocytes stained with antibodies from panel 3 as the FMOs generated from whole blood experiments were used throughout. FMOs were also not performed for the antibodies CD14-V500 and CD16-APC-H7 in all panels for as the FMOs generated from the whole blood FMO experiments were used. FMOs were performed on Annexin V-FITC and 7AAD-PerCP. The section below details the monocyte isolation protocol followed for all the isolated monocyte work.

3.7.2.2.1 Monocyte isolation

3.7.2.2.1.1 *RosetteSep® Negative Selection (enrichment)*

Approximately 10ml of whole blood that had been stored overnight in a 4°C fridge was decanted into a labelled 15ml BD Falcon ® centrifugation tube (BD Biosciences, San Jose, California); RosetteSep® Human Monocyte Enrichment Cocktail was added to the tubes at 50µl/ml of whole blood. The tubes were mixed well and incubated for 20 minutes at room temperature. RosetteSep® media was made up as per manufacturer's instructions i.e. , Phosphate Buffered Saline (PBS) (InvitroGen, San Diego, USA), 2% Fetal Bovine Serum (FBS) (InvitroGen, San Diego, USA) and 1mM EDTA (Sigma Aldrich, St Louis, Missouri, USA). After the incubation period the sample was diluted with an equal volume of RosetteSep® Media, approximately 10ml. The tube contents were then thoroughly mixed. The mixture was then layered onto 15ml of Ficoll™-Histopaque (BD Biosciences, San Jose, California) in a 50ml BD Falcon™ tube (BD Biosciences, San Jose, California). The tubes were centrifuged for 20 minutes at 1200g at room temperature with the brake off. The enriched cells were removed from the Ficoll™ density medium: plasma interface along with a small quantity of the Ficoll™ to ensure all the enriched cells were removed. The cells were then placed into a 50ml BD Falcon™ (BD Biosciences, San Jose, California) tube and RosetteSep® media was added to the 35ml mark. The contents were gently mixed and the tubes were centrifuged again at 1200g at room temperature with the brake off for 10 minutes. After centrifugation the supernatant was discarded, the cell pellet resuspended and another 5ml RosetteSep® media was added. Before the tubes were centrifuged again, 50µl of the cell suspension was removed and added to 50µl of Turks staining solution (Sigma Aldrich, St Louis, Missouri, USA) in a 96-well plate with the lid labelled appropriately for each cell culture well being used (Corning Incorporated, Corning, NY). The tubes were then centrifuged again at

1200g at room temperature with the brake off for 10 minutes to wash them. During the centrifugation step, 10µl of the cell/Turk solution suspension was removed from the 96-well plate for cell count use. A Neubauer Bürker haemocytometric counting chamber was used to perform the cell count (Celeromics, Valencia, Spain), the cell count for each cell culture well was recorded. The average (n = 21) monocyte cell count at the end of the RosetteSep® enrichment step was $2.02x10^6$ cells/ml out of the original input of 10ml. After the centrifugation step the supernatant was discarded and the pellet re-suspended in the residual volume approximately 150µl. This volume was then transferred to a 5ml BD Falcon™ Polystyrene Round-Bottom tube (BD Biosciences, San Jose, California) for use during the EasySep® procedure.

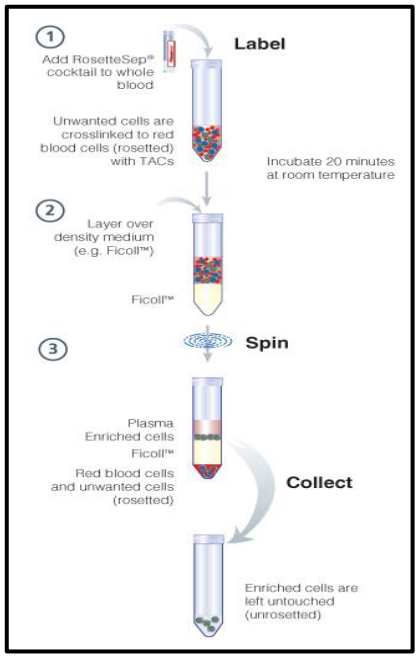

Figure 3- 3: RosetteSep® protocol diagram (www.stemcell.com)

3.7.2.2.1.2 *EasySep® Human CD14 Selection Kit (Positive selection)*

EasySep® Positive Selection Cocktail was added at 100µl/ml; e.g. for 2ml of cells, 200µl of cocktail was added. The contents were mixed well and incubated at room temperature for 15 minutes. During this period the EasySep® Magentic Nanoparticles were thoroughly mixed to ensure uniform suspension. After the incubation period, the particles were added at 50µl/ml cells. The contents were mixed well and incubated at room temperature for 10 minutes. During this time the monocytes were labelled by the magnetically CD14-linked nanoparticles, see **Figure 3-**

4. The total volume was then brought to 2.5ml using RosetteSep® media and the cells mixed by gently pipetting up and down 2-3 times. The tube was then placed into the Purple EasySep® magnet (Catalogue # 18000) and set aside for five minutes. After the five minutes, the supernatant fraction of the tube was discarded by inverting the magnet and the tube. The magnetically labelled monocytes remained in the tube. The tube was then removed from the magnet and the volume brought up to 2.5ml using RosetteSep® media and mixed by gently pipetting. The magnetic separation and supernatant decanting repeated. The volume was brought up to 2.5ml again and the magnetic separation and supernatant decanting repeated a third time. This resulted in a total of 3 x 5 minute magnetic separations. After the third supernatant decanting, the volume was brought up to 1ml with TCM and 50µl was removed and added to 50µl Turks solution (Sigma Aldrich, St Louis, Missouri, USA) in an appropriately labelled 96-well culture plate. The 1ml cell suspension was set aside and another cell count was performed using the Turks/cell suspension mixture as previously detailed. A schematic of how the positive selection kit works is found in **Figure 3-5** and illustration of protocol followed in **Figure 3-6**.

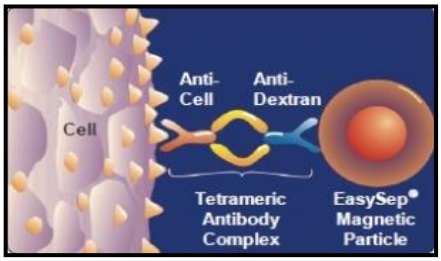

Figure 3- 4: Schematic of EasySep® Tetrameric Antibody Complexes (TAC) labelling of human cells (www.stemcell.com)

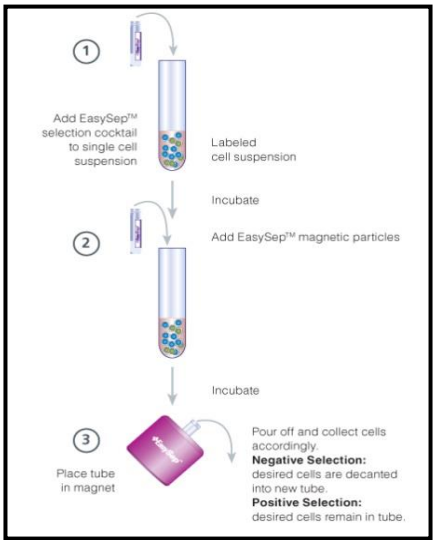

Figure 3- 5: EasySep® cell selection protocol diagram (www.stemcell.com)

Once the final cell count was performed, the isolated monocytes were placed into 5ml BD FalconTM Polystyrene Round-Bottom tubes (BD Biosciences, San Jose, California) at an average (n=21) concentration of $4.62x10^5$ cells/ml. 250µl of this cell suspension was added to each four tubes, two for panel 3 and two for panel 4, during *ex vivo* LPS stimulation at an average (n=20) concentration of $1,53x10^5$cells/ml [range $2,00x10^4$-$5,50x10^5$].

Apoptosis had to be induced before FMOs for panel 4 could be generated. This was done by treatment with Camptothecin (Sigma Aldrich, St Louis, Missouri, USA). Isolated monocytes were stimulated with 2.5µl/ml Camptothecin (Sigma Aldrich, St Louis, Missouri, USA) in appropriately labelled 5ml BD Falcon™ Polystyrene Round-Bottom tubes (BD Biosciences, San Jose, California) for 4 hours at 37°C, 5% CO2. The cells were then stained as described below. The cells were washed twice with cold staining buffer (PBS + 2% FBS) at 200g at room temperature for 5 minutes with the brake off. The cells were then re-suspended in Annexin V Binding Buffer. Antibodies were added to the tubes, volumes can be found in **Table 3-2**. The cells were gently vortexed and allowed to incubate at room temperature for 15 minutes. Thereafter, 400µl of Annexin V Binding Buffer was added and flow cytometry performed.

3.7.3 Flow cytometry colour compensation

Compensation experiments are performed to decrease spill-over of fluorescent signal from one detector channel to another. Emission spectra of fluorochromes may overlap and compensation ensures this overlap does not lead to spurious data generation. The purpose of compensation is to reduce this spill-over of a fluorochrome into a detector that is reserved to identify another fluorochrome that is in the same antibody panel. Compensation also aligns populations that are affected by spill over. This alignment results in increased accuracy of interpretation and analysis of data (Technical Bulletin, BD Biosciences, San Jose, California). Compensation experiments were not performed for all 21 antibodies but for every flourochrome, namely FITC, PE etc. The exception being tandem dyes (e.g. Pe-Cy7) which needed to have individual compensation set for each antibody tandem dye combination, irrespective of whether in different panels. The reason for this is that tandem dyes spectral spill-over varies amongst tandem lots (Johanssen & Macey, 2013). The compensation experiments were performed as described below. Compensation experiments were performed based on compensation beads and not cells.

5ml BD Falcon™ Polystyrene Round-Bottom tubes (BD Biosciences, San Jose, California) were labelled for each antibody. 100µl of staining buffer was added to each tube. BD CompBead Plus (BD Biosciences, San Jose, California) bottles were vortexed thoroughly before use. One drop of BD CompBead Plus Negative Control (BD Biosciences, San Jose, California) and 1 drop of BD CompBead Plus Anti-Mouse IgG1, κ beads was then added to each tube. BD CompBead Plus Anti-Rat IgG2a, κ beads were used where appropriate. Following this, the optimum volume of antibody was added to the appropriately labelled tube. The contents of the tubes were gently mixed, then incubated for 30 minutes in the dark at room temperature. After incubation, 2ml of staining buffer was added to each of the tubes, which were then centrifuged for 10 minutes at 200g. The supernatant was discarded and the pellet re-suspended in 500µl of staining buffer and the tube vortexed. The tubes were run on the BD FACS CANTO II flow cytometer (BD Biosciences, San Jose, California) with BD FACS Diva software (BD Biosciences, San Jose, California). The compensation values were calculated and set by the flow cytometer. Compensation settings were checked when experimental samples were acquired to ensure that bead-based compensation were suitably adjusted. This compensation procedure was repeated before every batch of study samples were acquired on the flow cytometer.

3.8 *In vitro* LPS-stimulation[2]

3.8.1 Whole blood

24-well flat bottomed Cell Culture Cluster plates (Corning Incorporated, Corning, NY) were used during the stimulation assay. Whole blood from donors either remained un-stimulated or was stimulated with 1µg/ml LPS (Clone O111:B4: Invivogen, San Diego, California, USA). Two wells were used per donor, appropriately labelled with donor identification number and whether the cells in the well were to be stimulated or remain un-stimulated. The total volume in each well was 1.2ml. This was obtained by adding 600µl of whole blood to each well. In the well labelled "stimulated" 120 µg/ml LPS was added to 480µl tissue culture media (TCM), that consisted of RPMI 1640 + GlutaMAX™ (InvitroGen, San Diego, USA) and 5% FBS (InvitroGen, San Diego, USA) as well as 0.8µl GolgiPlug™ (BD Biosciences, San Jose, California). In the well labelled

[2] In addition to fresh whole blood, isolated monocytes were also stimulated with LPS and stained with antibodies from panel 3 as a quality control for the use of isolated monocytes in panel 4.

"unstimulated", 600µl TCM and 0.8µl GolgiPlug™ (BD Biosciences, San Jose, California) was added to whole blood. The plate was then incubated at 37°C, 5% CO2 for four hours. After stimulation 120µl of EDTA (Sigma Aldrich, St Louis, Missouri, USA) was added to the wells and the contents gently mixed by pipetting up and down slowly. The cells were set aside for 10 minutes after which they were stained with monoclonal antibodies, see section **3.9.** for antibody staining protocol and **Figure 3-6** for workflow.

3.8.2 Isolated monocytes – Panel 3 & 4

Purified monocytes were added to 5ml BD FalconTM Polystyrene Round-Bottom tubes (BD Biosciences, San Jose, California), at an average concentration (n=20) of $1.53x10^5$cells/ml [2,00x104-5,50x105]. Two tubes per panel were labelled "stimulated" and "unstimulated". The protocol detailed above for whole blood was followed to induce LPS stimulation in isolated monocytes but adjustments to volumes were made. 50µl of 1µg/ml LPS (Clone O111:B4: Invivogen, San Diego, California, USA), 0.8µl of GolgiPlug™ (BD Biosciences, San Jose, California) were added to the tubes labelled "stimulated" and only 0.8µl of GolgiPlug™ (BD Biosciences, San Jose, California) were added to the tubes labelled "un-stimulated". The tubes were then placed in an incubator as detailed above. After incubation, 50µl of EDTA (Sigma Aldrich, St Louis, Missouri, USA) was added to all four tubes. The tubes were then set aside for 10 minutes and thereafter they were stained with antibodies, see section **3.9.** for antibody preparation protocol and **Figure 3-6** for workflow.

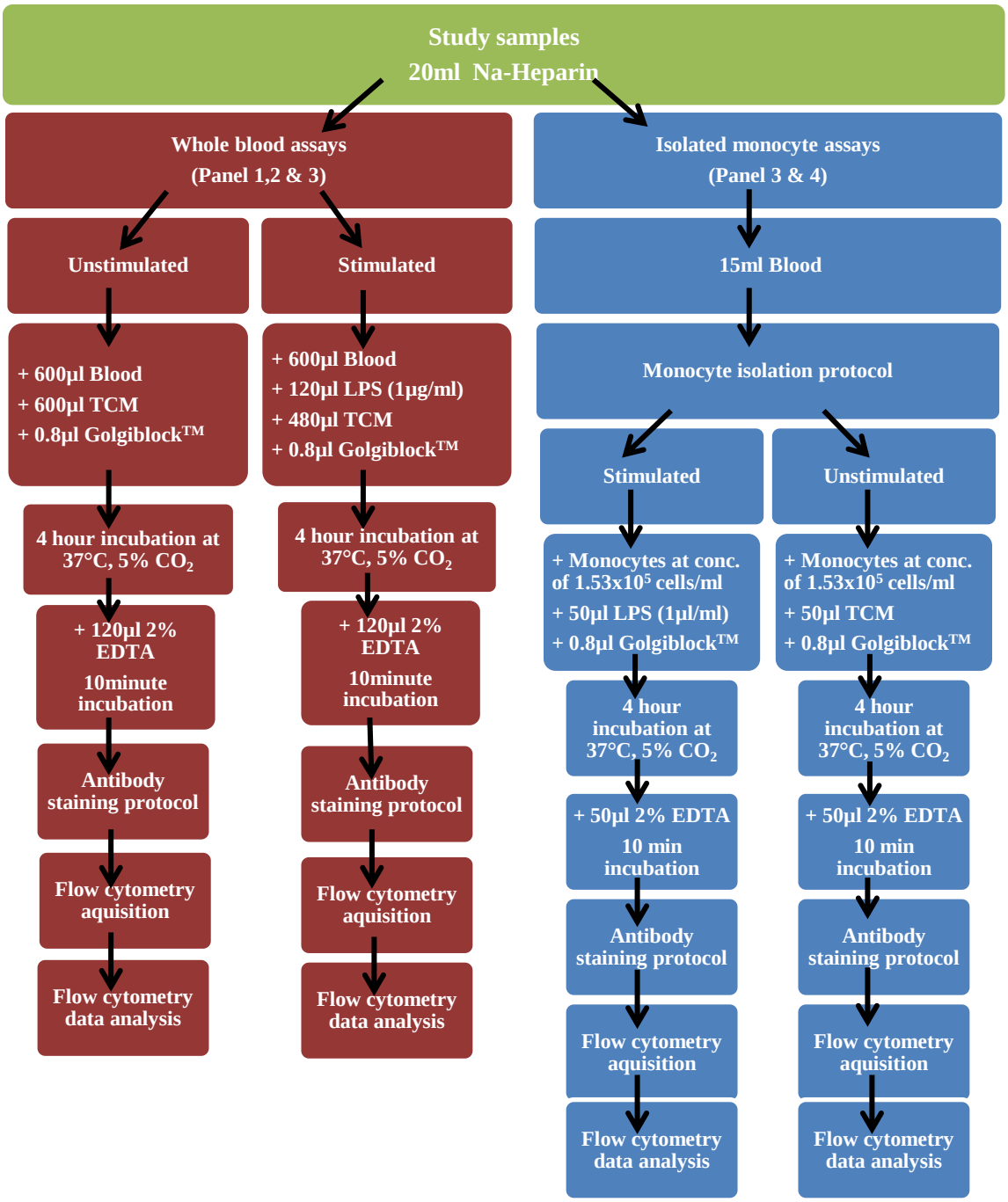

Figure 3- 6: Illustration of workflow followed. Protocol for whole blood assays found in red and isolated monocytes in blue. Protocols for monocyte isolation, antibody staining and flow cytometry acquisition can be found in sections 3.7.2.2, 3.9.1 and 3.10, respectively.

3.9 Preparation of Antibody Staining Mix

Monoclonal antibodies were taken out of storage at 4°C and placed in tissue culture laminar flow hood prior to usage in panel assembly.

Table 3- 1: The composition of the 4 flow cytometry panels used during this study as well as the placement of the 19 antibodies into the relevant panels.

Panel name	FITC	PE	PerCP	Pe-Cy7	APC	APC-H7	BV421	V500
(1)Phenotypic	CCR5	CCR1	CCR9	CX3CR1	CCR2	CD16	CCR7	CD14
(2)Activation & Apoptosis	CD69	TRAIL-R1	HLA-DR	CD62L	TNFR1	CD16	CD95	CD14
(3)Functional & Response	CD116	Bcl-2	VPAC-2		IL-10	CD16	TNF-α	CD14
(4)Apoptotic Potential	AnnexinV		7AAD			CD16		CD14

PerCP = Peridinin chlorophyll protein; APC = Allophycocyanin; PE = Phycoerythrin; FITC = Fluorescein isothiocyanate; APC-H7 = Allophycocyanin-Hilite®7; PE-Cy7 = Phycoerythrin-Cyanine7; TNF-α = Tumour necrosis factor-alpha; TNF-R1 = Tumour necrosis factor-receptor 1; TRAIL-R1 = TNF-related apoptosis-inducing ligand-receptor 1; 7AAD = 7-Aminoactinomycin; VPAC-2 = Vasoactive intestinal peptide receptor 2.

Antibody panels consisted of both cellular surface and intracellular markers. The staining procedure outlined below was the protocol followed during the staining of cells after *in vitro* stimulation. The details of all the antibodies, the titrated volumes and manufacturer details are presented in **Table 3-2**.

3.9.1 Surface marker antibody staining

3.9.1.1 Whole blood - Panel 1, 2 & 3

5ml BD Falcon™ Polystyrene Round-Bottom tubes (BD Biosciences, San Jose, California) were appropriately labelled with the antibody panel number and whether cells were stimulated or unstimulated. 100µl of cell incubation mixture and the correct volume of appropriate antibodies were added to each tube. The tubes were incubated in the dark for 30 minutes at room temperature. Thereafter, 2ml of 10x BD Lysing™ solution (BD Biosciences, San Jose,

California) was added to each tube and the tubes incubated for a further 10 minutes at room temperature. After the incubation period the tubes were centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was decanted using 3ml sterile Pasteur pipettes (Lasec, Ndabeni, Cape Town, South Africa), the cell pellet was resuspended and 2ml of BD CellWashTM (BD Biosciences, San Jose, California) was added to each tube. The tubes were centrifuged again at 375g for 5 minutes with the brake off at 18°C. The supernatant was decanted, the pellet resuspended and 400 µL of 1x BD Fixative™ solution (BD Biosciences, San Jose, California) was added. The tubes were stored in a 4°C fridge until flow cytometry acquisition the following day.

3.9.1.2 Isolated monocytes - Panel 4

After the last incubation step (see section **3.8.1** and **3.8.2**), cells were washed twice with cold BioLegend Cell Staining Buffer (BioLegend Inc., San Diego, CA), and then re-suspended in Annexin V Binding Buffer. 5µl of AnnexinV-FITC and 5µl of 7AAD-PerCP was added to the tubes. The tubes were gently mixed and incubated for 15 min at room temperature in the dark. Afterwards, 400 µL of 1x BD Fixative TM solution (BD Biosciences, San Jose, California) was added. The tubes were stored in a 4°C fridge until flow cytometry acquisition the following day.

3.9.2 Intracellular marker antibody staining – Panel 3

3.9.2.1 Whole blood

The staining protocol for cellular surface staining was followed for tubes from antibody panel 3, which is the only antibody panel that included intracellular staining (for TNF-α, IL-10 and Bcl-2). The standard protocol was adhered to until the second centrifugation and supernatant decanting step. In the place of BD Fixative™ (BD Biosciences, San Jose, California) being added, 100µL of BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, California) was added to the tubes and placed in a 4°C fridge to incubate for 20 minutes. After 20 minutes, 300µl of 1x PermWash™ (BD Biosciences, San Jose, California) was added, the tubes were mixed gently and then centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was removed, cell pellet resuspended and the intracellular antibody mix was added. The tubes were then incubated at 4°C for 30 minutes in the dark. After 30 minutes, 300µl of PermWash™ (BD

Biosciences, San Jose, California) was added and the tubes centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was discarded and 400µl of BD Fixative™ (BD Biosciences, San Jose, California) was added and the tubes stored in a 4°C fridge until flow acquisition the next day.

3.9.2.2 Isolated monocytes

The staining protocol for cellular surface staining (**3.10.1.2**) was followed until the second centrifugation and supernatant decanting step. In the place of BD FixativeTM (BD Biosciences, San Jose, California) being added to the tubes, 100µL of BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, California) was added to the tubes and placed in a 4°C fridge to incubate for 20 minutes. After 20 minutes 300µl of 1x PermWash™ (BD Biosciences, San Jose, California) was added, the tubes were mixed gently and then centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was removed, the pellet resuspended and the intracellular antibody mix was added. The tubes were then incubated at 4°C for 30 minutes in the dark. After 30 minutes, 300µl of PermWash™ (BD Biosciences, San Jose, California) was added and the tubes centrifuged at 375g for 5 minutes with the brake off at 18°C. The supernatant was discarded, the pellet resuspended and 400µl of BD Fixative™ (BD Biosciences, San Jose, California) was added and the tubes stored in a 4°C fridge until flow acquisition the next day.

Table 3- 2: Titrated antibodies volumes, manufacturer information and Isotype used (refer to Table 3-1 for panel design)

Antibodies	Manufacturer	Volume (µl)	Isotype (Clone)
CCR5-FITC	BioLegend Inc. , San Diego, CA	2.5	Rat IgG2a, κ (HEK/1/85a)
CCR1-PE	R & D Systems, Minneapolis, MN	5	Ms IgG2b (53504)
CCR9-PerCP	R & D Systems, Minneapolis, MN	5	Ms IgG2b (112509)
CX3CR1- Pe-Cy7	BioLegend Inc. , San Diego, CA	2.5	Rat IgG2b, κ (2A9-1)
CCR2- APC	R & D Systems, Minneapolis, MN	5	Ms IgG2b (48607)
CD16- APC-H7	BD Biosciences, San Jose, CA	2.5	Ms IgG1, κ (3G8)

CCR7 BV421	BioLegend Inc. , San Diego, CA	2.5	Ms IgG2a, κ (G043H7)
CD69-FITC	BD Biosciences, San Jose, CA	10	Ms IgG1, κ (FN50)
TRAIL- R1- PE	BioLegend Inc. , San Diego, CA	10	Ms IgG1, κ (DJR1)
HLA-DR- PerCP	BioLegend Inc. , San Diego, CA	2.5	Ms IgG2a, κ (L243)
CD62L- PeCy7	R & D Systems, Minneapolis, MN	2.5	Ms IgG1, κ (DREG-56)
TNFR1- APC	R & D Systems, Minneapolis, MN	5	Ms IgG1 (16803)
CD95- BV421	BioLegend Inc. , San Diego, CA	5	Ms IgG1, κ (DX2)
CD14- V500	BD Biosciences, San Jose, CA	2.5	Ms IgG2a, κ (M5E2)
CD116-FITC	BioLegend Inc. , San Diego, CA	10	Ms IgG1, κ (4H2)
Bcl-2 PE	BD Biosciences, San Jose, CA	10	Ms IgG1, κ (Bcl-2/100)
VPAC-2- PerCP	Constituted in Medical Virology	5	IgG (SP235)
IL-10- APC	BioLegend Inc. , San Diego, CA	10	Rat IgG2s, κ (JES3-19F1)
TNF-α- BV421	BioLegend Inc. , San Diego, CA	5	Ms IgG1, κ (MAb11)
AnnexinV-FITC	BioLegend Inc. , San Diego, CA	5	N/A
7AAD-PerCP	BioLegend Inc. , San Diego, CA	5	N/A

3.10 Study sample flow acquisition and gating strategy

Study samples were acquired and analysed on a BD FACS Canto II flow cytometer with FACS Diva software (BD Biosciences, San Jose, California). Additional post-acquisition analysis was performed using the same FACS Diva software (BD Biosciences, San Jose, California). The acquisition limit was set at 10 000 events per a tube but the average cell events during the study recorded was approximately 4500 events. The gating strategy for study sample acquisition was similar to the one followed during antibody titration experiments. Acquired cellular events were gated according to FSC-H versus FSC-A to identify single cell (singlet) populations, this was set as population 1 (P1). The monocyte population (population 2, P2) was defined based on P1 and

using SSC versus CD14–V500. To assess the position of the monocyte population within the total cells a back-gate plot of SSC versus FSC-H was generated displaying population 3 (P3). A CD14-V500 versus CD16-APC-H7 dot plot was then created using P2 to visualise both the CD14-positive (+) and CD 16+ monocyte population. An example of the gating strategy used is presented in **Figure 3-7**. All other marker expression was based on this scheme, and individual marker display patterns are included in the **Chapter 4** below.

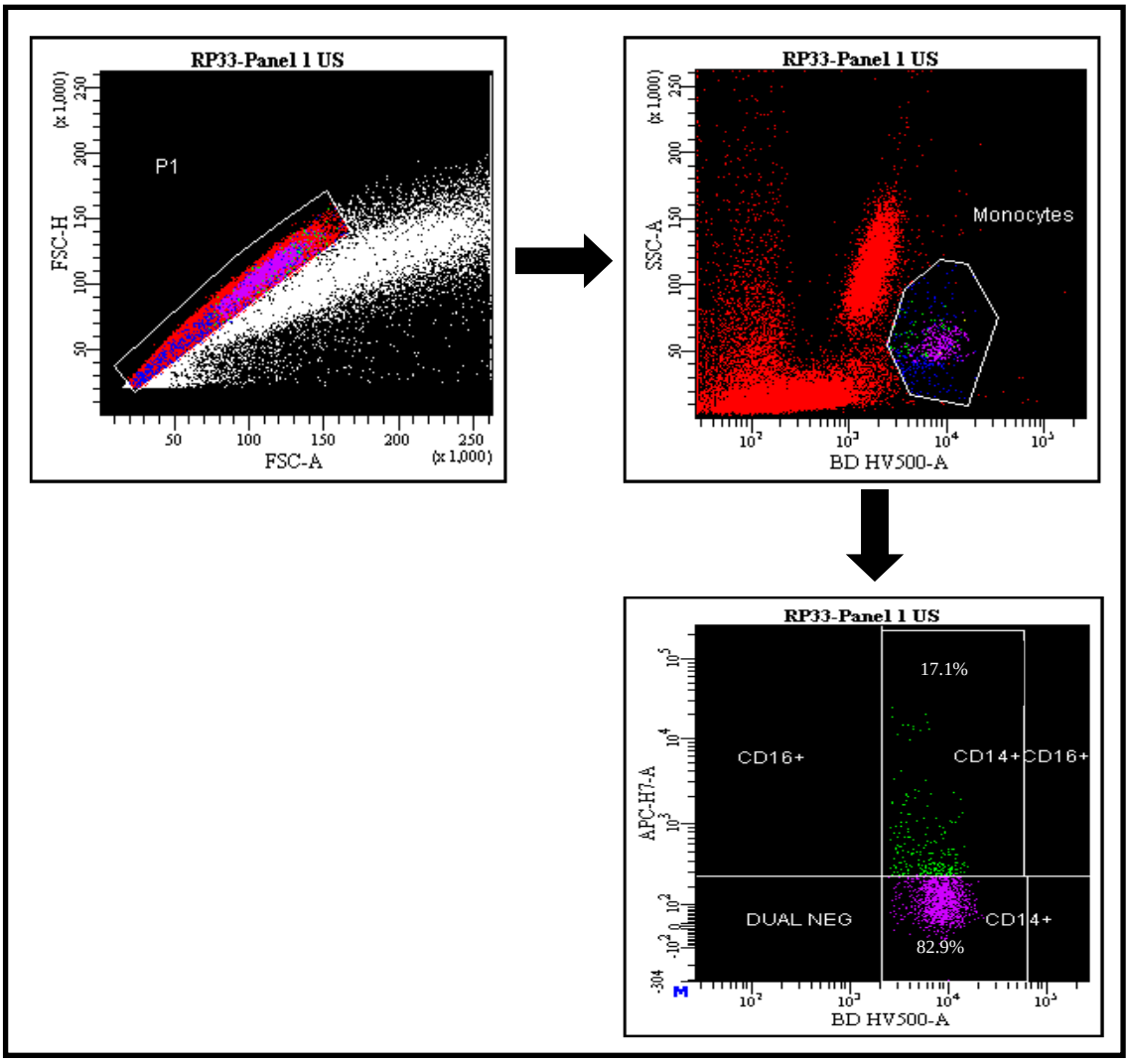

Figure 3- 7: Schematic of gating strategy used during flow cytometry acquisition. Singlet cells are first gated on using FSC-H vs. FSC-A. SSC-A vs. CD14-V500 is then used to distinguish the monocyte population. Finally CD14-V500 vs. CD16-APC-H7 is used to differentiate between the two monocyte subsets. APC-H7 represents CD16-APC-H7 and V500 represents CD14-V500.

3.11 Statistical Analysis

Percentage positive expression of each marker was generated using quadrant statistics from total gated events and was exported from FACS DIVA software into a Microsoft Excel spread sheet. Statistical analysis was performed using these exported percentage expression values with the use of GraphPad PRISM version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Student t-tests were used for intergroup comparisons. 2-Way Anova analyses were performed on marker expression between the two study groups, and determined which variable/s (HIV-1 infection, LPS stimulation, or an interaction between HIV-1 & LPS stimulation) were the cause of the significant marker alterations seen after LPS stimulation. A 2-Way Anova analysis compares the effect two independent variable (in this study, HIV-1 infection & LPS stimulation) has on a dependent variable (marker expression) (GraphPad Software, La Jolla California USA, www.graphpad.com). Therefore, performing a 2-Way Anova analysis in the context of the current will determine if the changes in marker expression in seen in the HIV+ group after LPS stimulation was dependant HIV-1 infection itself or LPS stimulation alone or whether it is these two variables intertwined (defined as an interaction).

Statistically significant correlations or differences were defined as having a p-value < 0.05, p-values between 0.05 and 0.005 were defined as highly significant and -values < 0.005 were defined as extremely significant. Post-analysis consultation with a statistician was used to ensure that all tests performed were appropriate and correctly applied for the data sets under investigation in this study.

Chapter 4: Results

This chapter presents the *in vitro* phenotyping data of both study groups pertaining to total monocytes, as well as CD14+CD16- and CD14+CD16+ subsets, plus the assessment of monocyte responsiveness to *in vitro* LPS stimulation. Whole blood samples and isolated monocytes were stimulated with LPS (1mg/µl) to assess the impact of LPS stimulation on monocytes in terms of cellular marker expression and intracellular cytokine production. All the data presented in this chapter represent mean percentage marker expression and are based on the CD14 positive gated events (i.e. total monocytes) acquired from flow cytometric analysis of whole blood and isolated monocytes. Unless otherwise stated, all data presented are based on whole blood assays. All data are presented graphically using median plus inter-quartile range box-and-whisker plots to illustrate data spread. In these figures, the middle line indicates the median, the box represents the interquartile range (25-75 percentiles), the whiskers indicate the 10-90 percentile and outliers are shown by dots. Mean data (± standard deviation) will be discussed in text, unless otherwise indicated. Any major discrepancies between mean and median data are discussed.

Student t-test analyses were used for intergroup comparisons. 2-Way Anova analyses were performed on marker expression between the two study groups (HIV+ & HIV-), and determined which variable/s (HIV-1 infection, LPS stimulation, or an interaction between HIV-1 & LPS stimulation) were the cause of the significant marker alterations seen after LPS stimulation. A 2-Way Anova analysis compares the effect two independent variable (in the current study, HIV-1 infection & LPS stimulation) has on a dependent variable (marker expression) (GraphPad Software, Inc., San Diego, CA, USA). Therefore, performing a 2-Way Anova analysis in the context of this study will determine if the changes in marker expression seen in the HIV+ group after LPS stimulation was dependent on HIV-1 infection itself or LPS stimulation alone or on these two variables effects combined (defined as an interaction).

All correlation tests of clinical markers with monocyte subsets and marker expression were performed within the HIV+ group only, summary tables of all correlations performed can be found in Appendix 1. Only significant correlations are stated in text, and a select few are represented graphically with XY data and linear regression, with relevant correlation coefficient r - and p-values.

Data was seen as significant if p-value < 0.05, highly significant if p-value < 0.01 and extremely significant if p-value < 0.005. Only for p-values > 0.005 will actual p-values be indicated, otherwise p-value < 0.005 will be used.

4.1. Patient demographics

A total of 66 individuals were recruited for this study, consisting of 40 HIV+ and 26 HIV- individuals. The mean age in years for the HIV- group was 31 (range 22-46) and for the HIV+ group it was also 31 (range 22-43). The HIV+ group had an excess of female donors (76.5%) compared to the HIV- (50%) but this difference was not statistically significant (p-value = 0.1218). The demographics of the study groups are summarized in **Table 4-1**.

4.2. CD4 count & viral load

Study participants were selected based on CD4 counts (> 300 cells/µl) at the time of, or prior to, collection of study samples. In a few cases the time between initial recruitment and sample collection was 3-6 weeks, which could allow for a drop in CD4 count as evidenced by the CD4 count range extending below the inclusion cut-off seen in **Table 4-1**. The mean CD4 count (cells/µl) for the HIV+ group was much lower than the HIV- group at 400.03 ±167.26, whilst CD4 count for the HIV- group was 931.32 ±438.73. Extremely significant differences were found between the CD4 counts of the two groups (p-value < 0.005), see **Figure 4-1**.

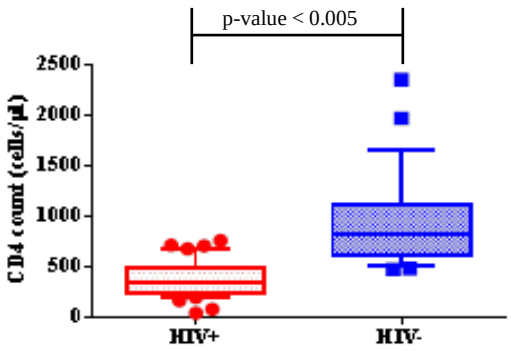

Figure 4- 1: Comparison of CD4 count across the two study groups, HIV+ (median 380.00cells/µl, range [203.00-763.00]) and HIV- (830.00, [481.00-2351.00]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

The mean viral load of the HIV+ group was $1.26x10^5$ copies/ml $\pm 2.10x10^5$. Interestingly, viral load showed a statistically significant positive relationship with CD38/8 expression (r-value = 0.3530; p-value = 0.0297) and a negative relationship between CD4 count (r-value = -0.6931; p-value < 0.005), see **Figure 4-2**. Correlation tests between CD4 count and CD38/8 were not significant (r-value = -0.1528; p-value = 0.4119). A summary of correlations can be seen in Appendix 1 (**Table 1**). A summary of viral load mean and median data can also be seen in **Table 4-1**.

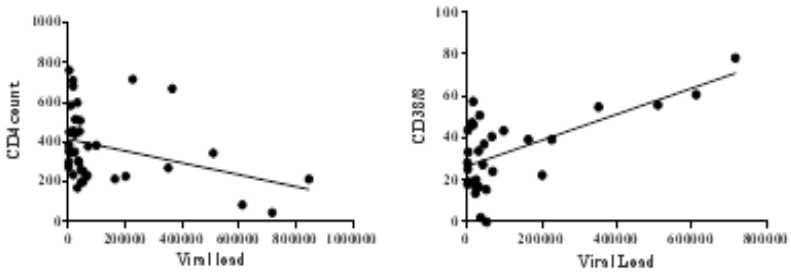

Figure 4- 2: Correlation and linear regression showing negative relationship between viral load and CD4 count (r-value = -0.6931; p-value < 0.005) (left) and positive relationship between viral load and CD38/8 expression (r-value = 0.3530; p-value = 0.0297) (right).

4.4. Immune activation status – CD38/8

CD38 expression on $CD8^+$ T lymphocytes is a well-defined marker of generalized immune activation (Liu *et al*., 1997). In this study this parameter was measured for comparative analysis of the immune activation and the monocyte markers assessed.

The HIV+ group was seen to have a significantly elevated activation status despite being generally healthy with stable CD4 counts (> 300cells/µl). The mean percentage of $CD8^+$ T-cells expressing CD38 was 31.75 ±17.58 for the HIV+ study group and 20.59 ±19.12 for the HIV- group (p-value = 0.0173), see **Figure 4-3** and **Table 4-1** for a summary of this data.

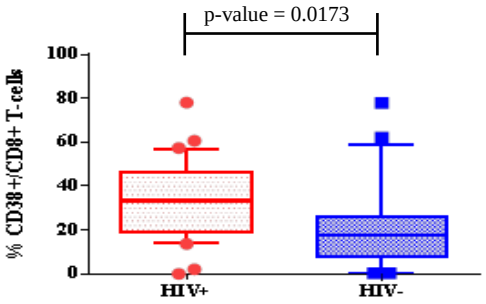

Figure 4- 3: Box-and-whisker plot showing % of CD38$^+$ CD8$^+$ T-cells in the two study groups, HIV+ (median 33.40%, range [0.04-78.24]) and HIV- (17.53, [0.25-78.05]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Table 4- 1: Study population demographic. HIV-: Healthy donors; HIV+: HIV-infected donors; SD: standard deviation

	HIV+	HIV-	P-values
Number of donors	40	26	N/A
Mean age[range]	31[22-43]	31[22-46]	0.9755
% Males	23.5	50	0.1218
% Females	76.5	50	0.1218
CD4 count median(cells/µl)[range]	356.00[203-263]	830.00[481-2351]	<0.005
CD4 count mean (cells/µl)[±SD]	385.53[±175.40]	931.32[±438.73]	<0.005
Viral load median (copies/ml)[range]	$3.50x10^5$[485-$8.46x10^5$]	N/A	N/A
Viral load mean(copies/ml)[±SD]	$1.26x10^5$[±$2.10x10^5$]	N/A	N/A
% CD38$^+$/CD8$^+$ median[range]	33.4[0.04-78.24]	17.53[0.25-78.05]	0.0173
% CD38$^+$/CD8$^+$mean[±SD]	33.70[±17.58]	20.59[±19.12]	0.0173

4.5. Monocyte subsets

Data on monocytes presented in this chapter are based on the total monocyte population (CD14+), the CD14+CD16- subset or the CD14+CD16+ subset. The total monocyte population refers to the percentage of the total leukocytes or total events analysed; see section **3.10** in Chapter 3 for a description and illustration of gating strategy. A summary of all the monocyte subset data can be found in **Table 4-2**.

4.5.1. Total monocyte population

The HIV+ group had a higher percentage of total gated monocytes compared to the HIV- group. The mean percentage of total monocytes gated in whole blood analyses was 2.87 ±1.43 for the HIV+ group and 2.14 ±1.27 for the HIV- study group (p-value = 0.0419), see **Figure 1** in Appendix 1 .

After LPS stimulation in whole blood; a mean 0.07% increase (delta percentage) was seen in the HIV+ group. In the HIV- group a 0.25% decrease was observed after stimulation (p-value < 0.005). These changes could be due to changes in other cell populations affecting the total events or due to increased adherence of monocytes to test tubes during stimulation. Correlations performed between the total gated monocyte population and clinical markers, CD4 count, viral load and CD38/8 expression showed no significant correlations, indicating that the overall increase in monocytes was likely due to a decrease in total leukocytes (and especially $CD4^+$ T cells) in the HIV group. A summary of these data can be seen in Appendix 1 (**Table 2**).

4.5.2. CD14+CD16- monocyte subset

A decrease in the percentage of the CD14+CD16- monocyte subset within the total monocyte fraction was seen in the HIV+ group. The mean percentage of monocytes within the CD14+CD16- monocyte subset was 83.43 ±8.94 for the HIV+ group and 86.82 ±7.19 for the HIV- group (p-value = 0.0448) see **Figure 4- 4**.

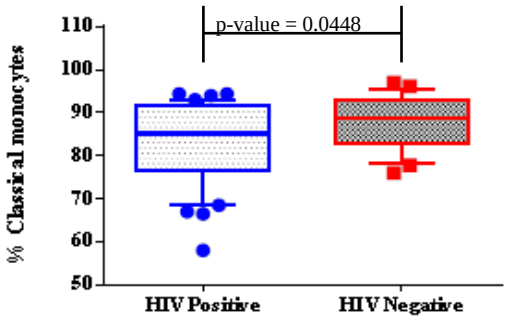

Figure 4- 4: Box-and-whisker plot showing comparison of the CD14+CD16- subset of both study groups, HIV+ (median 85.15%, range [56.00-96.40]) and HIV- (87.75, [66.30-97.00]) at baseline. HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Stimulation with LPS resulted in a statistically significant increase (p-value = 0.0233) in the mean percentage of CD14+CD16- monocyte subset in the HIV+ group. In the HIV- group no

statistically significant difference was found before and after stimulation (p-value = 0.8810). Correlations performed between the CD14+CD16- monocyte subset and clinical markers; yielded a significant negative correlation (r = -0.3884, p-value = 0.0308) between CD38/8 expression and CD14+CD16- monocyte subset percentage, see **Figure 4-5** below. A summary of these data can be seen in Appendix 1 (**Table 3**). A converse relationship was seen in the CD14+CD16+ monocyte subset, see section **4.5.3**. (**Figure 4-7**).

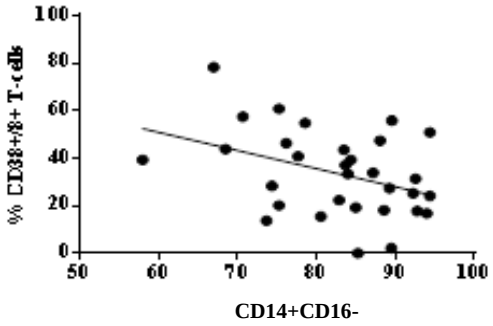

Figure 4- 5: Correlation, including linear regress ion, showing negative relationship (r = -0.3884, p-value = 0.0308) between the CD14+CD16- subset of HIV+ group with % CD38+/CD8+ T-cells at baseline.

4.5.3. CD14+CD16+ monocyte subset

In counterbalance to the decreased CD14+CD16- monocyte subset, a higher percentage of the CD14+CD16+ monocyte subset within the total monocyte fraction was seen in the HIV+ group at baseline. The mean percentage of CD14+CD16+ monocyte subset in the HIV+ group was 16.54 ±8.95, whilst in the HIV- group it was 13.15 ±7.12 (p-value = 0.0461) see **Figure 4-6**.

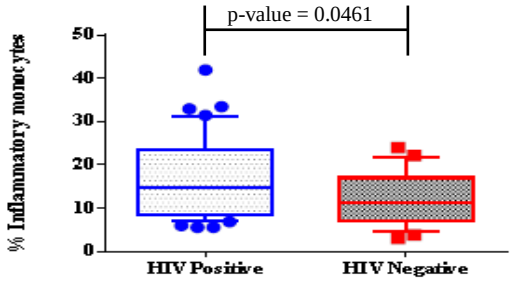

Figure 4- 6: Box-and-whisker plot comparing CD14+CD16+ subsets percentages across both study groups, HIV+ (median 14.85%, range [5.60-42.00]) and HIV-(12.25, [3.00-33.00]) at baseline. HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

LPS stimulation produced a statistically significant decrease (p-value = 0.0239) in the CD14+CD16+ monocytes in the HIV+ group. In the HIV- group stimulation did not result in any significant changes. Correlations performed between the CD14+CD16+ monocyte subset and clinical markers produced a significant positive correlation (r = 0.3862, p-value = 0.0319) between the CD14+CD16+ monocyte subset and CD38/8 expression, see **Figure 4-7** below. A summary of these data can be seen in Appendix 1 (**Table 4**).

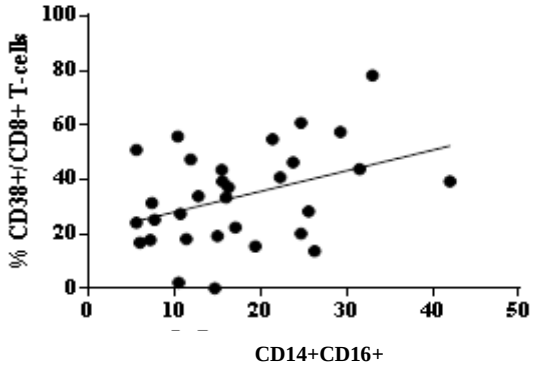

Figure 4- 7: Correlation, including linear regression, showing positive relationship (r = 0.3862, p-value = 0.0319) between CD14+CD16+subset of HIV+ group with % $CD38^{+}/CD8^{+}$ T-cells at baseline.

Table 4- 2: Summary of mean percentage data of the total gated monocyte population as well as monocyte subsets, at baseline and after stimulation in both study groups, * p –value < 0.05, *** p-value < 0.005

	Stimulus	HIV+ (mean ±SD)	HIV- (mean ±SD)
Total monocyte population (% of total events)	Unstimulated	2.43 (±1.43)*	2.14 (±1.27)*
	LPS	2.94 (±.52)***	1.89 (±1.21)***
CD14+CD16- monocyte subset (% total monocytes)	Unstimulated	83.43 (±8.94)*	86.32 (±7.19)*
	LPS	86.17 (±6.90)*	86.60 (±6.97)*
CD14+CD16+ monocyte subset (% total monocytes)	Unstimulated	16.54 (±8.95)*	13.15 (±7.12)*
	LPS	13.81 (±6.90)*	12.67 (±7.17)*

4.6. Phenotypic Marker expression on monocytes

4.6.1. CCR5

CCR5 is a cell surface chemokine receptor (G-protein coupled receptor) that is a well-known as the HIV co-receptor needed for productive infection of immune cells also expressing CD4

(Bucci, 2013). To test the potential susceptibility to HIV-1 infection CCR5 expression was assessed.

Total monocyte population

The HIV+ group had a higher expression of CCR5 at baseline compared to the HIV- group. The baseline mean percentage of CCR5 in HIV+ donors was 58.53 ±12.54, whilst for HIV- donors it was much lower at 11.47 ±8.41 (p-value <0.005), see **Figure 4-8** and **Figure 4-9** for flow representative data.

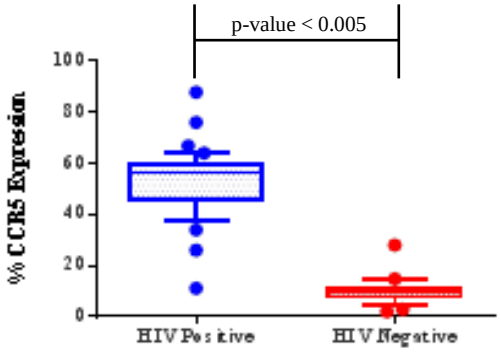

Figure 4- 8: Box-and-whisker plot showing comparison of CCR5 expression in the total monocyte population of study groups, HIV+ (median 56.35%, range [11.20-87.80]) and HIV-(10.00, [2.10-56.40]) at baseline. HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

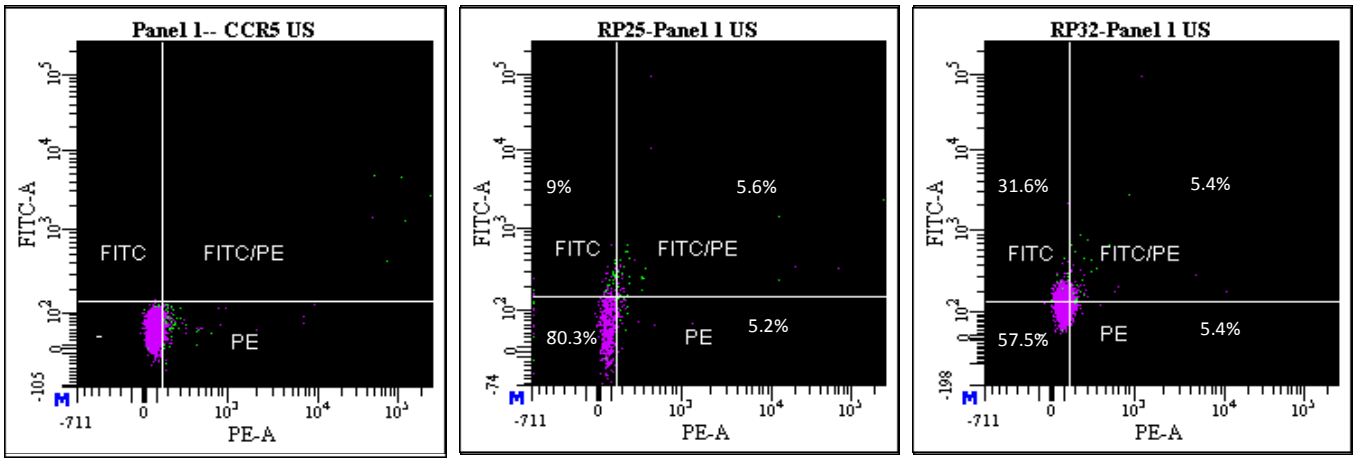

Figure 4- 9 Representative flow cytometry dot plots showing CCR5 (FITC) expression in the total monocyte population, (PE = CCR1). In the first box on the left (Panel 1 CCR5 US), the expression of CCR5 FITC vs. CCR1 PE is illustrated in a FMO sample excluding CCR5 marker (negative unstained control). In the middle and right boxes baseline expression of CCR5 in a HIV+ (RP32) vs. a HIV- (RP25) study sample is shown, respectively. Increased expression of CCR5 can be seen for RP32 vs. RP25, total CCR5 positivity for RP25 is 14.6%, and for RP32 it is 37%.

After stimulation, the expression in the HIV+ group decreased to 16.05 ±10.07 whereas the HIV- group's expression decreased only moderately to 9.33 ±6.36. 2-Way Anova analysis showed that this sharp decrease in the HIV+ group was due to a statistically extremely significant interaction between HIV infection and LPS stimulation (p-value <0.005) see **Figure 4-10**. A summary of correlations performed between the total monocyte population's CCR5 expression and clinical markers can be seen in Appendix 1 (**Table 5**).

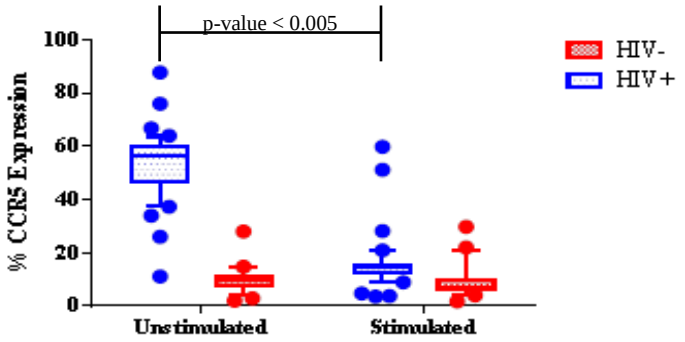

Figure 4- 10: The expression of CCR5 in the total monocyte population of both study groups before stimulation, HIV+ (median 56.35%, range [11.20-87.80]) vs. HIV-(10.00, [2.10-56.40]), and after LPS stimulation, HIV+ (14.90, [3.70-59.90]) vs. HIV-(6.80, [1.90-29.90]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

The mean percentage CCR5 expressing CD14+CD16- monocytes of the HIV+ donors were 12.07 ±18.71, whilst for HIV- donors it was 6.97 ±4.57. Despite the CCR5 expression in the CD14+CD16- monocyte subset of the HIV+ group observed to be almost double that of the HIV-group at baseline, no statistically significant difference was seen (p-value = 0.1933). LPS stimulation resulted in an increased CCR5 expression in both groups (p-value = 0.0131). Correlation tests performed between CCR5 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 6**).

CD14+CD16+ monocyte subset

CCR5 was expressed on a higher percentage of CD14+CD16+ monocytes than CD14+CD16- in both study groups. Statistically significant differences were seen between the CD14+CD16- subset and CD14+CD16+ subset for the HIV+ group (p-value < 0.005) and for the HIV- group (p-value < 0.005), see **Figure 4-11**.

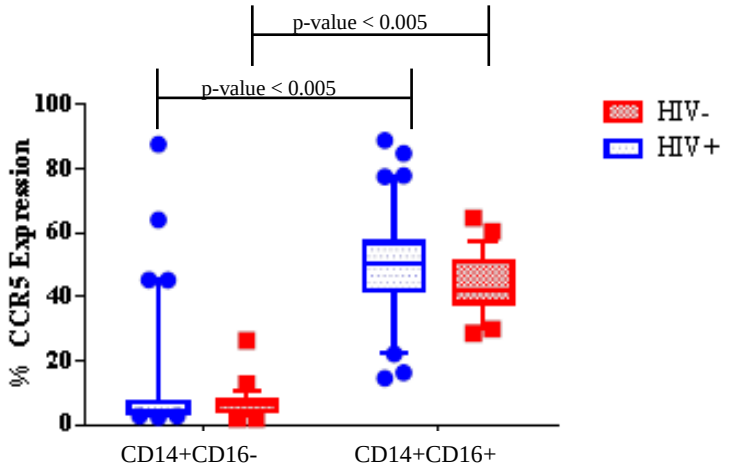

Figure 4- 11: Comparison of CCR5 expression across study group's monocyte subsets at baseline. HIV+ CD14+CD16- (median 4.4%, range [2.40-87.60]) vs. CD14+CD16+ (50.85, [14.50-88.90]) and HIV- CD14+CD16- (6.70, [1.80-26.30]) vs. CD14+CD16+ (42.20, [25.50-64.60]) HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

In the HIV+ group 49.63% ±17.13 were CCR5+ and 44.02% ±9.14 were CCR5+ in the HIV- group. These differences were not significant (p-value = 0.1420). After LPS stimulation CCR5 expression in the HIV+ group slightly decreased to 48.86 ±12.24 and, decreased more than 10%, in the HIV- group to 33.36 ±9.58. 2-Way Anova analysis showed a statistically significant interaction (p-value < 0.005) between HIV infection and LPS stimulation in causing only a slight decrease in the HIV+ group after stimulation, see **Figure 4-12**. Correlations performed between the CD14+CD16+ monocyte subset's CCR5 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 7**).

The dramatic difference in total percentage of monocytes expressing CCR5 not being reflected in the subsets appears strange, however it may reflect that this marker is up-regulated in both subsets in HIV and the cumulative percentage is significantly different to individual subsets.

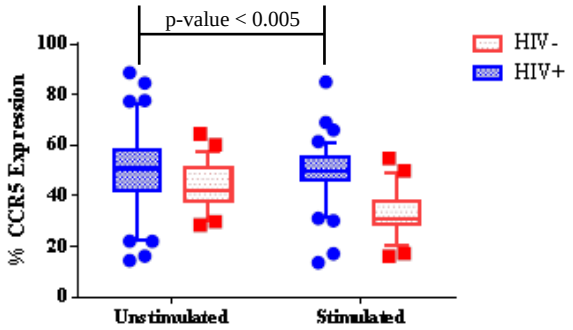

Figure 4- 12: Comparison of CCR5 expression in the CD14+CD16+ subset of both study groups before stimulation, HIV+ (median 50.85%, range [14.50-88.90]) vs. HIV-(42.20, [28.50-64.60]), and after stimulation, HIV+ (49.65, [13.70-85.20]) vs. HIV-(31.10, [16.20-54.90]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

4.6.2. CD116

CD116 or GM-CSF- receptor is involved in the stimulation of production of granulocytes and macrophages from the bone marrow. CD116 is also known to be involved with monocyte differentiation, survival and may play a role in enhancing viral replication during HIV-1 infection (Allavena *et al*., 1998; Kedzierska *et al*., 1998).

Total monocyte population

CD116 was found to be expressed in the majority of monocytes (>78%). Expression of CD116 in the total monocyte population of the HIV+ group was higher than in the HIV- group. At baseline the expression in the HIV+ group was 89.08 ±13.17 and 79.8 ±23.25 in the HIV- group (p-value = 0.0094), see **Figure 4-13**. After LPS stimulation, the expression for HIV+ group was seen to moderately increase and slightly decrease in the HIV- group. 2-Way Anova analysis showed the expression seen after stimulation resulted from LPS stimulation (p-value = 0.0219). Correlations performed between the total monocyte population's CD116 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 8**).

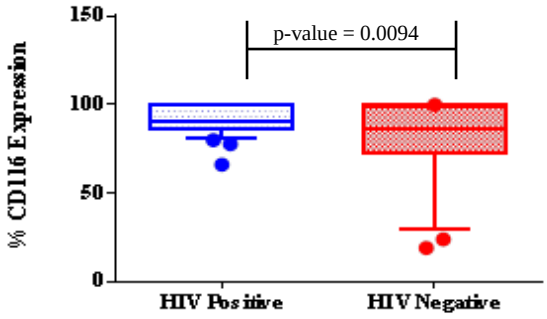

Figure 4- 13: Baseline comparison of CD116 expression in the total monocyte population of both study groups, HIV+ (median 90.25%, range [43.90-99.90]) and HIV-(86.30, [19.10-100]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

CD116 expression was minimally increased in the HIV+ group compared to the HIV- group at baseline, with expression at 88.28 ±17.13 and 83.26 ±23.70 in the HIV-group. This was however not statistically significant (p-value = 0.3502). Overall the expression was similar to the total monocyte population. Upon stimulation there was a statistically significant interaction (p-value < 0.005) between HIV infection and LPS stimulation which resulted in

the mean expression of CD116 increasing to 92.63 ±13.08 in the HIV+ group, whilst the HIV- group's mean expression decreased 10% after stimulation to 75.09 ±32.33. Although there was an increase in the mean percentage of the HIV+ group and a mean percentage decrease in expression in the HIV- group, this was not evident in the median data analysis, see **Figure 4-14**. Correlations performed between the CD14+CD16- monocyte subset's CD116 expression and clinical markers produced no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 9**).

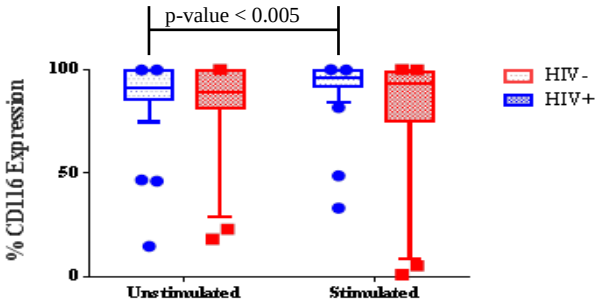

Figure 4- 14: Box-and-whisker plot showing CD116 expression in the CD14+CD16- subset across study groups before stimulation, HIV+ (median 91.05%, range [14.6-99.9]) vs. HIV-(89.1, [18-100]), and after stimulation, HIV+ (95.95, [33.1-100]) vs. HIV-(93.3, [0.9-100]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16+ monocyte subset

Baseline expression of CD116 was higher in HIV+ group, with expression seen to be 87.96 ±18.12 and 79.02 ±21.72 in the HIV- group, (p-value = 0.0941). No major differences in expression between the CD14+CD16- and CD14+CD16+ subsets in the HIV+ group were seen (p-value = 0.7698). But the difference were statistically significant in the HIV+ group (p-value = 0.0118)

An increase in CD116 expression to 90.93 ±15.05 and a decrease in excess of 10% in the HIV- group to 71.03 ±28.48 was seen after LPS stimulation. 2-Way Anova analysis showed a statistically significant interaction (p-value < 0.005) between HIV infection and LPS stimulation; see **Figure 4-15** and **Figure 2** in Appendix 1 for representative flow dot plots. This suggests that the slight increase in CD116 expression seen in the HIV+ group was caused by both HIV-infection and LPS stimulation since such a sharp decrease was not observed in the HIV- group.

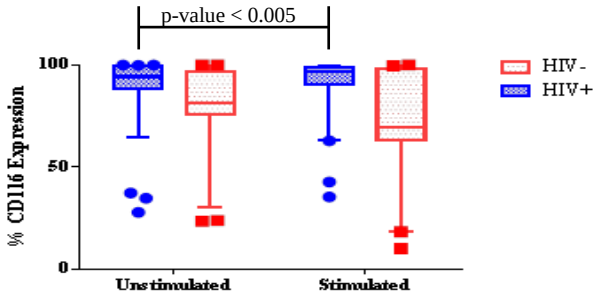

Figure 4- 15: Comparison of CD116 expression in CD14+CD16+ subset of both study groups before stimulation, HIV+ (median 94.25%, range [27.80-100]) vs. HIV-(81.60, [23.40-100]) and after stimulation, HIV+ (96.90, [35.40-100) vs. HIV-(69.4, [10-100]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Correlations performed between the CD14+CD16+ monocyte subset's CD116 expression and clinical markers produced no significant relationships. A summary of these data can be seen in Appendix 1 (**Table 10**).

4.6.3. VPAC-2 (Vasoactive Intestinal Peptide Receptor 2)

Vaso-active intestinal peptide (VIP), the ligand of VPAC-2, is known to mediate anti-inflammatory effects and has been shown to inhibit certain pro-inflammatory activities and LPS-induced inflammatory pathways. VPAC-2 has been noted for its immune-regulatory role and is known to inhibit viral replication (Smalley *et al*., 2009). See section **2.9.** in Chapter 2 for more information on VPAC-2. To investigate the potential of monocyte as therapeutic target expression of VPAC-2 was assessed.

Total monocyte population

Baseline expression in HIV+ donors was higher but not significantly different to expression in HIV- donors. Expression was 5.86 ±6.27 in the HIV+ donors and 3.32 ±2.97 in HIV- donors. Although the differences were not statistically significant, they were tending towards significance (p-value = 0.0774). After LPS stimulation an increase in expression levels was seen in both study groups. See Appendix 1 for representative flow dot plots (**Figure 4**) correlation tests performed between VPAC-2 expression and clinical markers (**Table 11**).

CD14+CD16- monocyte subset

The CD14+CD16- monocyte fraction of the total monocytes displayed a statistically higher baseline VPAC-2 expression. The mean percentage in the HIV+ group was 5.45 ±6.13 and 1.94 ±1.75 in the HIV- groups (p-value = 0.0065), see **Figure 4-16** below.

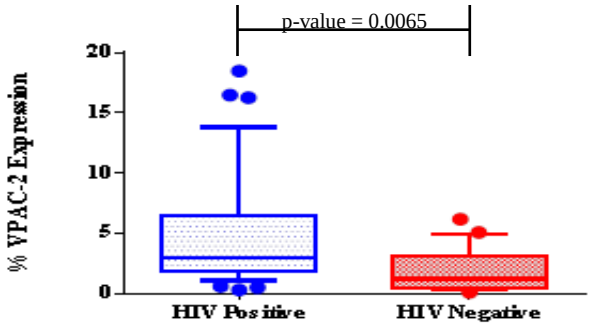

Figure 4- 16: Comparison of VPAC-2 expression in CD14+CD16- subset across both study groups, HIV+ (median 3.10%, range [0.30-31.20]) and HIV-(1.20, [0.10-6.20]), at baseline. HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Upon stimulation, there was a moderate increase in expression in the HIV+ group and a decrease in the HIV- group. 2-Way Anova analysis revealed the increase seen in the HIV+ group was due to HIV+ infection (p-value < 0.0011), see **Figure 4-17**. See Appendix 1 (**Table 12**) for correlations performed between the CD14+CD16- monocyte subset's VPAC-2 expression and clinical markers.

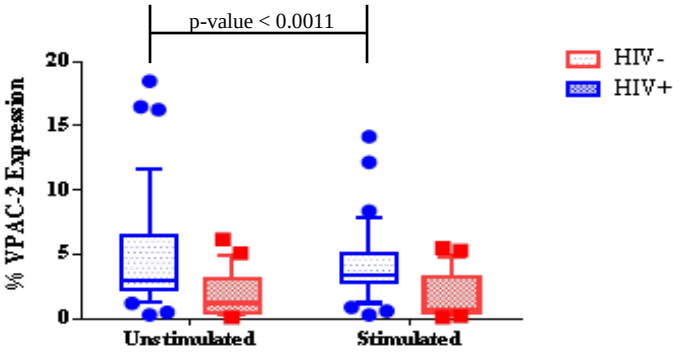

Figure 4- 17: Comparison of VPAC-2 expression in CD14+CD16- subset of both study groups before, (n=36) HIV+ (median 3.00%, range [0.30-18.50]) vs. (n= 32) HIV-(3.40, [0.30-14.20]) and after stimulation, HIV+ (1.20, [0.1-6.2]) vs. HIV-(0.70, [0.10-5.50]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16+ monocyte subset

In HIV+ donors VPAC-2 expression was 12.06 ±10.16 and 10.14 ±8.09 in HIV- donors (p-value = 0.4520). After LPS stimulation VPAC-2 expression increased in both groups. See Appendix 1 (**Table 13**) for correlation tests performed between the total monocyte population's VPAC-2 expression and clinical markers.

4.6.4. IL-10

IL-10 is a regulatory cytokine produced by several types of immune cells and has a primarily anti-inflammatory action. During the later stages of HIV-infection IL-10 production is known to be increased (Sozzani *et al*., 1997; Verani *et al*., 1997), see section **2.7.5.** in Chapter 2 for more information on IL-10. Intra-cellular IL-10-production was assessed to determine if production is affected during HIV-1 infection.

Total monocyte population

Expression of intracellular monocyte-associated IL-10 was almost six times higher in the HIV+ group than in the HIV- group. Baseline expression in HIV+ donors was 3.71 ±2.66 and 0.68 ±0.78 in HIV-donors (p-value < 0.005) see **Figure 4-18** and **Figure 6** in Appendix 1 for representative flow dot plot. Upon stimulation there was an increase in the IL-10 expression in the total monocyte population of both study groups. Using 2-way Anova analysis, it was seen that only LPS stimulation caused the responses seen (p-value-= 0.0202). Correlation tests performed between the total monocyte population's IL-10 expression and clinical markers produced no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 14**).

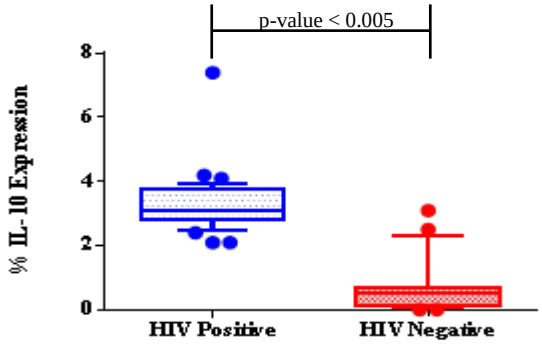

Figure 4- 18: Baseline comparison of IL-10 expression in the total monocyte population of both study groups, HIV+ (median 3.15%, range [2.10-19.00]) and HIV-(0.50, [0.00-3.10]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

The percentage of IL-10 expression in the HIV+ group's CD14+CD16- subset was slightly higher than in the HIV-1 group, but overall expression in this subset was low (<0.70%)compared to the total monocyte population and the CD14+CD16+ subset. The

baseline mean percentage in HIV+ donors was 0.63 ±0.35 and in HIV- donors it was 0.35 ±0.26 (p-value < 0.005) see **Figure 4-19**.

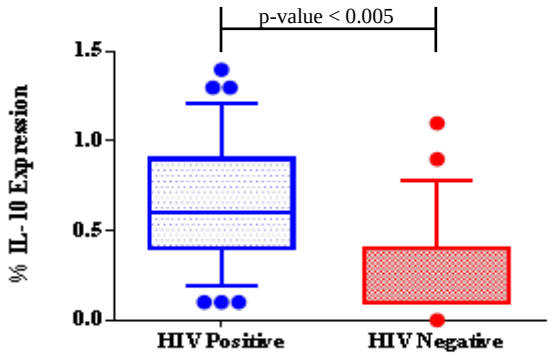

Figure 4- 19: Comparison of IL-10 expression in CD14+CD16- subset of both study groups HIV+ (median 0.60%, range [0.10-1.40]) and HIV-(0.04, [0.00-1.10]), at baseline. HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values

After LPS stimulation, there was a slight decrease of expression in the HIV+ group and a small increase in HIV- group. 2-Way Anova analysis revealed the slight decrease in the HIV+ group was due HIV infection (p-value < 0.005), the analysis also showed that the responses in the HIV+ group was tending towards an interaction between HIV-infection and LPS stimulation (p-value = 0.0547). Correlations performed between the CD14+CD16- monocyte subset's IL-10 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 15**).

CD14+CD16+ monocyte subset

Expression of IL-10 in this subset was more than 20 times higher than the CD14+CD16- subset; this difference was also statistically significant (p-value < 0.005).

Expression of IL-10 in the CD14+CD16+ subset of the HIV+ group was almost three and a half times more than in the HIV- group. Baseline expression of IL-10 in the HIV+ group was 12.66 ±2.91, and 3.99 ±2.38 in the HIV- group (p-value <0.005), see **Figure 4-20** and **Figure 8** in Appendix 1 for representative flow plots, which shows the higher expression of IL-10 in the HIV+ groups compared to HIV- group. After LPS stimulation, there was a slight increase in expression in both study groups, these responses were attributed to LPS stimulation (p-value < 0.005).

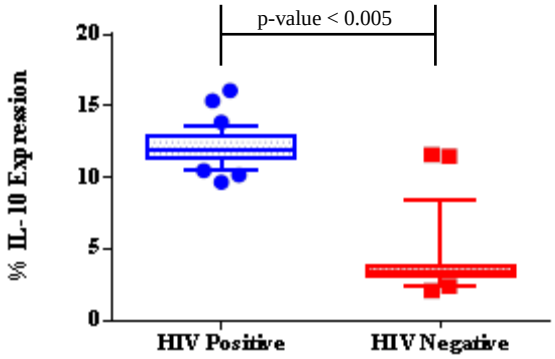

Figure 4- 20: Comparison of IL-10 expression in CD14+CD16+ subset of both study groups at baseline, HIV+ (median 12.05%, range [9.70-28.60]) and HIV-(3.4, [2.10-11.60]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values

Correlations performed between the CD14+CD16+ monocyte subset's IL-10 expression and clinical markers showed no significant correlation. A summary of these data can be seen in Appendix 1 (**Table 16**).

4.7. Homing Markers

All the markers presented in this section are migration associated. This includes chemokine receptors (CCR1, CX3CR1, CCR2, CCR7 and CCR9) and L-selectin (CD62L). These markers are either involved in the recruitment of leukocytes to sites of infection and inflammation or to lymphoid organs or tissues such as the GALT or lymph nodes themselves; see section **2.7.** in Chapter 2 for more information on these processes (Kedzierska & Crowe, 2002). These markers were examined to determine if monocytes are primed in HIV-1 infection to migrate to sites of infection, lymph nodes or sites of inflammation and thus also to exit the circulation.

4.7.1. CCR1

CCR1 is a chemokine receptor / migration marker involved with the recruitment and priming of monocytes to exit systemic circulation and travel to sites of infection (Shi & Pamer, 2011).

Total monocyte population

More than double the amount of monocytes expressed CCR1 in the HIV+ group vs. the HIV- group. At baseline expression in the HIV+ group was 6.48 ±3.20 and in the HIV- group was 3.21 ±1.53 (p-value < 0.005), see **Figure 4-21 and Figure 4-9** for representative flow dot plot. Upon stimulation the CCR1 expression decreased in both study groups, this was as a

result of LPS stimulation (p-value = 0.0299). Correlations performed between the total monocyte population's CCR1 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 17**).

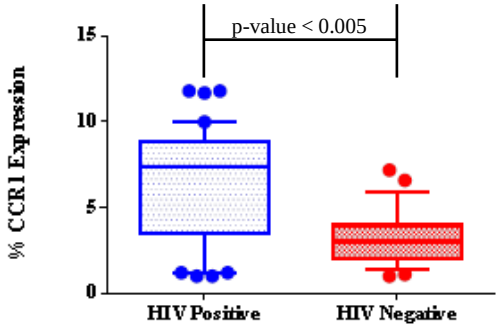

Figure 4- 21: Box-and-whisker plot showing baseline CCR1 expression in the total monocyte population of both study groups, HIV+ (median 7.35%, range [1.00-16.80]) and HIV-(3.00, [1.00-7.20]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

Overall percentage CCR1 expression patterns were similar; if not slightly lower, than the total monocytes. Baseline expression in the HIV+ group was 3.92 ±8.23 and 1.66 ±0.67 in HIV- group. The expression of CCR1 in the HIV+ group was almost double the expression in the HIV- group; however, the differences were not statistically significant (p-value = 0.557) but were tending to statistical significance. Upon stimulation, expression in both groups decreased slightly. Correlation performed between the CD14+CD16- monocyte subset's CCR1 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 18**).

CD14+CD16+ monocyte subset

Baseline expression in the CD14+CD16+ monocyte subset was more than double than the CD14+CD16- subset in both study groups. These differences were observed to be statistically significant in both study groups, with p-value < 0.005 for both study groups.

The HIV+ group displayed slightly higher CCR1expression at 11.16 ±11.33 than the HIV- group at 9.52 ±4.24 in the CD14+CD16+ subset at baseline (p-value = 0.5002). After stimulation there was a slight decrease in both study groups. Correlation tests performed between the CD14+CD16+ monocyte subset's CCR1 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 19**).

4.7.2. CX3CR1 (Fractalkine receptor)

CX3CR1 is a migration marker that primes leukocytes to exit circulation and migrate towards sites of infection; it is also known to be involved in monocyte survival. Expression of CX3CR1 is known to counterbalance the expression of chemokine receptor CCR2, which is also a migration marker. These receptors have the same function but their expression is usually inverse to each other, i.e. when CX3CR1 expression is increased CCR2 is normally decreased and vice versa ((Kedzierska & Crowe, 2002). The priming of monocytes to exit circulation by investigating the expression of both CXCR1 and CCR2 was assessed. In the current study this trend was observed; CX3CR1 expression was generally lower than CCR2 expression in both study groups. However, CX3CR1 was still higher when comparing expression between study groups. The same trend was seen for CCR2 expression between the study groups, see section **4.7.3**.

Total monocyte population

The baseline expression of CX3CR1 was almost three times lower in the HIV+ group compared to the HIV- group. Expression in the HIV+ group was 3.70 ±1.30, whereas in the HIV- group it was 9.01 ±3.21 (p-value < 0.005), see **Figure 4-22** and **Figure 5** in Appendix 1 for representative dot plots.

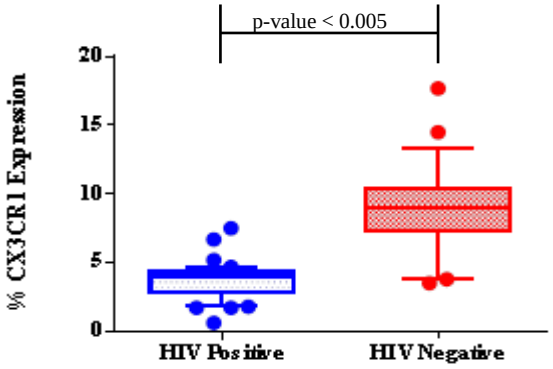

Figure 4- 22: Box-and-whisker plot showing comparison of CX3CR1 expression in the total monocyte population across both study groups at baseline, HIV+ (median 4.00, range [0.60-7.50]) and HIV-(9.00, [3.50-17.70]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

After LPS stimulation expression in the HIV+ group decreased minimally to 3.61 ±1.80, whilst in the HIV- group expression decreased moderately to 7.47 ±2.85, these responses were due to LPS stimulation alone (p-value = 0.0178). Correlations performed between the

total monocyte population's CX3CR1 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 20**).

CD14+CD16- monocyte subset

In the CD14+CD16- monocyte subset the baseline mean percentage expression of CX3CR1 was the same as general expression in monocytes. In the HIV+ group expression was 3.70 ±1.88 and in the HIV- group it was almost twice as high at 6.65 ±2.04 (p-value <0.005), see **Figure 4-23**. After LPS stimulation expression was decreased in both groups, these decreases observed were caused by LPS stimulation only (p-value < 0.005).

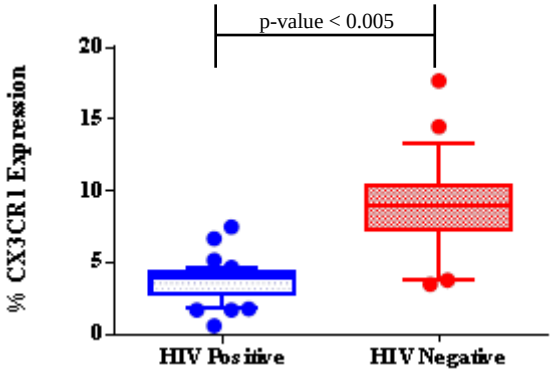

Figure 4- 23: Box-and-whisker plot showing comparison of CX3CR1 expression in CD14+CD16- subset between both study groups at baseline, HIV+ (median 3.80%, range [0.30-8.20]) and HIV-(7.00, [2.60-12.20]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Correlations performed between CX3CR1 expression and clinical markers produced a significant negative relationship between CD38/8 expression and CX3CR1 after LPS stimulation (r -value = -0.4916, p-value = 0.0050), see **Figure 4-24**. A summary of these data can be seen in Appendix 1 (**Table 21**).

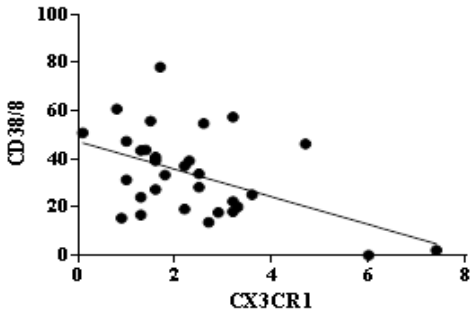

Figure 4- 24: Correlation, including linear regression, showing negative relationship of CX3CR1 expression in CD14+CD16- subset with CD38/8 expression after LPS stimulation (r = -0.4961, p-value = 0.0050).

CD14+CD16+ monocyte subset

CX3CR1 expression was more than three times higher in the CD14+CD16+ subset than the CD14+CD16- subset. These differences were statistically significant in both study groups, with p-value < 0.005 in both study groups. The expression was twice as low in the HIV+ group as in the HIV group. Baseline expression of CX3CR1 in the HIV+ group was 11.13 ±2.97 and in the HIV- group it was 23.46 ±7.60 (p-value <0.005), see **Figure 4-25**.

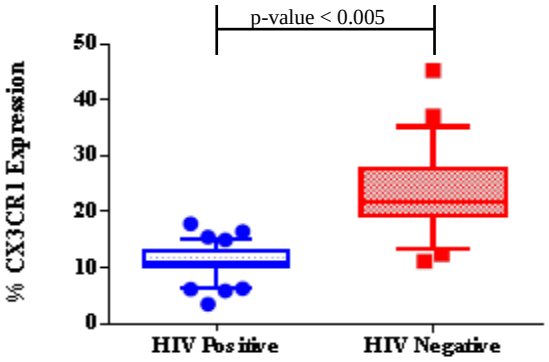

Figure 4- 25: Baseline comparison of CX3CR1 expression in CD14+CD16- subset across both study groups, HIV+ (median 10.95%, range [3.50-17.90]) and HIV-(21.80, [11.20-45.30]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Stimulation resulted in an increased CX3CR1 expression in the HIV+ group to 14.91 ±5.43 and to 23.96 ±7.33 in the HIV- group. Using 2-Way Anova analysis it was seen that the increase seen in the HIV+ group was due to an interaction between HIV infection and stimulation (p-value = 0.0187), see **Figure 4-26**.

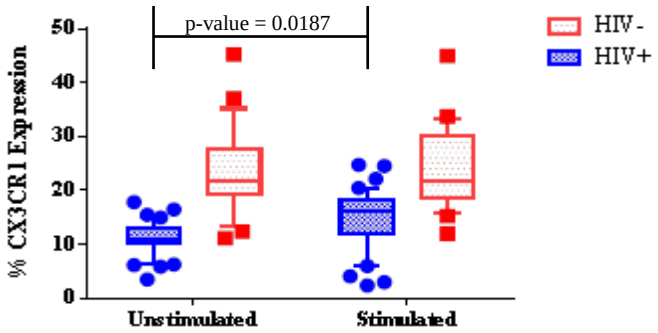

Figure 4- 26: Box-and-whisker plot showing comparison of CX3CR1 expression in CD14+CD16+ subset across both study groups before stimulation, HIV+ (median 10.95%, range [3.50-17.90]) and HIV-(21.80, [11.20-45.30]) and after stimulation, HIV+ (16.25, [2.40-24.80]) vs. HIV-(21.80, [12.00-45.00]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Correlations performed between the CD14+CD16+ monocyte subset's CX3CR1 expression and clinical markers showed no significant correlations, summary of these data can be seen in Appendix 1 (**Table 22**).

4.7.3. CCR2

CCR2 is a migration marker that primes leukocytes to exit systemic circulation and journey towards sites of infection or inflammation (Kedzierska & Crowe, 2002). As mentioned in section **4.7.2.**, there appears to be an inverse relationship with the expression patterns of CX3CR1 and CCR2. From the data below, CCR2 expression was observed to be higher in the total monocyte population and both the subsets of the HIV+ group compared to the HIV- group. However, it appears that CCR2 is expressed at higher levels than CX3CR1 in the HIV+ group. An important observation is that CCR2 expression appears to be higher in the CD14+CD16+ monocyte subset of the HIV+ group and the HIV- group irrespective of disease state. This is in contrast to reports by Mir *et al*. (2012) that state CX3CR1 is normally expressed in higher levels in the CD14+CD16+ monocyte subset. Despite the trend of inverse CX3CR1/CCR2 expression seen, correlation tests between these marker's expression in both monocyte subsets showed that only expression in the CD14+CD16+ subset exhibited a statistically negative correlation (r-value = -0.4401; p-value < 0.005), in the HIV+ group. See **Table 23** and **Table 24** in Appendix 1 for a summary of the correlation tests performed within the CD14+CD16- subset and CD14+CD16+ subset, respectively.

Total monocyte population

The mean baseline percentage for HIV+ donors was 51.97 ±21.25 and for HIV- donors, 33.37 ±16.21 (p-value < 0.005), see **Figure 4-27** and **Figure 3** in Appendix 1 for representative dot plots. Upon stimulation expression decreased in both study groups, this was due to LPS stimulation (p-value < 0.005). Correlations performed between the total monocyte population's CCR2 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 25**).

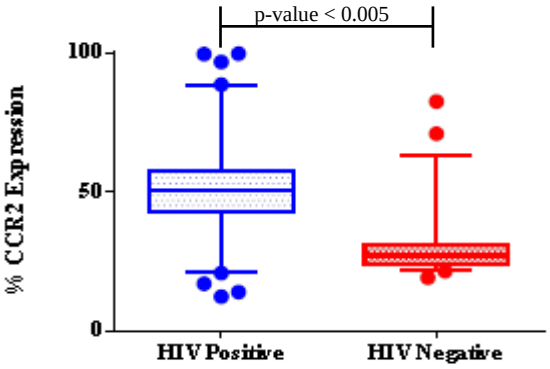

Figure 4- 27: Baseline comparison of CCR2 expression in the total monocyte population across both study groups at baseline, HIV+ (median 50.55%, range [12.40-99.90]) and HIV-(27.10, [19.20-82.60]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

Expression of CCR2 in the HIV+ group was elevated compared to the HIV- group, with overall similar range of values as in the total monocytes. In the HIV+ group expression was 53.95 ±21.85 and for the HIV-group it was 34.5 ±15.42. (p-value < 0.005), see **Figure 4-28**. After stimulation, expression in the HIV+ group decreased to 51.42 ±21.20 and in the HIV- group expression 31.76 ±15.75. 2-Way Anova analysis suggests that these differences are due to stimulation only (p-value < 0.005). Correlations performed between the CD14+CD16- monocyte subset's CCR2 expression and clinical markers showed a significant correlation between CCR2 expression and CD4 count (r -value = 0.3309, p-value = 0.0396). A summary of these data can be seen in Appendix 1 (**Table 26**).

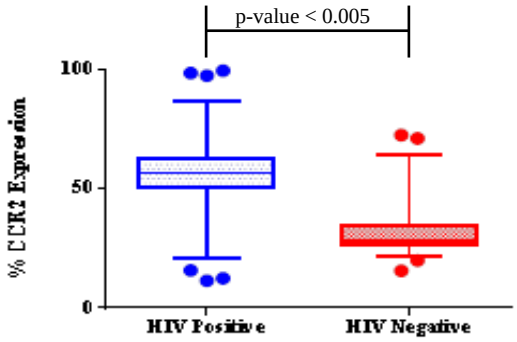

Figure 4- 28: Box-and-whisker plot showing comparison of CCR2 expression in CD14+CD16- subset across both study groups at baseline, (median 56.80% [range 11.50-99.90]) and HIV-(28.40, [15.70-72.80]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16+ monocyte subset

The CD14+CD16+ monocyte subset expressed slightly higher levels of CCR2 than the CD14+CD16- subset in HIV+ groups but these differences were not statistically significant (p-value = 0.3110). However, in the HIV- group the difference in CCR2 expression between the monocyte subsets was statistically significant (p-value < 0.005), see **Figure 4-29**. However, it is interesting to note that CCR2 expression in the HIV+ group was elevated (> 50%) despite disease status.

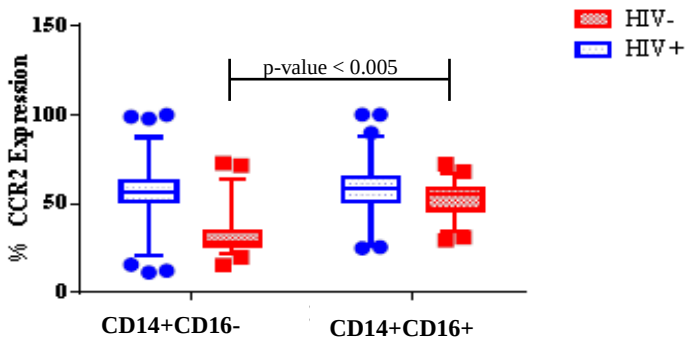

Figure 4- 29: Comparison of CCR2 expression across study group subsets at baseline. HIV+ CD14+CD16- (median 56.3%, range [11.5-99.90]) vs. CD14+CD16+ (58.60, [25.20-100.00]) and HIV- CD14+CD16- (25.4, [15.70-72.80]) vs. CD14+CD16+ (55.10, [29.60-72.20]). Statistically significant differences are indicated by bars with p-values.

Baseline expression of CCR2 in the CD14+CD16+ subset of HIV+ donors was 57.96 ±17.99 and 53.24 ±10.90 in HIV- donors (p-value = 0.2492). Upon LPS stimulation expression in HIV+ donors increased slightly to 58.31 ±17.08 and in HIV- donors the expression decreased to 52.08 ±13.06. Correlations performed between the CD14+CD16+ monocyte subset's CCR2 expression and clinical markers showed significant positive correlation between CD38/8 expression and CCR2 (r -value = 0.5946, p-value < 0.005), summary of these data can be seen in Appendix 1 (**Table 27**).

4.7.4. CCR7

CCR7 is a gut-homing marker that primes monocytes to migrate towards sites of infection, particularly in the gastrointestinal tract or GALT (Saleh *et al.*, 2007). To assess whether monocytes are primed to migrate towards the GALT, CCR7 expression was investigated.

Total monocyte population

High levels of CCR7 expression were observed in both study groups (>75%). However, baseline CCR7 expression in the total monocyte population of the HIV+ group was significantly higher than in the HIV- group. Expression was 85.85% ±13.99 in the HIV+ group and 77.93% ±13.92 in the HIV- group (p-value < 0.005), see **Figure 4-30** and **Figure 3** in Appendix 1 for representative dot plots. After LPS stimulation, the expression increased in both study groups. Correlations performed between the total monocyte population's CCR7 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 28**).

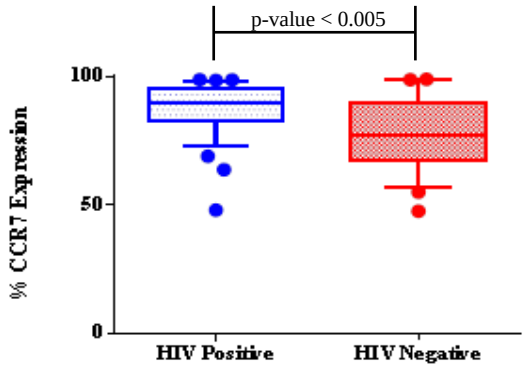

Figure 4- 30: Box-and-whisker plot showing CCR7 expression in the total monocyte population of both study groups at baseline, (median 89.6%, range [28.1-98.9]) and HIV-(77.1, [47.6-99]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

Expression of CCR7 was similar in both study groups as baseline. CCR7 expression in the HIV+ group was 77.13 ±13.64 and in the HIV- group it was 76.60 ±13.41 (p-value = 0.8815). Upon stimulation there was an increase in CCR7 expression in both study groups. Correlations performed between the CD14+CD16- monocyte subset's CCR7 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 29**).

CD14+CD16+ monocyte subset

Overall, CCR7 expression was observed in a higher percentage of CD14+CD16+ monocytes than CD14+CD16- monocytes ones. This difference was statistically significant in both the HIV+ and HIV- study groups (p-value< 0.005 for both study groups).

The mean percentages of CCR7 in both study groups were observed to be very similar at baseline. Expression in the HIV+ group was 90.40 ±9.64 and 90.45 ±8.98 in the HIV- group (p-value = 0.9827). After LPS stimulation expression increased in both study groups. Correlations performed between the CD14+CD16+ monocyte subset's CCR7 expression and clinical markers produced no significant correlation. A summary of these data can be seen in Appendix 1 (**Table 30**).

4.7.5. CCR9

CCR9 is a lymph-node homing marker that it also expressed on dendritic cells. Expression of this marker primes cells to migrate towards lymph nodes where they may be able to prime other immune cells into initiating an adaptive immune response cascade (Haas *et al*., 2011). An interesting trend was that CCR9 expression was increased in the total monocyte population and across monocyte subsets of the HIV+ group compared to the HIV- group but no statistical difference was found. Priming of monocytes, via CCR9, for migration to lymph nodes was assessed.

Total monocyte population

The mean percentage baseline expression in the HIV+ group was 6.56 ±7.93 and in the HIV- group it was 5.44 ±1.46 (p-value =0.1707). Upon stimulation the expression decreased in both groups. A summary of correlation data can be seen in Appendix 1 (**Table 31**) and **Figure 5** for representative dot plots.

CD14+CD16- monocyte subset

At baseline the expression in the HIV+ group was approximately double than that observed in the HIV- group. Expression in the HIV+ group was 7.98 ±16.70 and 3.66 ±1.56 in the HIV- group (p-value = 0.2082). After stimulation the expression decreased in both the groups. Correlation performed between the CD14+CD16- monocyte subset's CCR9 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 32**).

CD14+CD16+ monocyte subset

The CD14+CD16+ subset again displayed significantly higher expression than the CD14+CD16- subset for both study groups, p-value = 0.0073 for HIV+ group and p-value < 0.005 for HIV- group.

The baseline mean percentage in the HIV+ group was slightly higher at 16.34 (±14.36) and in the HIV- group the expression it was lower at 13.86 (±4.07) (p-value = 0.4200). After LPS stimulation there was an increase in expression across both groups. Correlation performed between the CD14+CD16+ monocyte subset's CCR9 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 33**).

4.7.6. CD62-L (L-Selectin)

CD62-L, also known as L-selection, is a homing marker that is involved in the migration of leukocytes from systemic circulation through blood vessels into sites of inflammation and infection (Ancuta *et al*., 2003). To determine if monocytes are primed to exit systemic circulation during HIV-infection CD62-L expression was investigated

Total monocyte population

At baseline CD62-L expression in the HIV+ group was almost double the expression seen in the HIV- group. Expression in the HIV+ group was 29.57 ±14.12 and 16.12 ±15.75 in the HIV- group (p-value < 0.005), see **Figure 4-32** and **Figure 4-31** for representative flow data. Mean data analysis, however, did not reflect the expression seen in the HIV- group.

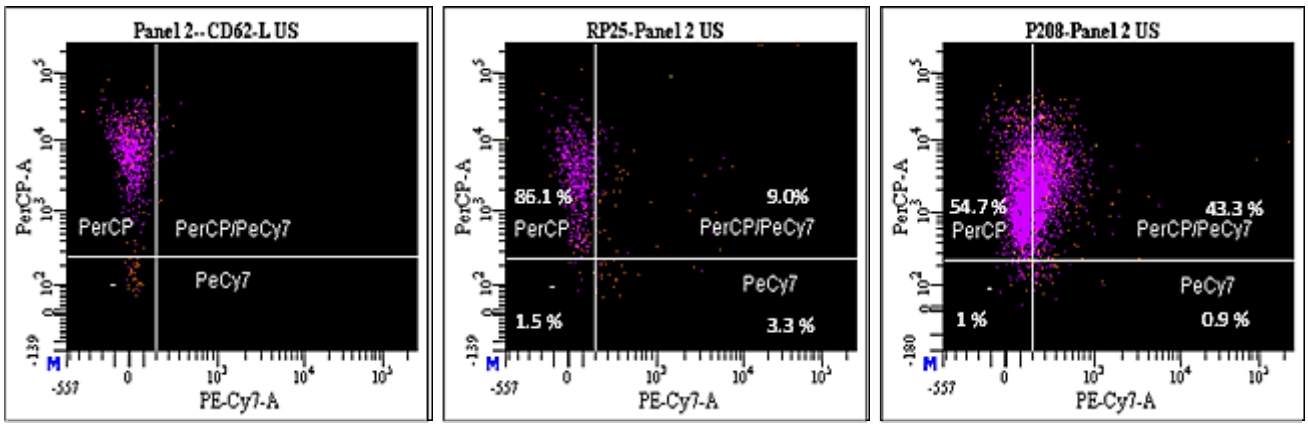

Figure 4- 31: Representative flow cytometry dot plots showing CD62-L (PE-Cy7) expression in the total monocyte population, (PerCP = HLA-DR). In the first box on the left (Panel 2 CD62-L US), the expression of CD62-L-PE-Cy7 vs. HLA-DR-PeCP is illustrated in a FMO sample excluding CD62-L-marker (negative unstained control). In the middle and right boxes, baseline expression of CD62-L- in a HIV- (RP25) vs. a HIV+ (P208) study sample is shown, respectively. Increased expression of CD62-L can be seen for P208 vs. RP25, total CD62-L positivity for RP25 is 12.30%, and for P208 it is 44.20%.

Upon LPS stimulation CD62-L expression decreased in both study groups, in the HIV+ group expression decreased to11.90 ±10.01 and in the HIV- group it expression decreased slightly to 13.90 ±11.9. The dramatic decrease in CD62-L expression in the HIV+ group was observed to be as a result of an interaction between HIV-1 infection and LPS stimulation (p-value < 0.005). Despite the decrease in mean percentages seen in the HIV- group, the median data analysis did not reflect this decrease, see **Figure 4-32**.

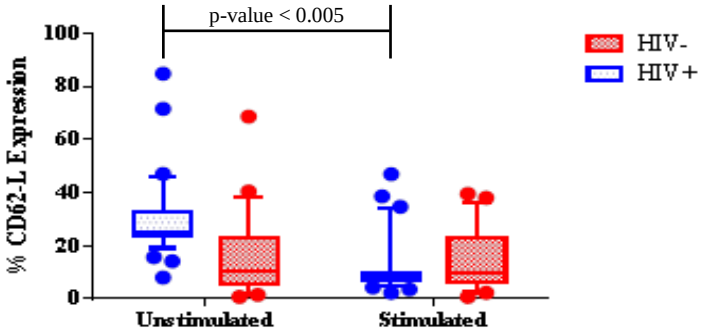

Figure 4- 32: Box-and-whisker plot showing CD62-L expression in the total monocyte populations of both study groups before stimulation, HIV+ (median 25.00%, range [8.10-84.90]) vs. HIV-(10.70, [0.90-68.70]) and after stimulation, HIV+ (8.50, [2.40-47.00]) vs. HIV-(10.00, [0.30-39.60]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

Correlations performed between the total monocyte population's CD62-L expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 34**).

CD14+CD16- monocyte subset

Expression of CD62-L was higher in the HIV+ group compared to the HIV- group. Baseline expression in the HIV+ group was 15.23 ±15.46 and 8.43 ±8.99 in the HIV- group (p-value = 0.0820). Upon stimulation the percentage expression decreased in both groups, this was caused by LPS stimulation only (p-value = 0.0073). Correlations performed between the CD14+CD16- monocyte subset's expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 35**).

CD14+CD16+ monocyte subset

As with several other markers, CD62-L expression was higher in the CD14+CD16+ subset than the CD14+CD16- subset and this difference was statistically significant in both study groups (p-value< 0.005 for both study groups).

Expression was slightly higher in the HIV+ group compared to the HIV- group. Baseline expression was 37.10 ±18.48 in the HIV+ group and 31.30 ±14.03 in the HIV- group, but not significantly (p-value = 0.2247). After LPS stimulation CD62-L expression decreased in both groups. Correlations performed between CD14+CD16+ monocyte subset's CD62-L expression and clinical markers showed no significant correlation. A summary of these data can be seen in Appendix 1 (**Table 36**).

4.8. Activation Markers

Monocyte activation status was assessed by investigating the expression of activation markers, CD69 and HLA-DR.

4.8.1. CD69

CD69 is a marker of cellular activation and expression usually increases as a cell is exposed to stimuli. CD69 expression has also well-described in T- cell activation (Funderburg & Sieg, 2012). To measure the level of monocyte activation, CD69 expression was assessed. Statistical differences were only found between the CD14+CD16+ monocyte subsets of the two study groups despite CD69 expression always being higher in the HIV+ group compared to the HIV- group.

Total monocyte population

Baseline expression of CD69 was double in the HIV+ group when compared to the HIV- group. Expression was 10.47 ± 14.20 in the HIV+ group and 5.25 ±2.26 in the HIV- group. However, the differences were not statistically significant (p-value = 0.1059). After LPS stimulation, expression was increased minimally in HIV+ donors but slightly decreased in HIV- donors. Correlations performed between the total monocyte population's CD69 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 37**).

CD14+CD16- monocyte subset

CD69 expression in the CD14+CD16- monocyte subset was observed to be very similar to general expression in monocytes. Expression was again more than double in the HIV+ group than in the HIV- group. Baseline expression of CD69 in the HIV+ group was 9.11 ±13.94 and 4.17 ±2.35 in the HIV- group (p-value =0.1189). Upon stimulation expression increased

slightly in the HIV+ group to 10.37 ±16.19 but decreased in the HIV- group to 3.11 ±1.15. This increase in the HIV+ group was as a result of HIV infection (p-value = 0.0090). Correlations performed between the CD14+CD16- monocyte subset's CD69 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 38**).

CD14+CD16+ monocyte subset

Significantly higher CD69 expression was observed in the CD14+CD16+ subset across both study groups when compared to the CD14+CD16- counterparts (p-value < 0.005 for both study groups). Expression of CD69 was more than double in the HIV+ group than in the HIV- group. Expression in the HIV+ group was 34.04 ±15.74 and 16.32 ±8.90 in the HIV- group (p-value < 0.005), see **Figure 4-33** and **Figure 4-34**for flow representation of baseline CD69 expression between the two study groups.

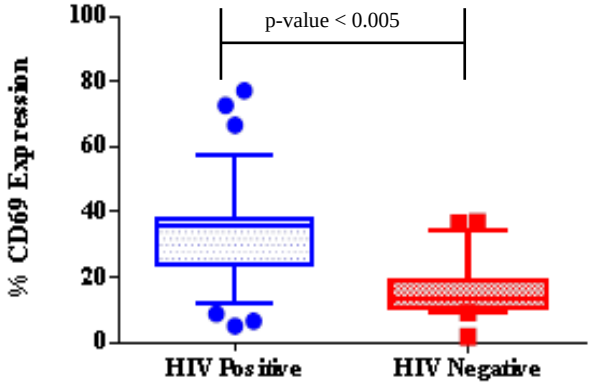

Figure 4- 33: Box-and-whisker plot showing CD69 expression in the CD14+CD16+ subset of both study groups at baseline, HIV+ (median 35.60, range [5.10-77.40]) and HIV-(13.40[1.80-37.00]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

After LPS stimulation, expression decreased in both study groups, this was due to stimulation only (p-value = 0.0164). A summary of correlation data can be seen in Appendix 1 (**Table 39**).

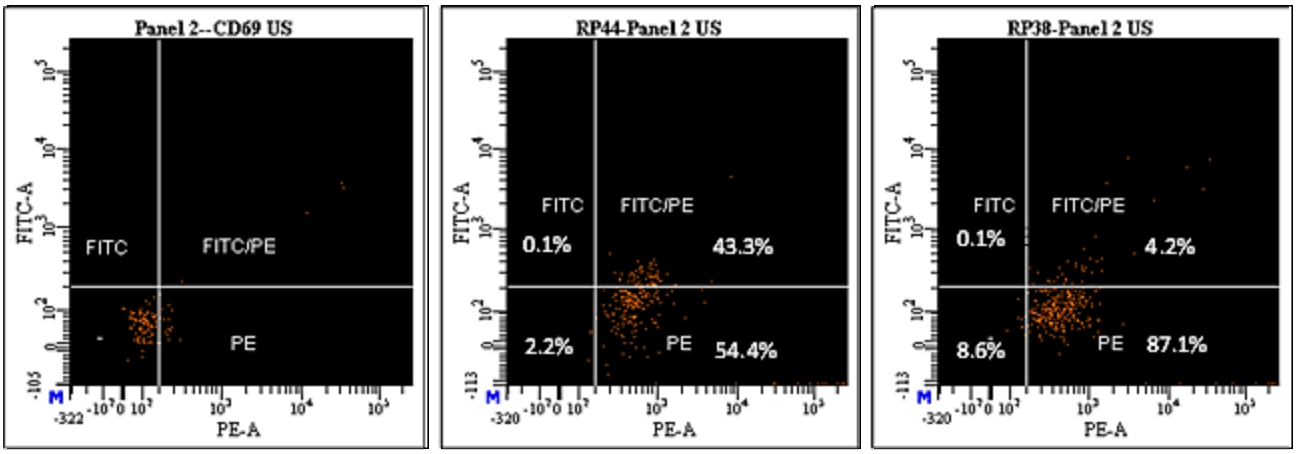

Figure 4- 34: Representative flow cytometry dot plots showing CD69 (FITC) expression in the CD14+CD16+ subset, (PE = TRAIL-R1). In the first box on the left (Panel 1 CD69 US), the expression of CD69 FITC vs. TRAIL-R1 PE is illustrated in a FMO sample excluding CD69 marker (negative unstained control). In the middle and right boxes baseline expression of CD69 in a HIV+ (RP44) vs. a HIV-(RP38) study sample is shown, respectively. Increased expression of CD69 can be seen for RP44 vs. RP38, total CD69 positivity for RP44 is 43.40%, and for RP38 it is 4.30%.

4.8.2. HLA-DR (MHC class II)

HLA-DR, a component of the MHC class II, is also a marker of activation that is constitutively expressed (usually >80% of cells) on all antigen-presenting cells, including monocytes (Cheadle *et al*., 1991). Monocyte activation defined by HLA-DR expression was assessed.

Total monocyte population

HLA-DR appeared to be constitutively expressed by both groups as expression was high. Baseline expression in the HIV+ group was 86.41 ±16.00 and 87.83 ±14.68 in the HIV- group (p-value = 0.7823), see **Figure 4-31** for representative flow dot plots. Upon stimulation, there was an increase of expression across both groups, these responses were due to stimulation (p-value = 0.0330). Correlation tests performed show a negative relationship between HLA-DR expression and CD4 count after LPS stimulation (r-value = -0.3506; p-value = 0.0168). A summary of correlation data can be seen in Appendix 1 (**Table 40**).

CD14+CD16- monocyte subset

In the CD14+CD16- monocyte subset HLA-DR was also constitutively expressed by both study groups. Expression at baseline was 87.89 ±16.62 for the HIV+ group and 87.08 ±12.27 for HIV- group (p-value = 0.8480). After stimulation, expression increased in both groups. A negative relationship between HLA-DR expression and CD4 count at baseline (r-value = -

0.3629; p-value = 0.0232). After stimulation this relationship switched to a positive relationship (r-value = 0.3325; p-value = 0.0452). A summary of correlation data can be seen in Appendix 1 (**Table 41).**

CD14+CD16+ monocyte subset

HLA-DR was also constitutively expressed in the CD14+CD16+ monocyte subset by both study groups. Interestingly, HLA-DR expression was lower on the CD14+CD16+ subset than the CD14+CD16- in the HIV+ group, but this difference was not statistically significant (p-value = 0.1103). The differences in HLA-DR expression between monocyte subsets in the HIV- group was also not statistically significant (p-value = 0.4595).

Expression of HLA-DR in the CD14+CD16+ monocyte subset of the HIV+ group was 82.02 ±15.79 and 84.20 ±14.48 in HIV- group (p-value = 0.6117). After LPS stimulation, expression increased in both groups. Correlations performed between the CD14+CD16+ monocyte subset's HLA-DR expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 42**).

4.9. Apoptotic Potential

The apoptotic potential of monocytes was assessed by investigating the expression of apoptosis-related markers, TRAIL-R1, TNR1, CD95, Bcl-2 and 7AAD/AnnexinV dual expression.

4.9.1. TRAIL-R1 (TNF-Related Apoptosis Inducing Ligand Receptor 1)

TRAIL-R1 is one of 4 different cellular receptors that binds the apoptosis-inducing ligand TRAIL and results in cells becoming sensitive to apoptosis through this pathway (Yang *et al*., 2003; Cerutti, 2009) .TRAIL-R1 expression was high in the total monocyte population and both monocyte subsets. The observed high levels of expression (higher than majority of published studies) may be due to brightness of flourochrome marker (PE), and inadequate compensation adjustment (all compensation was bead-based, not cell based). To determine monocytes sensitivity to apoptosis TRAIL-R1 expression was assessed.

Total monocyte population

TRAIL-R1 expression was higher in the HIV+ group compared to the HIV- group. At baseline expression in the HIV+ group was 74.91 ±15.96 and 56.70 ±23.26 in the HIV- group (p-value < 0.005), see **Figure 4-35** and **Figure 4-34** for representative dot plots. After LPS stimulation, expression in the HIV+ group decreased slightly to 71.13 ±14.82 and in the HIV- group increased to 67.35 ±22.90, these responses were due to stimulation (p-value < 0.005). Correlations performed between the total monocyte population's TRAIL-R1 expression and clinical markers showed no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 43**).

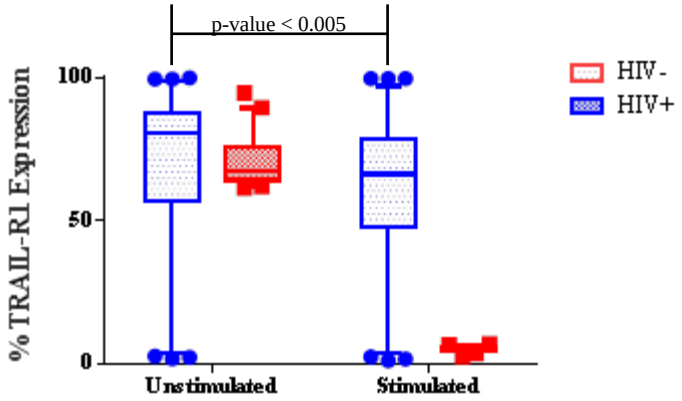

Figure 4- 35: Baseline comparison of TRAIL-R1 expression in total monocyte population in both study groups, HIV+ (median 74.70%, range [21.00-100.00]) and HIV-(58.30, [15.00-94.60]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

Expression was slightly but insignificantly lower in the HIV+ group compared to the HIV- group. Baseline expression of TRAIL-R1 was 67.42 ±33.42 in the HIV+ group and 71.21 ±9.58 in the HIV- group (p-value = 0.6194). Upon stimulation there was an abrupt decrease in TRAIL-R1 expression in the HIV- donors was observed as compared to the HIV+ donors. The moderate decrease observed in the HIV+ group appears to be caused by an interaction between HIV-infection and LPS stimulation (p-value <0.005), see **Figure 4-36**.Correlations performed between the CD14+CD16- monocyte subset's TRAIL-R1 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 44**).

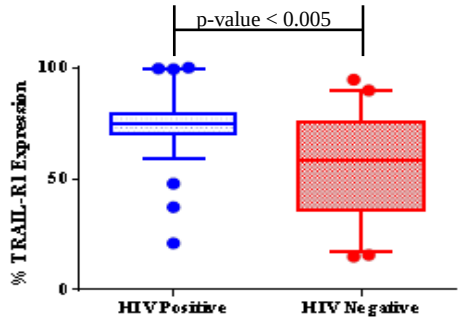

Figure 4- 36: Box-and-whisker plot showingTRAIL-R1 expression in CD14+CD16- subsets of both study groups before stimulation, HIV+ (median 80.50%, range [1.70-100.00]) vs. HIV-(67.20, [61.40-94.70]) and after stimulation, HIV+ (66.30, [1.00-99.80]) vs. HIV-(5.00, [2.40-65.60]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16+ monocyte subset

In the CD14+CD16+ monocyte subset the baseline expression of TRAIL-R1 in HIV+ donors was 71.79 ±19.51 and much lower in HIV- donors at 39.12(±34.31 (p-value <0.005), see **Figure 4-37**. Compared to CD14+CD16- monocytes the HIV+ group was increased (p-value = 4415), but the most dramatic difference was the low expression in the HIV- group (p-value< 0.005).

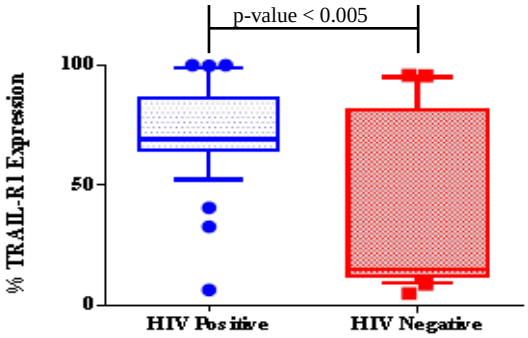

Figure 4- 37: Baseline comparison of TRAIL-R1 expression in the CD14+CD16+ subsets of both study groups, HIV+ (median 69.00%, range [6.4.00-100.00]) and HIV-(15.10, [4.90-95.80]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars withpP-values.

Upon LPS stimulation there was an increase in the TRAIL-R1 expression in HIV+ donors to 77.95 ±11.59, compared to a decrease to 37.18 ±32.67 in HIV- donors. This increase in the HIV+ group was as a result of an interaction by HIV-infection and LPS stimulation (p-value = 0.0490). Correlations performed between the CD14+CD16+ monocyte subset's TRAIL-R1 expression and clinical markers produced no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 45**).

4.9.2. TNFR1 (TNF Receptor-1)

TNFR1 (together with TNFR2) represents the cellular receptor for TNF-α. TNFR1 is primarily involved in cell survival whereas TNFR2 is associated with cell death. The signal transduction pathways of each receptor do however interlink, so this is not a hard-and-fast rule. When TNFR1 binds to its ligand, TNF-α, the cell usually becomes more resistant to the apoptosis-related functions of TNF-α (Herbein & Khan, 2008; Herbein & Varin, 2010). Resistance of monocytes to apoptosis during HIV-1 infection was investigated by assessing TNFR1 expression.

Total monocyte population

Baseline expression of TNFR1 in the HIV+ group was almost half the expression seen in the HIV- group. Expression was 2.44 ±1.43 in the HIV+ group and 4.14 ±3.14in the HIV- group (p-value = 0.0077), see **Figure 4-38** and **Figure 7** in Appendix 1 for representative dot plots. Upon stimulation there was a decrease in expression in both groups, this was due to stimulation only (p-value = 0.0139). Correlations performed between TNFR1 expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 46**).

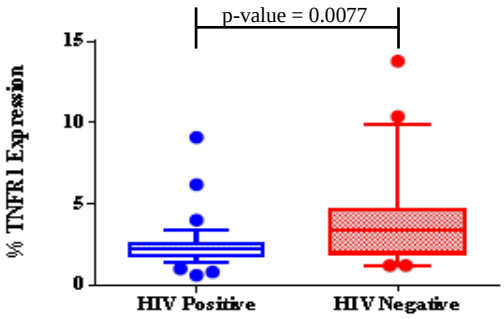

Figure 4- 38: Box-and-whisker plot showing TNFR1 expression in total monocyte populations of both study groups at baseline, HIV+ (median 2.20%, range [0.60-9.10]) and HIV-(3.40, [1.20-13.80]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

CD14+CD16- monocyte subset

The baseline expression was very similar for both study groups. Expression of TNFR1 in the HIV+ group was 1.15 ±1.12 and 1.31 ±0.66 in the HIV- group (p-value = 0.5571). Upon stimulation there was a decrease in expression in both groups. Correlations performed between TNFR1 expression and clinical markers produced a significant positive correlation

with CD4 count (r -value = 0.3789, p-value = 0.0174). A summary of these data can be seen in Appendix 1 (**Table 47**).

CD14+CD16+ monocyte subset

The CD14+CD16+ subset displayed significantly higher TNFR1 expression as compared to the CD14+CD16- subset, p-value< 0.005 for both study groups.

The trend of HIV+ infection resulting in decreased expression also applied here. In the CD14+CD16+ monocyte subset the baseline expression of TNFR1 was slightly lower in the HIV+ group at 10.19 ±3.65 compared 13.53 ±6.93 in the HIV- group (p-value = 0.0216), see **Figure 4-39**.

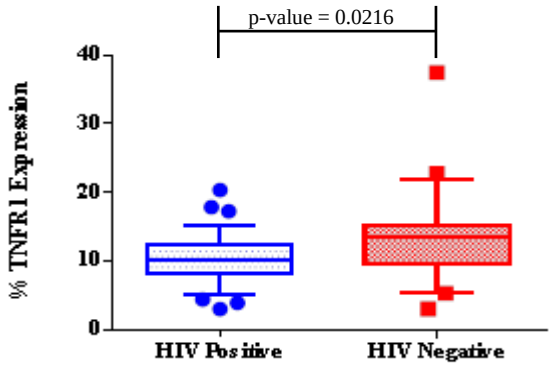

Figure 4- 39: Baseline comparison of TNFR1 expression in CD14+CD16+ subsets of both study groups, HIV+ (median 10.00%, range [10.70-20.40]) and HIV-(13.50, [3.00-32.50]). HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values

After stimulation expression increased in both groups, these increases in both groups were due to LPS stimulation (p-value = 0.0449). However, the increase in the HIV+ group was also seen to also be affected by HIV-infection (p-value = 0.0086), no interaction was seen as significant (p-value = 0.550). Correlations performed between the CD14+CD16+ monocyte subset's TNFR1 expression and clinical markers produce no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 48**).

4.9.3. CD95 (Fas) - Death marker and TNF-α (Tumor necrosis factor-alpha) - Pro-inflammatory cytokine

The expression percentages obtained for CD95 and TNF-α after flow cytometry acquisition were very high (> 90%) and were in conflict with current literature. Flow data analysis was performed when laboratory work had concluded. After reviewing the controls set for both markers during their respective FMO experiments, it was seen that whilst the negative

controls were set correctly, expression of CD95 and TNF-α seen in tubes containing the antibodies from their FMO experiments were similar to the expression patterns seen in both HIV+ and HIV- samples after acquisition, see **Figure 4-40** for CD95 and **Figure 4-41** for TNF-α, respectively.

Attempts were made to salvage the data. The titration experiments for both CD95 and TNF-α were reviewed with hopes that a more accurate expression would be seen compared to the patient samples; both markers were plotted against SSC-A to try to eliminate interference from other flourochrome markers; CD95 and TNF α were plotted against different markers from their respective panels (Panel 2 and 3, respectively), again attempting to eliminate interference from the marker they were plotted against during acquisition and analysis. Unfortunately, these attempts were unsuccessful. These errors could have occurred due to high expression of the flourochrome (BV421), it is interesting to note that both these markers make use of BV421; problematic adjusting of bead compensation to cells, especially for monocytes as a minority population; flow cytometer malfunctioning, the flow cytometer used during the early stages of this study broke and was replaced, however, the new flow cytometer also exhibited some problematic functioning; or human error. It was concluded that these results were unusable. Future studies should include detailed analysis of a few samples to check settings are correct.

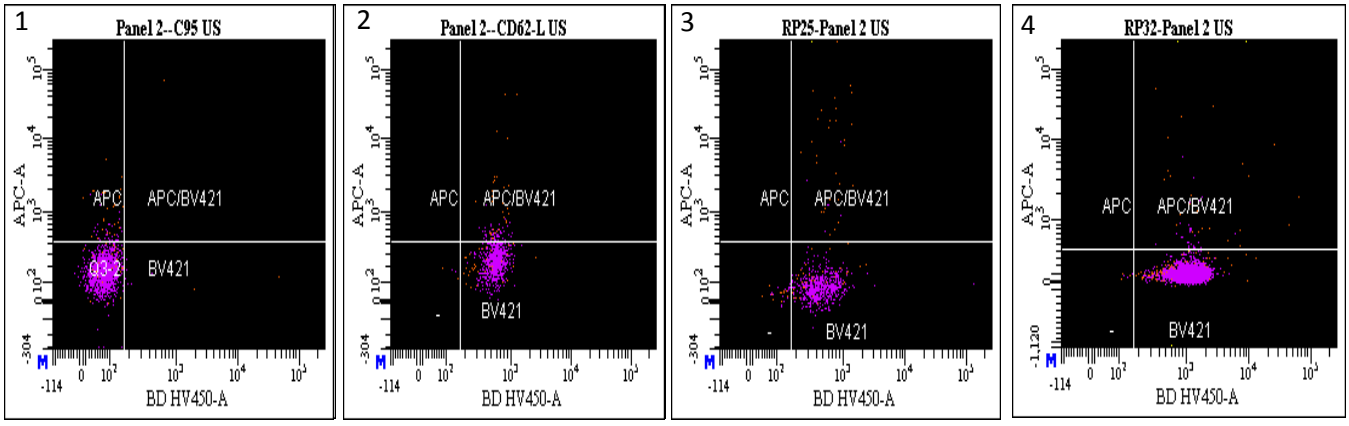

Figure 4- 40: Representative flow cytometry dot plots showing CD95-BV421 (shown here as BD HV450*) expression in the total monocyte population, (APC = TNFR1). In the first dot plot on the left (Panel 2 C95 US), the expression of CD95 BV421 vs. TNFR1-APC is illustrated in a FMO sample excluding C95 marker (negative unstained control). The second dot plot (Panel 2 CD62-L US) shows CD95 expression in CD62-L's FMO experiment. The second dot plot from the right (RP25 Panel 2 US) shows CD95 expression in a HIV- study sample and the dot plot on the far right (RP32 Panel 2 US) shows CD95 expression in HIV+ study sample (RP32). From the dot plots it can be seen that in plot 2 CD95 expression shifts completely to the right when CD95-BV421 is present in the tube. A similar pattern can be seen in both the HIV+ and HIV- study samples.*CD95-BV421 is shown as BD HV450 due to the settings of the FACS DIVA software that was used during analysis, see section 3.10. in Chapter 3 for study sample flow acquisition.

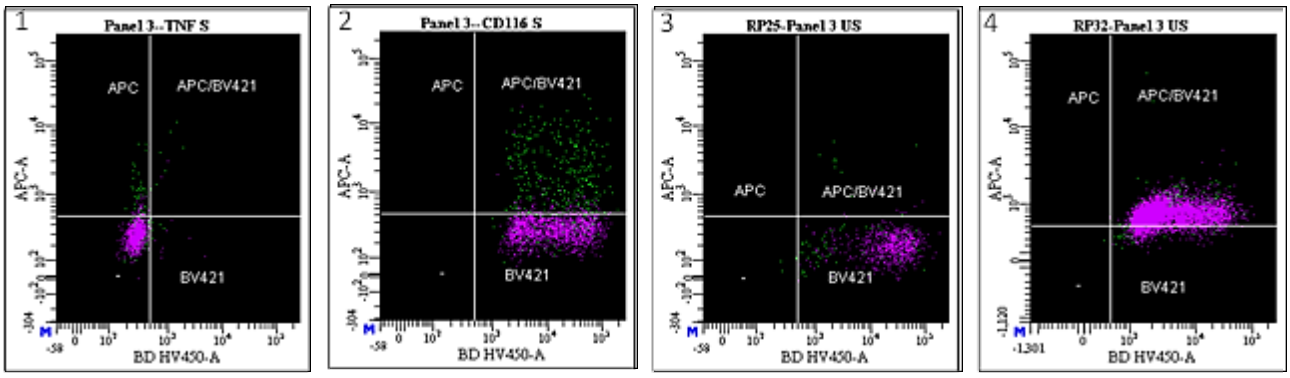

Figure 4- 41: Representative flow cytometry dot plots showing TNF-α- (BV421) expression in the total monocyte population, (APC = IL-10). In the first dot plot on the left (Panel 3 TNF-α S), the expression of TNF-α BV421 vs. IL-10-APC is illustrated in a FMO sample excluding C95 marker (negative unstained control). The second dot plot (Panel 3 CD116 S) shows TNF-α expression in CD116's FMO experiment. The second dot plot from the right (RP25 Panel 3 US) shows TNF-α expression in a HIV- study sample and the dot plot on the far right (RP32 Panel 3 US) shows TNF-α expression in HIV+ study sample (RP32). From the dot plots it can be seen that in plot 2 TNF-α expression shifts completely to the right when TNF-α -BV421 is present in the tube. A similar pattern can be seen in both the HIV+ and HIV- study samples.

4.9.4. Bcl-2- Anti-apoptotic protein

Bcl-2 is an anti-apoptotic protein that is involved in protecting cells from apoptosis, specifically associated with the intrinsic death pathway (Gautier *et al*., 2009). Bcl-2 expression assessed to determine if monocytes are resistant to apoptosis during HIV-1 infection. Although Bcl-2 expression was increased in the total monocyte population and in both monocyte subsets in the HIV+ group, no statistically significant differences were found between study groups. This could possibly be as a result of data spread, sample size, variation in study participants and outliers.

Total monocyte population

Highest expression was seen in the CD14+CD16+ subset. Baseline expression of Bcl-2 was more than twice as high in the HIV+ group compared to the HIV- group. Expression was 7.44 ±13.31 in the HIV+ donors and 3.93 ±3.17 in the HIV- donors (p-value = 0.2071). Upon stimulation there was a moderate decrease in expression for HIV+ donors to but a slight increase in HIV- donors. Correlations performed between Bcl-2 expression after LPS stimulation and clinical markers produced a significant positive correlation with CD4 count (r -value = 0.3573, p-value = 0.0256). A summary of these data can be seen in Appendix 1 (**Table 49**).

CD14+CD16- monocyte subset

In the HIV+ group baseline expression was much higher than in the HIV- group. Expression was 9.22 ±16.85 in the HIV+ group and 3.13 ±2.81 in the HIV- group (p-value = 0.0989). After LPS stimulation the expression increased in both groups. Correlations performed between the CD14+CD16- monocyte subset's Bcl-2 expression and clinical markers yielded a significant positive correlation with CD4 count (r -value = 0.3551, p-value = 0.0265). A summary of these data can be seen in Appendix 1 (**Table 50**).

CD14+CD16+ monocyte subset

Bcl-2 expression was higher in the CD14+CD16+ subset compared to the CD14+CD16- subset, these difference were statistically significant for both study groups, p-value< 0.005 for both study groups.

The baseline expression in CD14+CD16+ monocyte subset of HIV+ donors was 12.52 ±17.63 and 7.48 ±5.86 in HIV- donors (p-value = 0.1712). LPS stimulation resulted in an increase of expression across both groups. Correlations performed between Bcl-2 expression and clinical markers produced a significantly positive correlation with CD4 count (r -value = 0.4138, p-value < 0.005), summary of these data can be seen in Appendix 1 (**Table 51**).

4.9.5. 7AAD/AnnexinV dual expression

7AAD/AnnexinV dual expression is an indication of late apoptosis in cells (Lecoeur *et al*., 1997; Dong *et al*., 2009). Expression patterns for dual expression were investigated on a small subset of study samples using isolated monocytes. The percentage of monocytes undergoing late apoptosis was assessed using 7AAD/AnnexinV dual expression. Overall, it was seen that a higher percentage of monocytes in the HIV+ group were in the late stages of apoptosis compared to the HIV- group, but the differences in expression between the two study groups were not statistically significant. Possible reasons for this could be loss of monocytes during the isolation process as well as adherence of cells to test tubes during stimulation.

Total monocyte population

Baseline mean percentage expression in HIV+ donors was 1.71 ±1.01 and in HIV- donors it was 1.23 ±0.18 (p-value = 0.3987). LPS stimulation resulted in an increased expression

across both groups (p-value < 0.005). Correlations performed between the total monocyte population's 7AAD/AnnexinV expression and clinical markers yielded no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 52**).

CD14+CD16- monocyte subset

Baseline expression in the CD14+CD16- monocyte subset was higher in the total monocyte population com than in the CD14+CD16- monocyte subset. Expression in the HIV+ donors was 0.52 ±0.12 and 0.48 ±0.11 in the HIV- donors (p-value = 0.5548). Upon stimulation there was an increase in expression across both groups, these responses were due to LPS stimulation only (p-value < 0.005). A summary of correlation data can be seen in Appendix 1 (**Table 53**)

CD14+CD16+ monocyte subset

7AAD/AnnexinV dual expression was, like other markers, increased in the CD14+CD16+ subset than in the CD14+CD16- subset. The differences seen between the CD14+CD16- subset and CD14+CD16+ subset for the HIV+ group were statistically significant (p-value < 0.005), see **Figure 4-42**. Statistically significant differences between the two subsets were seen for the HIV- group as well, (p-value < 0.005**)**. 7AAD/AnnexinV dual expression in the HIV+ group was 3.94 ±0.88 and 3.96 ±0.18 in the HIV- group (p-value = 0.5221). LPS stimulation caused an increase in the both the groups 7AAD/AnnexinV expression (p-value < 0.005). Correlations performed between the CD14+CD16+ monocyte subset's 7AAD/AnnexinV expression and clinical markers produced no significant correlations. A summary of these data can be seen in Appendix 1 (**Table 54**).

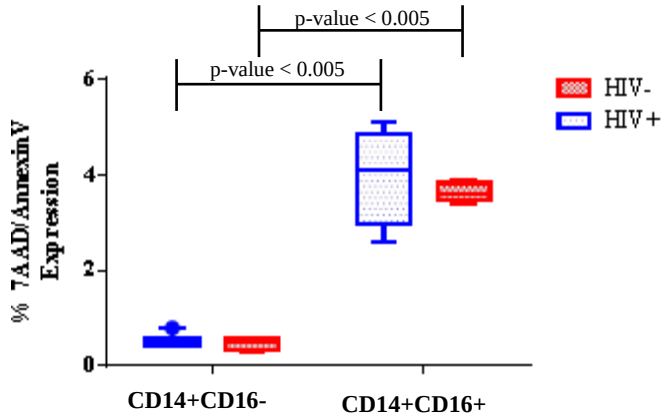

Figure 4- 42: Comparison of 7AAD/AnnexinV dual expression across study group subsets at baseline. HIV+ CD14+CD16- (median 0.50%, range [0.40-0.80]) vs. CD14+CD16+ (4.10, [2.60-5.10]) and HIV- CD14+CD16- (0.50, [0.30-0.60]) vs. CD14+CD16+ (3.60, [3.40-3.90]). Statistically significant differences are indicated by bars with p-values

4.10. Results Summary

Overall during HIV-1 infection monocytes produced higher levels of IL-10 as well as appear to be primed to exit systemic circulation and migrate towards the GALT. Monocytes are also at a higher cellular activation status during HIV-infection compared to HIV- monocytes. These trends are especially true for the CD14+CD16+ subset, which generally displayed higher levels of expression and cytokine production that the CD14+CD16- subset. This higher expression trend in the CD14+ CD16+ subset was also observed between the HIV- group's subset, despite the difference in expression between study groups. This would suggest that monocyte function is generally still intact. LPS-induced responses were similar across both study groups. Overall, monocyte phenotype was altered *in vitro* during HIV-1 infection, and *in vitro* LPS stimulation, lead to an enhancement of pre-existing differences.

Chapter 5: Discussion

The hypothesis of this study was that HIV-1 infection induces changes in the monocyte cell population that affects their phenotype and functional responsiveness to LPS stimulation. Phenotypic changes encompass changes to activation status, homing receptor expression and apoptotic sensitivity. To test the hypothesis, expression of unspecified phenotypic markers (CCR5, IL-10, CD116 & VPAC-2), homing markers (CCR1, CCR2, CX3CR1, CCR7, CCR9 & CD62-L) and activation markers (CD69 & HLA-DR), as well as markers related to apoptosis (TRAIL-R1, TNFR1, CD95, TNF-α, Bcl-2 & 7AAD/AnnexinV) were assessed, and the findings compared to the those of uninfected healthy controls. The implications of the changes observed and how they relate to current literature are discussed in the sections below. Strengths and limitations of the study follow afterwards.

The current study met the objectives and answered the research questions put forth at the beginning. Through this the study was able to achieve its aim and prove the hypothesis of the study.

HIV+ individuals have a higher immune activation status despite CD4 counts > 300cells/µl

In the current study infection with HIV-1 is associated a significant decrease in CD4 count and a higher immune activation status (measured by CD38/8 expression), phenomena well-described in numerous other studies. This was observed despite individuals having CD4 counts > 300cells/µl. HIV infection was also associated with changes to the total monocyte population (as a percentage of total blood events) and of the monocyte subsets. There appears to be disagreement in the literature with regard to the exact role of each monocyte subset. In **Chapter 2**, it was put forward that CD14+CD16+ monocytes are involved in promoting inflammation, whereas the CD14+CD16- subset are repair oriented (Kedzierska & Crowe, 2002). This model seems restrictive as others have said CD14+CD16+ monocytes are involved in repair and CD14+CD16- in priming acquired immunity through their APC function (Gautier *et al.*, 2009). Data from the current study shows that the CD14+CD16+ monocytes are expanded, activated, producing higher levels of IL-10 compared to CD14+CD16- monocytes and primed for migration during HIV-1 infection. This may be due to the HIV+ group in the current study having CD4 counts greater 300cells/µl and being

treatment naive. If CD14+CD16+ monocytes are also involved in repair, this could indicate the initiation of the fibrosis pathway at this stage as well.

CD14+CD16+ monocyte subset is increased during HIV-1 infection

During HIV-1 infection an overall increase in the circulating total monocyte population was observed, however this may reflect decreases in other blood cell types, especially $CD4^+$ T-cells. There also may be an element of production from the bone marrow but precise indicators of bone marrow emigration were not examined and therefore it is difficult make a comment with certainty. This, however, does not detract from the subsets changes, which are real as they are a component of the total monocyte population and as such subset shifts indicate real population changes.

There was an increase in the CD14+CD16+ monocyte subset and a corresponding decrease in the CD14+CD16- monocyte subset in the HIV+ group. Various studies have also observed this preferential expansion of the CD14+CD16+ monocyte subset during HIV-1 infection (Ziegler-Heitbrock *et al*., 1993; Tacke & Randolph, 2006; Ancuta *et al*., 2008; Tallone *et al*., 2011; Hijdra *et al*., 2012). Increases in the CD14+CD16+ monocyte subset are known to be accompanied by an increase in pro-inflammatory cytokines and a general shift towards M1-activated macrophages that generate a pro-inflammatory milieu in the surrounding tissues. However, in this study it was observed that the CD14+CD16+ produced higher levels of IL-10, compared to the CD14+CD16- subset. Ancuta *et al*. (2006) demonstrated that the CD14+CD16+ monocyte subset actively promotes HIV-1 replication during differentiation into macrophages and dendritic cells. CD14+CD16+ monocytes have also been suggested as a potential reservoir for HIV-1 even though a very low percentage of monocytes are thought to be infected with HIV-1 (Ancuta *et al*., 2006). However, since monocytes are known to be resistant to the cytopathic effects of HIV-1 infection, it's possible that this characteristic allows monocytes to serve as reservoir cells if they do become infected with HIV-1 (Kedzierska & Crowe, 2002).

Correlations of viral load and CD38/8 (immune activation) with CD14+CD16+ monocyte subset changes indicate that during HIV-infection an increase of the CD14+CD16+ monocyte subset is related to increases in viral replication and immune activation. Similarly related is the decrease in the CD14+CD16- monocyte subset. Furthermore, if monocytes were additionally stimulated with LPS (in this case *ex vivo*) then these relationships become more

evident. Since the CD14+CD16- monocyte subset and the CD14+CD16+ monocyte subset together constitute the total monocyte population, any increase in one subset should be mirrored by a decrease in the other. The increased CD14+CD16+ monocyte subset observed in HIV+ individuals as well as the significant relationships this subset has with well-known clinical markers highlight the importance of the CD14+CD16+ monocyte subset in HIV disease. The various functions CD14+CD16+ monocytes are suggested to perform, such as production of TNF-α and possible role as viral reservoir, furthermore emphasize the importance of this monocyte subset during HIV-1 infection.

Dramatic increase in CCR5 expression observed in HIV-infection that may be related to, or facilitated by, increased IL-10 production.

Possible increased susceptibility of monocytes to HIV-1 infection was evidenced by elevated expression of HIV co-receptor, CCR5, in HIV+ individuals. CCR5 expression was consistently elevated in the HIV+ group compared to the HIV- group. Within the HIV+ group, the CD14+CD16+ monocyte subset also expressed much higher levels of CCR5 than the CD14+CD16- monocyte subset. It has been suggested that increased CCR5 expression on monocytes/macrophages could stem from being exposed to a chronically inflammatory environment as a result of increased plasma LPS levels, known to occur during HIV-1 infection as a result of gut leakage (Chang & Altfeld, 2009; Munsaka *et al*., 2009; Sandler & Douek, 2012). In contrast to this, Sozzani *et al*. (1997) suggests that IL-10 production by monocytes may maintain CCR5 expression in mucosal tissues and that this could enhance the dominant role of this co-receptor in R5-tropic HIV infection. Despite the multitude of possible reasons behind increased CCR5 expression, it is known that increased expression may eventually result, and has been suggested to facilitate, infection of these cells with R5-tropic virus (Naif *et al*., 1998; Munsaka *et al*., 2009; Herbein & Varin, 2010). After *ex vivo* stimulation with LPS, CCR5 expression in the total monocyte population decreased dramatically compared to the HIV uninfected group. This decrease could be attributed to an interaction between HIV-1 infection and LPS stimulation. A minimal decrease of CCR5 expression was also observed in the CD14+CD16+ monocyte subset after stimulation. However, in the CD14+CD16- monocyte subset CCR5 expression increased, albeit minimally, after stimulation. Franchin *et al*. (2000) also reported a down-regulation of CCR5 after LPS stimulation, which the authors indicate to be a sign of resistance to infection. This

is in contrast to a previously mentioned study, which suggests increased plasma LPS levels result in increased CCR5 expression (Munsaka *et al*., 2009).

It is very interesting to note that together with high levels of CCR5 expression, IL-10 production was also increased in the total monocyte population and monocyte subsets in the HIV+ group compared to the HIV- group. Again, the CD14+CD16+ monocyte subset was seen to express higher levels of IL-10 than the CD14+CD16- monocyte subset. Live virus and viral glycoproteins such as HIV-1 Tat, have been shown to induce IL-10 production in monocytes, which together with macrophages have been shown to be the main producers of IL-10 (Redpath *et al*., 2001; Coleman & Wu, 2009). LPS, however, has also been implicated as an inducer of IL-10 production (Mocellin *et al*., 2004). After *ex vivo* LPS stimulation, IL-10 expression increased in the total monocyte population and CD14+CD16+ monocyte subset whilst expression decreased minimally in the CD14+CD16- monocyte subset of the HIV+ group. The decrease in expression observed in the CD14+CD16- subset could be due to a loss of cells (resulting from adherence to tubes and/or apoptosis) or active down-regulation of production in this subset, which may suggest that the CD14+CD16+ subset is the primary producer of IL-10. IL-10 production has also been suggested to increase levels of soluble sCD14 and high levels of sCD14 have been associated with reduced responses of monocytes to LPS (Redpath *et al*., 2001; Coleman & Wu, 2009).

Herbein & Varin (2010) proposed a model for the HIV-1 viral life cycle where they expanded the classification of macrophages to include not only macrophage activation type, M1 (Classical/Inflammatory tissue), and M2 (Alternative/repair), but also a new macrophage activation type, which they termed, “deactivated macrophage”. The authors suggest that when in the deactivated macrophage state viral entry is increased in the presence of high CCR5 and IL-10 expression. It is interesting to note, that Sozzani *et al*. (1997) have shown that monocytes are most readily infected with HIV BaL, a R5 viral strain, following stimulation with IL-10 and CCR5 expression was also seen to be increased. The authors associated this susceptibility to infection with the increase in CCR5 expression and suggest that infection by HIV BaL was facilitated by the increased CCR5 expression and resulted in increased viral entry. These authors also proposed that increased CCR5 expression results from an elevated IL-10 expression. Coleman & Wu (2009) proposed that secretion of IL-10 by monocytes and IL-10’s immuno-regulatory properties may result in inadequate responses of monocytes to HIV-1 infection and LPS stimulation and could facilitate progression to AIDS. The proposed ability of opportunistic infections, like CMV, to utilize induced IL-10 production to delay

immune responses while it establishes an infection supports the hypothesis suggested by Coleman & Wu (Redpath *et al*., 2001). Although Herbein & Varin (2010) discuss activation states of macrophages, evidence from Sozzani (1997) and Coleman & Wu (2009) suggest that a similar "deactivated" state, which facilitates viral entry, could be a present in monocytes as well. IL-10 however has many effects and may inhibit HIV replication depending on the pro-inflammatory cytokines it interacts with, such as TNF-α and IL-6 (Sozzani *et al*., 1997; Verani *et al*., 1997).

From correlation tests performed, no relationship between the increased CCR5 expression and IL-10 production in monocytes during HIV-infection was seen.

Monocytes are primed to exit circulation and migrate towards sites of infection

An increased migration profile was observed in monocytes in HIV+ individuals as evidenced by up-regulation of several migration-associated markers. These include CCR2, CCR1 and CD62-L; and also the gut- and lymph node-homing markers, CCR7 and CCR9. This suggests monocytes are primed to exit circulation and travel towards sites of inflammation and infection. Monocytes may migrate to the GALT, where due gut leakage, microbial translocation occurs, contributing to immune activation through the release of cytokines and recruitment of various immune cells. Alternatively, monocytes could migrate towards lymph nodes where they may prime naïve T-cells (as monocyte-derived DCs) or memory or effector (as macrophages) through antigen presentation activity, thereby promoting the adaptive immune response.

Migration out of systemic circulation to lymph nodes, the GIT and sites of infection/inflamed tissues can be facilitated through the chemokine receptors CCR2 and CX3CR1 which respond to their respective ligands, CCL2(MCP-1) and fractalkine, to assist in endothelial migration (Iijima *et al*., 2011). CX3CR1 and CCR2 are thought to have an inverse expression relationship, meaning that when CCR2 expression is high, CX3CR1 expression is low and vice versa. The CD14+CD16- monocyte subset is thought to express high levels of CCR2 and low levels of CX3CR1, whilst in the CD14+CD16+ monocyte subset it's thought that the opposite is observed (Ancuta *et al*., 2003). Our results show that in the CD14+CD16- monocyte subset of both study groups, the $CCR2^{high}/CX3CR1^{low}$ expression trend was observed *in vivo* but the expected $CCR2^{low}/CX3CR1^{high}$ pattern in the CD14+CD16+

monocyte subset was not. Instead a $CCR2^{high}/CX3CR1^{low}$ expression trend was seen the CD14+CD16+ monocyte subset *in vivo* and CCR2 expression was higher in the CD14+CD16+ subset compared to the CD14+CD16- subset of both study groups. But despite CX3CR1 expression being lower than CCR2 expression, CX3CR1 was still elevated in the CD14+CD16+ monocyte subset compared to the CD14+CD16- monocyte subset in both study groups. These results challenge the notion that only the CD14+CD16- monocyte subset displays a $CCR2^{high}/CX3CR1^{low}$ expression trend, suggesting that expression of both these markers need to be investigated further. Correlation tests suggest that during HIV-infection a high CD38/8 expression results in an increase in CCR2 expression in the CD14+CD16+ monocyte subset and a decrease in CCR9 expression in the total monocyte population. This could explain why an unusually increased expression of CCR2 was seen in the CD14+CD16+ subset. Statistical correlations also showed that a higher viral load may lead the decreased CX3CR1 expression seen in the CD14+CD16- monocyte subset of HIV-1 infected individuals, which could supports the expression trend suggested by Ancuta *et al*. (2003).

A review by Kedzierska & Crowe (2002), stated that monocytes of HIV-infected individuals experience decreased chemotactic ability as a result of a down-regulation of CCR2 and that they display defective migratory responses to chemotactic stimuli. This down-regulation of CCR2 was not observed during the current study; rather an increased expression of CCR2 in the total monocyte and both monocyte subsets was observed. It should be noted that the down-regulation of CCR2 was reported in AIDS patients, this is a stark comparison with this study's participants who had CD4 counts >300cells/µl. The increased CCR2 expression reported in this study could indicate a heightened sensitivity of monocytes in HIV-1 infection to CCL2 and therefore a potentially enhanced chemotactic ability compared to monocytes in uninfected individuals. Furthermore, the increased CCR2 expression in the CD14+CD16+ monocyte subset could indicate a preferential priming of CD14+CD16+ monocytes over the CD14+CD16- monocyte subset to exit circulation in response to elevated CCL2 levels (rather than increased fractalkine levels). However, comment cannot be made whether migratory responses were affected as only phenotypic characterization was studied. A study by Williams *et al*. (2013) also showed an up-regulation of CCR2 but in HIV-infected persons with neurocognitive impairment. The authors state that increased transmigration of HIV-infected monocytes across the blood-brain-barrier was seen in the presence of high levels of CCL2 and speculate that this was in part due to the elevated levels of CCR2 and enhanced sensitivity to CCL2 compared to uninfected individuals. Furthermore, Ancuta *et al*. (2003)

suggests the expansion of the CD14+CD16+ monocyte subset during HIV-infection and its migration to tissues facilitates the dissemination of HIV-1, if these cells are infected, into the lymph nodes, brain and intestine and may therefore promote infection of $CD4^+$ T-cells. It is also known that CCR2 is required for the migration of monocytes from the bone marrow (Iijima *et al*., 2011). This suggests that the increase in the monocyte population seen during HIV, mentioned earlier, could result from a higher migration of monocytes from the bone marrow.

The generalized pattern of an increased migration profile is further supported by the increased CD62-L expression, a marker whose role in the recruitment of leukocytes has been well-documented. A study by Hayes *et al*. (1999) that investigated leukocyte adhesion molecule profiles in HIV-1 infection showed also showed a decreased CD62-L expression in HIV-1 infected individuals with CD4 counts ≥ 200cells/µl compared uninfected controls. However, this study measured mean fluorescence intensity (MFI), whilst the current study measured mean percentage expression. Furthermore, eventhough participants were also treatment naive blood was collected in potassium Heparin tubes for the Hayes *et al*. (1999) study, which could account for differences seen. This is in contrast to the current study's findings of an elevated CD62-L expression in HIV-1 individuals in the total monocyte population and both monocyte subsets. Furthermore, Ancuta *et al*. (2003) suggested that CD62-L expression in the CD14+CD16+ monocyte subset is lower than in the CD14+CD16- monocyte subset. However in the current study an increased expression of CD62-L in the CD14+CD16+ monocyte subset compared to the CD14+CD16- monocyte subset in both study groups was seen.

The chemokine receptor CCR1 is known to also be involved in the recruitment of monocytes to and movement within inflamed tissues and mediates the arrest of monocytes in shear flow. In addition to this, CCR1 has been shown together with CCR5 to facilitate the movement of monocytes towards CCL5 via trans-endothelial migration in mice models (Shi & Pamer, 2011). Furthermore, these receptors are known to respond to MCP-1α (Kaufmann *et al*., 2001). Sozzani *et al*. (1997), showed enhanced chemotactic ability of monocytes stimulated with IL-10 compared to control cells. The authors also showed that this increased chemotactic ability was possibly due to the ability of IL-10 to up-regulate CCR1 and CCR2 receptors. In the current study an increased expression of CCR1 in the total monocyte population and in both monocyte subsets was observed compared to the HIV- group. Within the HIV+ group, the CD14+CD16+ monocyte subset was observed to express higher CCR1

levels compared to the CD14+CD16- monocyte subset. These results, in addition to the increased CCR5 expression seen and the increased IL-10 expression, and increased CCR2 expression indicate that monocytes and specifically the CD14+CD16+ monocyte subset, are strongly primed to exit the circulation.

Increased expression of CCR7 and CCR9 was seen in the total monocyte population and both monocyte subsets in the HIV+ group compared to the HIV- group. In the HIV+ group the CD14+CD16+ monocyte subset showed higher expression of both CCR7 and CCR9 than the CD14+CD16- monocyte subset. CCR7 and CCR9 are homing markers which facilitate the movement of monocytes to secondary lymphoid tissues, including GALT and lymph nodes (Scandella *et al*., 2002; Linton *et al*. 2012). Damås *et al*. (2009) showed that CCR7 ligands, CCL19 & CCL21, have been linked to viral persistence and inflammation. The authors concluded that the CCL19/CCL21/CCR7 system may be in a pathogenic loop with HIV and may play a role during infection by contributing to abnormal inflammation. These results were observed in both treated and untreated HIV-infected individuals. So although CCR7 works with its homeostatic ligands to regulate lymphocyte migration to lymph nodes it may also play a role in persistent HIV infection by promoting viral persistence through inducing replication in activated T-cells and facilitating infection of resting $CD4^+$ T-cells as well as establishing latency (Saleh *et al*., 2007). This together with the current study's results could signify that monocytes and in particular, CD14+CD16+ monocytes are primed to migrate towards secondary lymph nodes, such as the GALT, using CCR7 ligands as opposed to CCR9 ligands. However, this can be explained by the negative correlation found between CCR9 expression in the total monocyte population and CD38/8 expression *ex vivo*, which suggests a decrease in expression CCR9 as immune activation increases, making monocytes less sensitive/responsive to CCR9 ligands.

Ex vivo LPS stimulation, as stated in the begging of this chapter, suggests that HIV-1 infection does not alter the expression of migration-associated markers of monocytes with respect to CCR1 and CD62-L. Expression of CX3CR1, CCR2 and CCR9 in the CD14+CD16+ monocyte subset appeared to be conserved and even enhanced during HIV-infection, since CX3CR1, CCR2 and CCR9 expression decreased in the total monocyte population and CD14+CD16- monocyte subset after LPS stimulation. The same expression patterns were seen in the HIV- group, with the exception of CCR2 expression, where the CD14+CD16+ monocyte subset CCR2 expression also decreased after LPS stimulation. It is

interesting to note that CCR7 expression was the only migration marker to have increased after LPS stimulation in both study groups.

These results, together with the aforementioned functions of the respective migration and gut-homing markers suggest that during HIV-1 infection (and with continual LPS stimulation), monocytes and specifically CD14+CD16+ monocytes could be primed to exit circulation and migrate towards the gut. As stated in Chapter 2 a study done in mice by Gautier *et al*. (2009) showed the behaviour of the CD14+CD16+ monocyte subset is context dependent, therefore more studies are needed to fully elucidate the role that the CD14+CD16+ monocytes subset plays during HIV-1 infection in particular.

Monocytes have a higher activation status during HIV-1 infection

CD69 expression was increased in the total monocyte population and in both monocyte subsets compared to HIV uninfected individuals *in vivo*. A statistically significant difference in CD69 expression in the CD14+CD16+ monocyte subset of HIV-infected individuals was observed. It has previously been shown that CD69 expression is increased in monocytes during HIV-1 infection (Funderburg & Sieg, 2012). This trend towards an increased CD69 expression indicates that monocytes of HIV-infected individuals are at a higher activation state than monocytes in HIV- individuals. This higher state of activation may be facilitated through increased plasma LPS levels. A role for elevated LPS levels in driving monocyte activation in disease progression has been suggested by Ancuta *et al*. (2003), and is now generally accepted.

The suggestion that monocytes have a higher activation status during HIV-1 infection is further supported but the high HLA-DR expression seen at baseline. HLA-DR was, however, constitutively expressed in both study groups (> 80% expression), although in the total monocyte population and CD14+CD16+ monocyte subset expression was marginally lower. There are however conflicting reports with regards to its expression on monocytes in HIV-1 infection (Lochner *et al*., 1994). The expression of HLA-DR is thought to be important for the recognition of foreign antigen. T-helper cells are dependent on surface expression of both HLA-DR and foreign antigen for induction of a response and proliferation (Cheadle *et al*., 1991). This, together with the HLA-DR expression observed in this study, would indicate that the ability of monocytes in HIV-infected individuals to stimulate an adaptive immune response and recognize foreign antigen is as good as in monocytes from healthy individuals.

Ideally, MFI values should have been compared; this however, depends on meticulous cross-experimental standardization that was not implemented during the current study. This could be investigated in future studies.

Expression of HLA-DR was similar in the HIV+ group and HIV- group after stimulation *in vitro*. CD69 expression on the other hand increased in the HIV+ group's total monocyte population and CD14+CD16- monocyte subset after stimulation. Expression appeared to decrease in the CD14+CD16+ monocyte subset of the HIV+ group and in both the monocyte subsets and the total monocyte population of the HIV- group. This loss in CD69 expression could be explained by adherence of monocytes to test tubes during stimulation and or apoptosis, as most activated cells may be primed to mature in macrophages or to die. The increased HLA-DR expression, together with increased CD69 expression in the total monocyte and CD14+CD16- monocyte subset suggests that stimulation with LPS could result in an activated cellular status during HIV-1 infection. This would support the hypothesis that microbial translocation and microbial products are major contributors to immune activation as stated by Brenchley *et al*. (2006).

Based on statistical correlations, HLA-DR expression increases in both the total monocyte population and CD14+CD16- monocyte subset as CD4 count decreases, after LPS stimulation and at baseline respectively. After stimulation the negative relationship in the CD14+CD16- subset switched to a positive relationship. The negative relationship between the total monocyte population and CD4 count after LPS, suggests a stimulation of antigen presenting function during HIV-1 infection The switch to a positive relationship with CD4 count in the CD14+CD16- monocyte subset could indicate a desensitization of this monocyte to stimuli as HIV-1 infection progresses and CD4 T-cell numbers become more depleted without the intervention of ART.

Changes in apoptosis-associated markers in HIV-infection

Both activated monocytes and other cells, such as T-cells, express and secrete TRAIL, the ligand for TRAIL-R1, under pro-CD14+CD16+ conditions. The amount of soluble TRAIL has been seen to increase during HIV-1 infection (Yang *et al*., 2003; Cerutti, 2009). Whilst TRAIL secretion was not measured in this current study per se, a high level of activation in monocytes (increased CD69 and HLA-DR), and also generalized activation (as evidenced by CD38 on $CD8^+$ T-cells) was observed. Together with this, increased TRAIL -R1 expression

in the HIV+ group's total monocyte population and the CD14+CD16+ monocyte subset was also observed. TRAIL-R1, however, was decreased in the CD14+CD16- monocyte subset.

Wei *et al*. (2010) have shown that despite high levels of TRAIL-R1 expression, monocytes displayed negligible sensitivity to TRAIL cytotoxity and that TRAIL does not induce death (through caspase activation) but rather monocyte migration. The authors also suggested that TRAIL exerts this non-apoptotic function using an alternative pathway, namely the "PI3K-RhoGTPase" pathway, see **Figure 5-1**. These findings were supported by Cummins & Badley (2009). However, a study by Huang *et al*. (2006), using recombinant human TRAIL (rhTRAIL), showed that rhTRAIL induced apoptosis in monocyte-derived-macrophages (MDM) and inhibited viral replication of HIV-1 infection, whilst rhTRAIL has little to no effect on uninfected MDM. They also suggested that the viral inhibition seen was mediated through inhibition of the AKT-1 pathway. Whilst, Secchiero *et al*. (2002), on the other hand, showed that TRAIL-R1 mediates monocyte maturation.

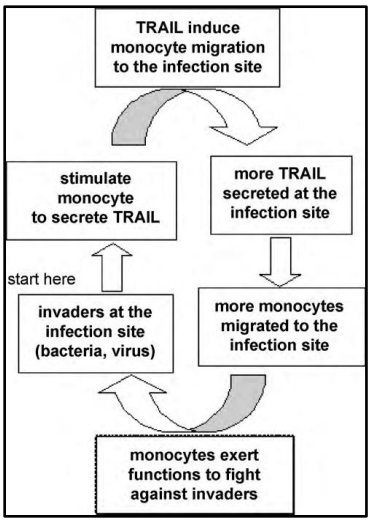

Figure 5- 1: Hypothesis of the positive feedback loop for monocytes to eliminate foreign invaders suggested by Wei *et al*., 2010. After infection by foreign invaders such as bacteria or virus, the F peptide and N36 peptide (from HIV) and LPS (from bacteria) could stimulate monocytes to secrete TRAIL. TRAIL would induce monocyte migration toward the infection site, resulting in increased TRAIL secretion by the recruited monocytes that would in turn increase the accumulation of monocytes at the infection site, where monocytes can eliminate foreign invaders (Wei *et al*., 2010).

LPS has been shown to increase TRAIL secretion by monocytes (Halaas *et al*., 2000). A possible preservation, or in the CD14+CD16+ monocytes case an enhancement, of TRAIL-R1 expression was observed. This suggests an increased migratory potential of monocytes, facilitated through TRAIL-R1, to sites of infection where monocytes may recruit more immune cells which HIV-1 could infect. Another role could be to not inhibit the

differentiation, possibly mediated by TRAIL-R1, of monocytes into macrophages which are more susceptible to HIV-1 infection.

HIV Tat has been shown to increase Bcl-2 expression which has been shown to inhibit apoptosis by TRAIL (Coleman & Wu, 2009; Cummins & Badley, 2009). Through an *in vitro* model, Coleman & Wu (2009) showed HIV-1 *Tat* has been shown to use TRAIL to promote monocyte survival in situations where apoptosis would normally occur. In the current study, Bcl-2 expression in HIV-1 infected individuals showed a trend towards up-regulation in both monocyte subsets and the total monocyte population compared to uninfected individuals (although not attaining statistical significance). It is interesting to note that Bcl-2 expression was also higher in the CD14+CD16+ subset compared to the CD14+CD16- monocyte subset in HIV+ individuals (but again not statistically significant, possibly due to sample size). These results are however noteworthy as they suggest a possible priming of monocytes towards survival in HIV-1 infection. This, in addition the increased CCR5 and IL-10 expression, could mean that HIV-1 infection could be inducing a prolonged life-span of monocytes during infection, which could enhance viral replication, and could suggest a possible role as a viral reservoir cell. Prolonged survival would also implicate these cells in promoting ongoing inflammation in this disease.

HIV-1 Tat protein also been shown to target the TNFR signaling pathway by modifying gene expression, which leads to immune suppression and the formation of viral reservoirs. Furthermore, HIV viral proteins have also been shown to mimic TNFR signals and in this way circumvent the induction of apoptosis (Herbein & Khan, 2008). In addition, TNFR1 has also been implicated in HIV viral replication which is facilitated by TNF-α (Herbein & Varin, 2010). TNFR1 expression was lower in the total monocyte population and in both monocyte subsets in HIV+ individuals. From the suggested role TNFR signal may play in resistance to apoptosis, the decreased expression could indicate an increased sensitivity to apoptosis and inhibition of viral replication mediated by the TNF-α mediated pathway. Further evidence for this suggested susceptibility to apoptosis during HIV-infection is the *in vivo* increase, albeit minimal, in expression of late apoptosis marker, 7AAD/AnnexinV, in both monocyte subsets and the total population in the HIV+ group compared to the HIV- group. After LPS stimulation expression TNFR1 increased moderately in both study groups. This indicated monocytes in HIV- individuals do not undergo apoptosis more readily than monocytes in HIV+ individuals would. Therefore, LPS stimulation *in vivo* may only be responsible for monocytes undergoing early apoptosis during HIV-infection. However, apoptosis was not

induced in the current which prevented investigation of apoptosis susceptibility in this way. This would clarify the mechanisms in play that affect apoptotic susceptibility in monocytes during HIV-infection.

CD116 was expressed at a high level on all monocytes. CD116 is used as a monocyte marker, and differences in the high level expression would have better been assessed using mean fluorescence intensity data (see above discussion on HLA-DR). CD116 has multiple effects on monocytes including differentiation into macrophages, maturation and is prolonging monocyte survival. CD116 is also known to increase monocyte response to anti-inflammatory mediators (Bagasra *et al*., 1992; Jung *et al*., 1995; Allavena *et al*., 1998; Kedzierska *et al*., 1998). It has been suggested that CD116's effect on monocyte differentiation may increase susceptibility of monocytes to HIV infection (Kedzierska *et al*., 1998). A review by Kedzierska *et al*. (1998) shows that there have been conflicting reports on the effect CD116 has during HIV-1 infection with some studies showing that it plays a role in increasing HIV replication, whilst others show it inhibits replication and some showing that it has no effect at all on replication. Although an increased CD116 expression in HIV+ individuals in this study, comment cannot be made on whether the increased CD116 expression observed has any effect on HIV-1 viral replication as only phenotypic characterization was performed. However, it is interesting that most published data suggests that CD116 does indeed increase HIV replication and it only has beneficial effects *in vivo* when patients were on anti-retroviral therapy (Pomerantz *et al*., 1990; Kedzierska *et al*., 1998). The increased CD116 expression observed in the HIV+ group, and specifically the CD14+CD16+ monocyte subset, could infer an increased resistance to apoptosis or maturation of the monocyte population, as a result of the multiple effects CD116 has on monocytes. Furthermore, stimulation with LPS could increase survival and maturation of monocyte subsets during HIV infection and this may result in the enhancement of HIV replication. Expression of CD116 increased in both the total monocyte population and the monocyte subsets in the HIV+ after LPS stimulation. Interestingly in the HIV- group, expression decreased after LPS stimulation. This is in contrast to the TNFR1 findings from the current study and thus more studies have to be conducted to elucidate the exact role of CD116 expression in monocytes.

VPAC-2 expression in monocytes and its potential as a therapeutic target.

Vaso-active intestinal peptide (VIP), the ligand of VPAC-2, is known to inhibit LPS-induced inflammatory pathways. VPAC-2 has been noted for its immune-regulatory role (neutralizing pro-inflammatory stimuli, inhibiting apoptosis) and is known to inhibit viral replication (Smalley *et al*., 2009). Temerozo *et al*. (2013) also showed that VIP promotes inhibition of HIV-1 viral replication through VPAC-2 rather than VPAC-1 and that VPAC-2 can be classified as an inhibition mediator, whilst VPAC-1 is an HIV production inducer. Bokaei *et al*. (2007) also reported that stimulation of VPAC-2 inhibits HIV-integration into host DNA and confers a strong resistance to infection. In this study VPAC-2 expression was investigated, as a first stage in investigating its usefulness as a putative target for therapeutic intervention – to down-regulate the pro-inflammatory functions of monocytes. An increased VPAC-2 expression in the total monocyte population and in both monocyte subsets of the HIV+ group compared to the HIV- group. Furthermore, the CD14+CD16- monocyte subset expressed lower levels than the CD14+CD16+ monocyte subset in the HIV+ group. The high expression of VPAC-2 in the HIV+ group and specifically by the CD14+CD16+ monocyte subset suggests an increased sensitivity to the effects of VIP and therefore its immune-regulatory effects. Moreover, increased expression of VPAC-2 was observed in the HIV+ group after stimulation. The increased expression observed both *in vivo* and *ex vivo* after stimulation, indicates that thesis receptor may be exploitable in a VIP-based immunotherapeutic setting. In particular, the CD14+CD16+ monocyte subset may represent a useful therapeutic target cell for investigation of the impact of the immune modulating effect of VIP during HIV-1 infection and its associated chronic immune activation. The positive correlation that VPAC-2 expression exhibits with CD4 count in the total monocyte population and both monocyte subsets after LPS stimulation suggests that immune-modulation via VPAC-2 with VIP may need to be administered with ART to ensure CD4 count is normalized and therefore VPAC-2 expression higher. Further work need to be performed on this marker, and a detailed assessment of its relationship to other monocyte markers of activation and to monocyte function needs investigation.

Strengths and limitations of the study

The current study had a number of limiting factors such as sample size, which largely affected statistical significance of results. This would have been potentially eliminated with a

larger number of study participants. The size of the study cohort was primarily restricted due to the fact that the majority of patients only present at clinics during advanced stages of HIV-infection, when their CD4 counts have dropped below the requirements of the study. Also, study participants were also lost due to failure to report for blood draw visits. The study design was cross-sectional due the difficulties in study participant recruitment mentioned would have meant that patient follow-up and adherence to the study would have been difficult, in addition to this time was also a constraint for the purposes of a Master's degree study. The current study was not able to investigate the changes of markers of interest over time to see fully clarify how they correlate to clinical markers. Standardization procedures for flow cytometry had to be repeated several times to major instrument modifications during the study. Whilst integrity of most markers was possible, inclusion of data on two markers originally included in the study design (TNF-α and CD95) was not possible. Post-acquisition analysis revealed that these two markers had generated spurious data to the extremely bright signal generated by positive-staining cells. Future studies should include both cell compensation procedures and direct post-acquisition analysis for all markers and panels. Finally, whole blood was also stored overnight for monocyte isolation in the morning which could have impacted responsiveness of monocytes to LPS in the purified monocyte experiments.

Despite these short-comings, the current study is the first to investigate the expression of migration markers, gut-homing markers, phenotypic markers and activation markers simultaneously in monocytes in South Africa. A multi-colour (8 Colour) flow cytometry was used, which allowed a large range of markers to be investigated. Expression of these markers was correlated to well-known clinical markers in an effort to highlight the importance of specific markers of interest. In addition to this, functioning of monocytes during HIV-infection using LPS stimulation was also assessed. Overall this study's findings highlight the important role that monocytes and specifically, CD14+CD16+ monocytes, play during HIV-infection, furthermore the potential therapeutic use of the CD14+CD16+ subset emphasizes the need to perform more research on these cells in future.

Chapter 6: Conclusion

Monocytes are integral members of the innate immune system and their functioning has direct effects on several other immune cell role players, including those within other branches of the immune system. Monocytes are one of the first cell types recruited to sites of inflammation, where they become major contributors to the inflammatory process and initiation of innate immunity. Monocytes are also important antigen presenting cells. Their ability to migrate from the blood to sites where they are needed is essential to their function. The recruitment of monocytes to sites of inflammation and infection is crucial for pathogen clearance, but it may also add to the damage associated with inflammation. Monocytes and macrophages are prime role players in several inflammation-associated degenerative diseases. HIV-1 infection, a chronic non-resolving viral infection, is thought to have a significant impact on the functioning of monocytes. The conflicting nature of many reports on monocyte function in HIV-1 may be due to the type of patients used (disease stage, sex etc.), the presence/absence of co-infection, whether they were on ART or not and also how monocytes were assessed (experimental methodology). In addition to the confusing nature of the literature, it is also important to remember that changes in monocytes in HIV infection may be due to direct infection, indirect viral-mediated effects (e.g. Tat) or bystander cell effects.

The current study has demonstrated that monocytes and in particular CD14+CD16+ monocytes appear to maintain functional integrity (in chronic stable HIV infection), display phenotypic and other marker changes indicative of priming for lymphoid tissue migration and are the main producers of IL-10. The positive relationships between the percentage of CD14+CD16+ monocytes and CD38/8 expression, the subset's CCR2 expression with CD38/8 expression, and its IL-10 expression and viral load emphasize the role these cells play in contributing to immune activation, and suggest immune activation and viral replication are linked. Furthermore, if monocytes continue to be stimulated with LPS (in this case *ex vivo*) then these relationships become more established. This is further supported by the negative relationships between the percentage of CD14+CD16- monocytes and CD38/8 and viral load.

Future work should address the findings of this study, particularly how the expanded, activated and migration primed CD14+CD16+ subset function. Is the CD14+CD16+ subset primarily phagocytic, pro-inflammatory and damage inducing, or is it involved in repair and/or fibrosis? It is imperative that further studies are conducted that will help to shed light

on monocyte function at different stages of disease, the impact of co-infections and ARV therapy and also how potential immune-modulatory approaches impact on these cells.

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Appendix 1

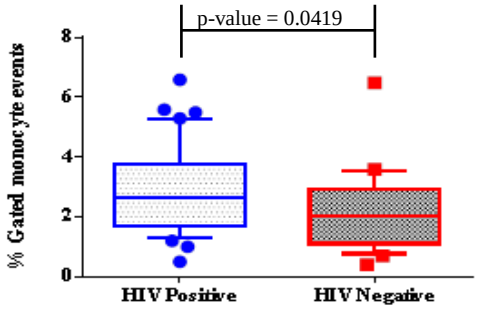

Figure 1: Box-and-whisker plot of total gated monocyte population across the two study groups, HIV+ (median 2.65%, range [0.50-6.60]) and HIV-(2.00, [0.40-6.50]) at baseline. HIV-: Uninfected donors; HIV+: HIV-infected donors. Statistically significant differences are indicated by bars with p-values.

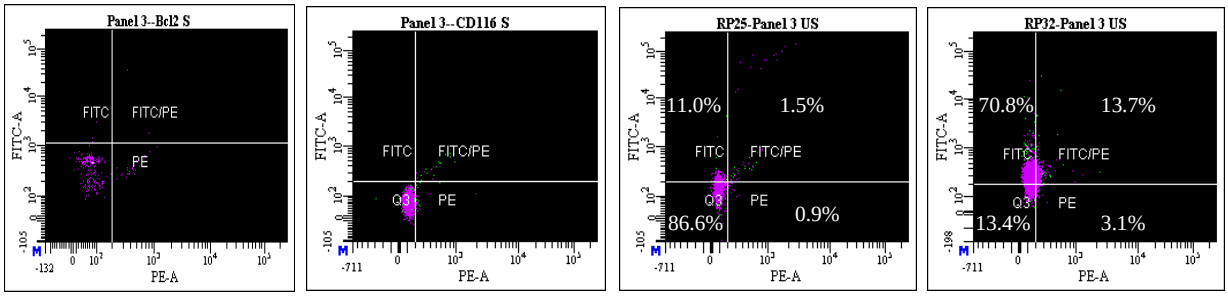

Figure 2: Representative flow cytometry dot plots showing CD116-FITC and Bcl-2-PE expression in the CD14+CD16+ monocyte subset. In the first dot plot on the left (Panel 3 Bcl-2 S), Bcl-2's FMO sample (negative unstained control) is illustrated. The second dot plot (Panel 2 CD116 S) shows CD116's FMO sample. The third dot plot from the left (RP25 Panel 3 US) shows the expression of both CD116-FITC (12.5%) and Bcl-2-PE (2.4%)in a HIV- study sample and the dot plot on the far right (RP32 Panel 3 US) shows the expression of both CD116-FITC (84.5%) and Bcl-2-PE (16.8%) in HIV+ study sample.

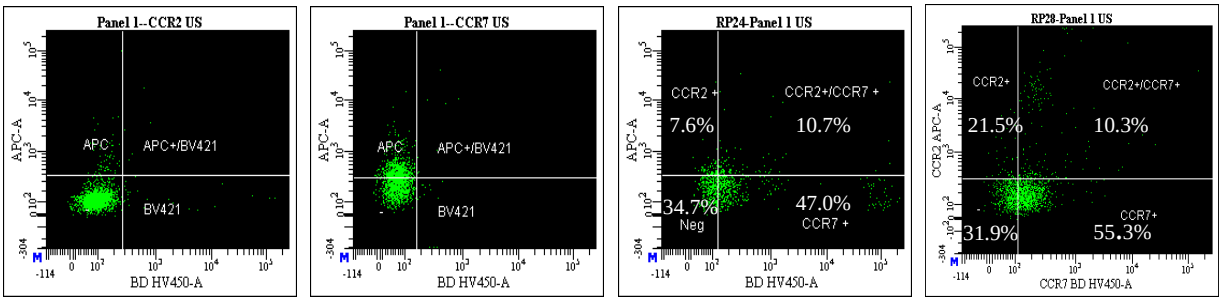

Figure 3: Representative flow cytometry dot plots showing CCR2-APC and CCR7-BV421 (shown here as BD HV450*) expression in the total monocyte population. In the first dot plot on the left (Panel 1 CCR2 US), CCR2's FMO sample (negative unstained control) is illustrated. The second dot plot (Panel 1 CCR7 US) shows CCR7's FMO sample. The third dot plot from the left (RP24 Panel 1 US) shows the expression of both CCR2-APC (18.5%) and CCR7-BV421 (57.7%) in a HIV- study sample and the dot plot on the far right (RP28 Panel 1US) shows the expression of both CCR2-APC (31.8%) and CCR7-BV421 (65.6%) in HIV+ study sample. CCR7-BV421 is shown as BD HV450 due to the settings of the FACS DIVA software that was used during analysis, see section 3.10. in Chapter 3 for study sample flow acquisition.

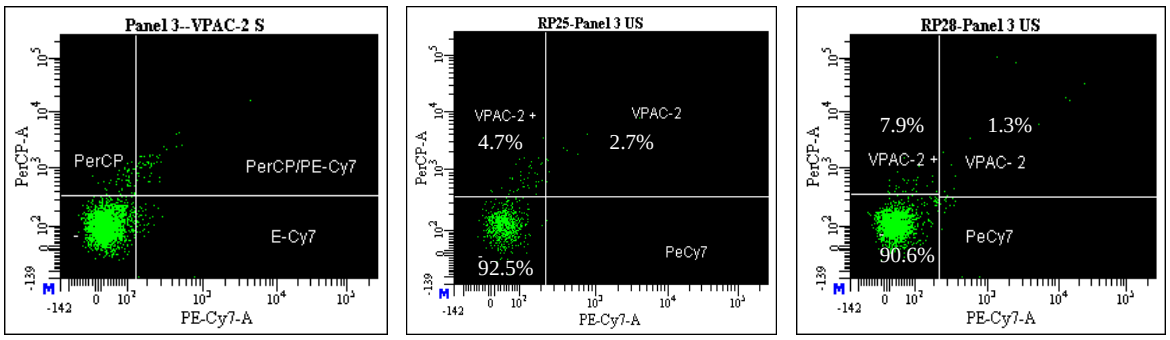

Figure 5: Representative flow cytometry dot plots showing VPAC-2-PerCP in the total monocyte population. In the first dot plot on the left (Panel 3 VPAC-2 S), VPAC-2's FMO sample (negative unstained control) is illustrated. The middle dot plot (RP25 Panel 3 US) shows the expression of VPAC-2-PerCP (7.4%) in a HIV- study sample and the dot plot on the far right (RP28 Panel 3 US) shows the expression of VPAC-2-PerCP (9.2%) in HIV+ study sample.

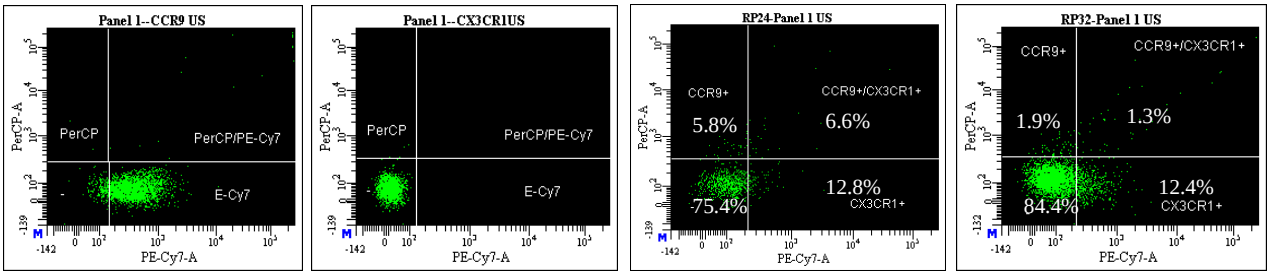

Figure 6: Representative flow cytometry dot plots showing CCR9-PerCP and CX3CR1-PE-Cy7 in the total monocyte population. In the first dot plot on the left (Panel 1 CCR9 US), CCR9's FMO sample (negative unstained control) is illustrated. The second dot plot (Panel 1 CX3CR1 US) shows CX3CR1's FMO sample. The third dot plot from the left (RP24 Panel 1 US) shows the expression of both CCR9-PerCP (12.4%) and CX3CR1-PE-Cy7 (19.4%) in a HIV- study sample and the dot plot on the far right (RP28 Panel 1 US) shows the expression of CCR9-PerCP (3.2%) and CX3CR1-PE-Cy7 (13.7%) in HIV+ study sample.

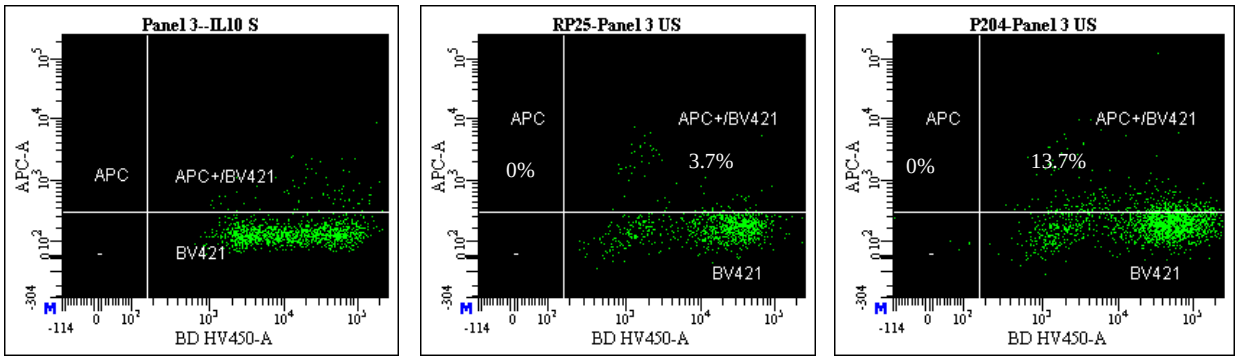

Figure 4: Representative flow cytometry dot plots showing IL-10-APC expression in the total monocyte population. In the first dot plot on the left (Panel 3 IL-10 US), IL-10's FMO sample (negative unstained control) is illustrated. The second dot plot (RP25-Panel 3 US) shows the expression of IL-10-APC (3.7%) in a HIV- study sample and the dot plot on the far right (P204 Panel 3 US) shows the expression of IL-10-APC (13.7%) in HIV+ study sample

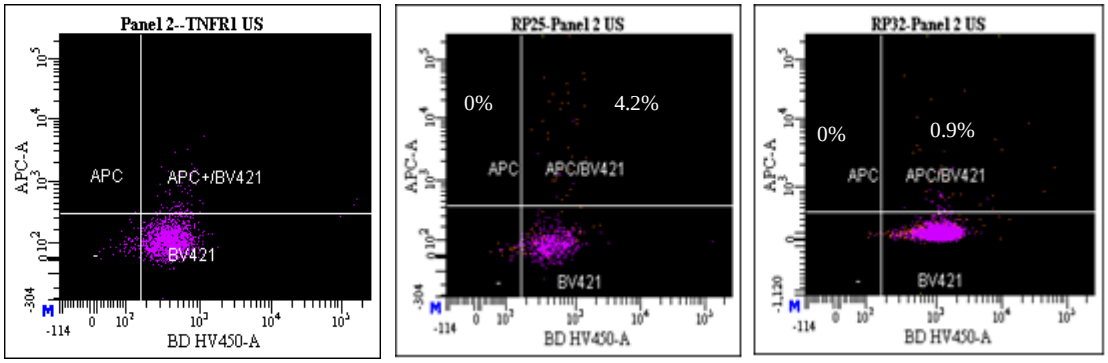

Figure 7: Representative flow cytometry dot plots showing TNFR1-APC expression in the CD14+CD16+ monocyte subset. In the first dot plot on the left (Panel 2 TNFR1 US), TNFR1's FMO sample (negative unstained control) is illustrated. The second dot plot (RP25-Panel 2 US) shows the expression of TNFR1-APC (4.2%) in a HIV- study sample and the dot plot on the far right (RP32 Panel 2 US) shows the expression of TNFR1-APC (0.9%) in HIV+ study sample.

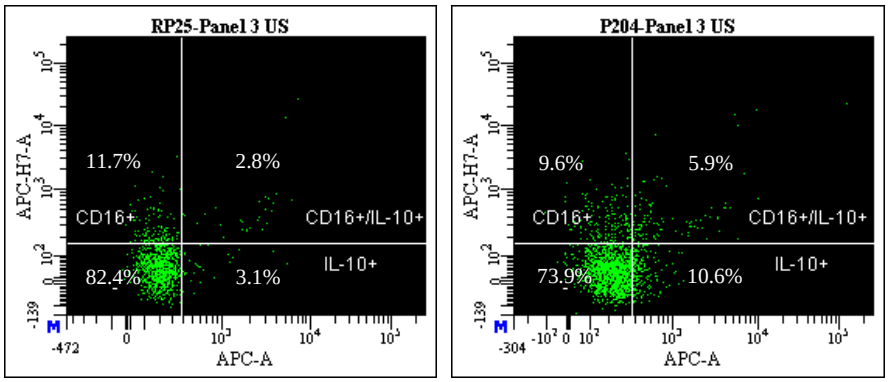

Figure 8: Representative flow cytometry dot plots showing CD16-APC-H7 and IL-10-APC expression in the CD14+CD16+ monocyte subset. The dot plot on the left (RP25-Panel 3 US) shows the expression of both CD16-APC-H7 (14.5%) and IL-10-APC (5.89%) in a HIV- study sample and the dot plot on the far right (P204 Panel 2 US) shows the expression of both APC-H7 (15.5%) and IL-10-APC (16.5%) in HIV+ study sample. It can be seen that the HIV+ sample displays a higher dual expression of CD16 and IL-10 (5.9%) compared to the HIV- sample (2.8%).

Table-1: Correlation between viral load and CD4 count and CD38/8 expression at baseline

Viral load vs.	r-value	P-value
CD4 count	-0.3530	0.0297
CD38/8	0.6391	<0.005

Table-2: Correlation between total monocyte population and CD4 count, viral load and CD38/8 expression in response to stimuli

Total monocyte population vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.06790	0.6772
	LPS	-0.05224	0.7350
CD38/8	Unstimulated	0.1119	0.5491
	LPS	0.1175	0.5289
Viral load	Unstimulated	0.1270	0.4473
	LPS	0.2855	0.0823

Table-3: Correlation between CD14+CD16- monocyte subset and CD4 count, viral load and CD38/8 expression in response to stimuli

CD14+CD16- monocyte subset vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.02947	0.8567
	LPS	0.1960	0.2256
CD38/8	Unstimulated	**-0.3884**	**0.0308**
	LPS	**-0.5486**	**0.0014**
Viral load	Unstimulated	-0.2417	0.1437
	LPS	**-0.3356**	**0.0394**

Table-4: Correlation between CD14+CD16+ monocyte subset and CD4 count, viral load and CD38/8 expression in response to stimuli

CD14+CD16+ monocyte subset vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.02894	0.8593
	LPS	-0.1958	0.2260
CD38/8	Unstimulated	**0.3862**	**0.0319**
	LPS	**0.5462**	**0.0015**
	Unstimulated	0.2422	0.1429

Viral load	LPS	**0.3362**	**0.0390**

Table-5: Correlation between total monocyte population's CCR5 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR5 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	**0.3511**	**0.0263**
	LPS	-0.1248	0.4491
CD38/8	Unstimulated	0.09356	0.6166
	LPS	0.05206	0.7809
Viral load	Unstimulated	-0.02152	0.8979
	LPS	-0.0007604	0.9964

Table-6: Correlation between CD14+CD16- monocyte subset's CCR5 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR5 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1948	0.2347
	LPS	0.1289	0.4309
CD38/8	Unstimulated	-0.09805	0.5997
	LPS	0.08825	0.6369
Viral load	Unstimulated	0.1091	0.5203
	LPS	0.1501	0.3752

Table-7: Correlation between CD14+CD16+ monocyte subset's CCR5 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR5 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.03489	0.8308
	LPS	-0.1792	0.2684
CD38/8	Unstimulated	-0.1349	0.4692
	LPS	-0.04156	0.8243
Viral load	Unstimulated	-0.1578	0.3441
	LPS	-0.06914	0.6800

Table-8: Correlation between total monocyte population's CD116 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD116 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1940	0.2433
	LPS	-0.0005419	0.9975
CD38/8	Unstimulated	0.1703	0.3596
	LPS	0.09953	0.5942
Viral load	Unstimulated	-0.03323	0.8474
	LPS	0.09841	0.5733

Table-9: Correlation between CD14+CD16- monocyte subset's CD116 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD116 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1579	0.3370
	LPS	0.02388	0.8853
CD38/8	Unstimulated	0.1440	0.4395
	LPS	0.3395	0.0617
Viral load	Unstimulated	-0.02803	0.8692
	LPS	0.07919	0.6413

Table-10: Correlation between CD14+CD16+ monocyte subset's CD116 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD116 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.3075	0.0604
	LPS	0.2243	0.1757
CD38/8	Unstimulated	0.1334	0.4745
	LPS	0.3537	0.0509
Viral load	Unstimulated	-0.1004	0.5602
	LPS	0.06668	0.6992

Table-11: Correlation between total monocyte population VPAC-2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

VPAC-2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.2573	0.1138
	LPS	**0.4493**	**0.0041**
CD38/8	Unstimulated	-0.3677	0.8445
	LPS	-0.1113	0.5570
Viral load	Unstimulated	-0.02788	0.8708
	LPS	-0.06189	0.7159

Table-12: Correlation between CD14+CD16- subset's VPAC-2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

VPAC-2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.08714	0.6021
	LPS	**0.3935**	**0.0132**
CD38/8	Unstimulated	-0.2512	0.1728
	LPS	-0.1276	0.4941
Viral load	Unstimulated	-0.1750	0.3073
	LPS	0.05762	0.7605

Table-13: Correlation between CD14+CD16+ subset's VPAC-2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

VPAC-2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1519	0.3628
	LPS	**0.4063**	**0.0114**
CD38/8	Unstimulated	-0.1166	0.5322
	LPS	-0.1870	0.3137
Viral load	Unstimulated	0.009277	0.9572
	LPS	-0.08357	0.6267

Table-14: Correlation between total monocyte population's IL-10 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

IL-10 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1793	0.2814
	LPS	0.04269	0.7991
CD38/8	Unstimulated	-0.06867	0.7136
	LPS	-0.05010	0.7890
Viral load	Unstimulated	0.1384	0.4209
	LPS	-0.03353	0.8461

Table-15: Correlation between CD14+CD16- monocyte subset's IL-10 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

IL-10 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1498	0.3965
	LPS	0.09004	0.5909
CD38/8	Unstimulated	-0.03185	0.8694
	LPS	-0.2671	0.1464
Viral load	Unstimulated	0.2200	0.1972
	LPS	0.01303	0.9399

Table-16: Correlation between CD14+CD16+ monocyte subset's IL-10 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

IL-10 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.2462	0.1418
	LPS	-0.2947	0.0725
CD38/8	Unstimulated	0.1433	0.4419
	LPS	0.1011	0.5884
Viral load	Unstimulated	0.1347	0.4404
	LPS	0.2868	0.0898

Table-17: Correlation between total monocyte population's CCR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.2213	0.1700
	LPS	0.1811	0.2633
CD38/8	Unstimulated	0.2469	0.1806
	LPS	0.1581	0.3956
Viral load	Unstimulated	0.1284	0.4423
	LPS	-0.1345	0.1811

Table-18: Correlation between CD14+CD16- monocyte subset's CCR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.04670	0.7771
	LPS	0.03854	0.8158
CD38/8	Unstimulated	0.06890	0.7127
	LPS	-0.08296	0.6573
Viral load	Unstimulated	-0.009020	0.9577
	LPS	-0.08935	0.5990

Table-19: Correlation between CD14+CD16+ monocyte subset's CCR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1306	0.4218
	LPS	0.01873	0.9087
CD38/8	Unstimulated	-0.02675	0.8821
	LPS	-0.1983	0.2327
Viral load	Unstimulated	-0.1967	0.2365
	LPS	-0.09762	0.6014

Table-20: Correlation between total monocyte population's CX3CR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CX3CR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.07702	0.6367
	LPS	0.02441	0.8811
CD38/8	Unstimulated	0.3255	0.0739
	LPS	0.03627	0.8464
Viral load	Unstimulated	0.6298	0.05882
	LPS	0.05078	0.7621

Table-21: Correlation between CD14+CD16- monocyte subset's CX3CR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CX3CR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.03367	0.8366
	LPS	0.001146	0.9559
CD38/8	Unstimulated	0.1530	0.4114
	LPS	**-0.4916**	**0.0050**
Viral load	Unstimulated	**0.7751**	**0.0388**
	LPS	-0.005454	0.7485

Table-22: Correlation between CD14+CD16+ monocyte subset's CX3CR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CX3CR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.3573	0.8267
	LPS	-0.06509	0.6099
CD38/8	Unstimulated	-0.2450	0.1841
	LPS	-0.1382	0.4586
Viral load	Unstimulated	-0.1947	0.2367
	LPS	-0.1160	0.4887

Table-23: Correlation between CD14+CD16- monocyte subset's CX3CR1 and CCR2 before and after stimulation

CX3CR1 vs.	Stimulus	r-value	P-value
CCR2	Unstimulated	-0.9408	0.5689
	LPS	-0.6416	0.6980

Table-24: Correlation between CD14+CD16+ monocyte subset's CX3CR1 and CCR2 before and after stimulation

CX3CR1 vs.	Stimulus	r-value	P-value
CCR2	Unstimulated	**-0.4401**	**0.0045**
	LPS	-0.1046	0.5205

Table-25: Correlation between total monocyte population CCR2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.07702	0.06367
	LPS	0.02441	0.8811
CD38/8	Unstimulated	0.09019	0.6294
	LPS	0.2088	0.2597
Viral load	Unstimulated	0.05882	0.7257
	LPS	0.05078	0.7621

Table-26: Correlation between CD14+CD16- monocyte subset's CCR2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	**0.3309**	**0.0396**
	LPS	0.2069	0.2063
CD38/8	Unstimulated	-0.02215	0.9059
	LPS	0.09593	0.6077
Viral load	Unstimulated	-0.1099	0.5171
	LPS	-0.1809	0.2840

Table-27: Correlation between CD14+CD16+ monocyte subset's CCR2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.003075	0.9850
	LPS	-0.2315	0.2103
CD38/8	Unstimulated	**0.5946**	**0.0004**
	LPS	**0.4801**	**0.0063**
Viral load	Unstimulated	0.2366	0.1526
	LPS	0.2527	0.1186

Table-28: Correlation between total monocyte population CCR7 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR7 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.03602	0.8277
	LPS	0.04310	0.7917
CD38/8	Unstimulated	0.1151	0.5448
	LPS	0.06645	0.7225
Viral load	Unstimulated	0.1779	0.2922
	LPS	-0.04947	0.7745

Table-29: Correlation between CD14+CD16- monocyte subset's CCR7 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR7 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1528	0.3530
	LPS	0.2460	0.1311
CD38/8	Unstimulated	-0.1502	0.4198
	LPS	-0.03848	0.8791
Viral load	Unstimulated	0.2542	0.1289
	LPS	0.1792	0.2886

Table-30: Correlation between CD14+CD16+ monocyte subset's CCR7 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR7 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.03237	0.8428
	LPS	0.02290	0.8885
CD38/8	Unstimulated	-0.1747	0.3473
	LPS	-0.2315	0.2103
Viral load	Unstimulated	-0.06196	0.7117
	LPS	-0.2627	0.1111

Table-31: Correlation between total monocyte population CCR9 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR9 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.02014	0.9018
	LPS	0.02441	0.8811
CD38/8	Unstimulated	**-0.4015**	**0.0252**
	LPS	0.03829	0.8379
Viral load	Unstimulated	-0.01985	0.9058
	LPS	-0.04941	0.7745

Table-32: Correlation between CD14+CD16- monocyte subset's CCR9 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR9 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.07416	0.6537
	LPS	0.2460	0.1311
CD38/8	Unstimulated	0.06246	0.7385
	LPS	-01041	0.5773
Viral load	Unstimulated	-0.1579	0.3507
	LPS	-0.1688	0.3417

Table-33: Correlation between CD14+CD16+ monocyte subset's CCR9 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CCR9 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1609	0.3213
	LPS	-0.1377	0.3969
CD38/8	Unstimulated	0.07247	0.6894
	LPS	0.1678	0.3668
Viral load	Unstimulated	-0.1020	0.5424
	LPS	-0.001311	0.9938

Table-34: Correlation between total monocyte population's CD62-L expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD62-L vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1008	1.5526
	LPS	0.03496	0.8373
CD38/8	Unstimulated	0.03885	0.8370
	LPS	-0.4428	0.8130
Viral load	Unstimulated	-0.1172	0.5027
	LPS	0.2401	0.1647

Table-35: Correlation between CD14+CD16- subset's CD62-L expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD62-L vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.07812	0.6364
	LPS	0.01950	0.9662
CD38/8	Unstimulated	-0.1508	0.5912
	LPS	-0.07437	0.6909
Viral load	Unstimulated	-0.1880	0.2657
	LPS	-0.1304	0.4416

Table-36: Correlation between CD14+CD16+ monocyte subset's CD62-L expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD62-L vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.05474	0.7476
	Stimulated	-0.2023	0.2299
CD38/8	Unstimulated	0.1547	0.4060
	Stimulated	0.1845	0.3204
Viral load	Unstimulated	-0.2502	0.1417
	Stimulated	0.1362	0.4355

Table-37: Correlation between total monocyte population's CD69 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD69 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1920	0.2401
	LPS	0.2032	0.2148
CD38/8	Unstimulated	0.08045	0.6671
	LPS	0.1255	0.5011
Viral load	Unstimulated	-0.01523	0.9287
	LPS	0.2380	0.1561

Table-38: Correlation between CD14+CD16- subset's CD69 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD69 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1314	0.4251
	LPS	0.03841	0.8164
CD38/8	Unstimulated	0.1295	0.4574
	LPS	0.1306	0.4839
Viral load	Unstimulated	0.009857	0.9537
	LPS	0.3232	0.0510

Table-39: Correlation between CD14+CD16+ monocyte subset's CD69 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

CD69 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.0774	0.6489
	LPS	-0.07557	0.6566
CD38/8	Unstimulated	0.1354	0.4777
	LPS	0.2010	0.2783
Viral load	Unstimulated	0.1811	0.4994
	LPS	0.2986	0.1052

Table-40: Correlation between total monocyte population's HLA-DR expression and CD4 count, viral load and CD38/8 expression in response to stimuli

HLA-DR vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1823	0.2666
	LPS	**-0.3506**	**0.0168**
CD38/8	Unstimulated	-0.1780	0.3381
	LPS	0.2471	0.1802
Viral load	Unstimulated	0.1242	0.4640
	LPS	0.2284	0.1739

Table-41: Correlation between CD14+CD16- subset's HLA-DR expression and CD4 count, viral load and CD38/8 expression in response to stimuli

HLA-DR vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	**-0.3629**	**0.0232**
	LPS	**0.3325**	**0.0452**
CD38/8	Unstimulated	0.1686	0.6347
	LPS	0.2027	0.2742
Viral load	Unstimulated	0.1771	0.2944
	LPS	0.1811	0.02385

Table-42: Correlation between CD14+CD16+ monocyte subset's HLA-DR expression and CD4 count, viral load and CD38/8 expression in response to stimuli

HLA-DR vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.008016	0.9624
	LPS	-0.05853	0.7308
CD38/8	Unstimulated	-0.1179	0.5277
	LPS	-0.09387	0.6155
Viral load	Unstimulated	0.113	0.5171
	LPS	0.09967	0.5689

Table -43: Correlation between total monocyte population's TRAIL-R1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

TRAIL-R1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.04925	0.7659
	LPS	-0.09843	0.5511
CD38/8	Unstimulated	-0.05200	0.7811
	LPS	-0.02210	0.9061
Viral load	Unstimulated	0.1124	0.9474
	LPS	0.1981	0.2400

Table -44: Correlation between CD14+CD16- subset's TRAIL-R1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

TRAIL-R1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1923	0.2409
	LPS	0.038880	0.8142
CD38/8	Unstimulated	-0.105	0.5647
	LPS	0.1530	0.4110
Viral load	Unstimulated	0.07786	0.6469
	Stimulated	0.04862	0.7751

Table-45: Correlation between CD14+CD16+ monocyte subset's TRAIL-R1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

TRAIL-R1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.1523	0.3681
	LPS	0.1171	0.4901
CD38/8	Unstimulated	0.07172	0.7014
	LPS	-0.04786	0.7982
Viral load	Unstimulated	0.1153	0.5097
	LPS	0.05716	0.7443

Table-46: Correlation between total monocyte population's TNFR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

TNFR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.03417	0.8409
	LPS	-0.07471	0.6603
CD38/8	Unstimulated	0.2427	0.1884
	LPS	0.06042	0.7468
Viral load	Unstimulated	-0.08329	0.6364
	LPS	-0.2432	0.1591

Table-47: Correlation between CD14+CD16- subset's TNFR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

TNFR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	**0.3789**	**0.0174**
	LPS	**0.4230**	**0.0073**
CD38/8	Unstimulated	-0.03758	0.9540
	LPS	-0.1199	0.5206
Viral load	Unstimulated	-0.2672	0.1099
	LPS	-0.1751	0.2999

Table-48: Correlation between CD14+CD16+ monocyte subset's TNFR1 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

TNFR1 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1345	0.0604
	LPS	0.2243	0.1757
CD38/8	Unstimulated	0.1348	0.4697
	LPS	0.06599	0.7243
Viral load	Unstimulated	-0.09545	0.5855
	LPS	-0.1763	0.3111

Table-49: Correlation between total monocyte population's Bcl-2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

Bcl-2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.2729	0.0928
	LPS	**0.3573**	**0.0256**
CD38/8	Unstimulated	0.1510	0.4176
	LPS	-0.2079	0.2617
Viral load	Unstimulated	-0.007106	0.9667
	LPS	-0.1873	0.2671

Table-50: Correlation between CD14+CD16- subset's Bcl-2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

Bcl-2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	**0.3551**	**0.0265**
	LPS	**0.3935**	**0.0132**
CD38/8	Unstimulated	0.1659	0.3724
	LPS	0.2049	0.2688
Viral load	Unstimulated	-0.02949	0.8624
	LPS	-0.03580	0.8334

Table-51: Correlation between CD14+CD16+ subset's Bcl-2 expression and CD4 count, viral load and CD38/8 expression in response to stimuli

Bcl-2 vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	**0.4138**	**0.0098**
	LPS	**0.4788**	**0.0024**
CD38/8	Unstimulated	0.1289	0.4895
	LPS	-0.04320	0.8175
Viral load	Unstimulated	-0.06007	0.7278
	LPS	-0.1278	0.4578

Table-52: Correlation between total monocyte population's 7AAD/AnnexinV expression and CD4 count, viral load and CD38/8 expression in response to stimuli

7AAD/AnnexinV vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	-0.1485	0.8515
	LPS	-0.2679	0.7321
CD38/8	Unstimulated	-0.1590	0.6820
	LPS	0.5215	0.1500
Viral load	Unstimulated	-0.3153	0.4085
	LPS	-0.3032	0.4277

Table-53: Correlation between CD14+CD16- subset's 7AAD/AnnexinV expression and CD4 count, viral load and CD38/8 expression in response to stimuli

7AAD/AnnexinV vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.2590	0.7410
	LPS	-0.8336	0.1614
CD38/8	Unstimulated	**-0.7373**	**0.0234**
	LPS	-0.09978	0.7984
Viral load	Unstimulated	**0.6967**	**0.0370**
	LPS	-0.3265	0.3912

Table-54: Correlation between CD14+CD16+ subset's 7AAD/AnnexinV expression and CD4 count, viral load and CD38/8 expression in response to stimuli

7AAD/AnnexinV vs.	Stimulus	r-value	P-value
CD4 count	Unstimulated	0.7742	0.2258
	LPS	0.5481	0.4519
CD38/8	Unstimulated	-0.2073	0.5926
	LPS	0.04952	0.8993
Viral load	Unstimulated	0.3831	0.3089
	LPS	0.3530	0.3514

Table-55: Correlation between viral load and IL10 and CCR5 expression at baseline in total monocyte population and monocyte subsets

IL-10 vs. CCR5	Stimulus	r-value	P-value
Total monocyte population	Unstimulated	0.02507	0.8813
CD14+CD16- monocyte subset	Unstimulated	-0.1906	0.2517
CD14+CD16+ monocyte subset	Unstimulated	-0.1249	0.4551