

Role of HAART in reconstituting T-cell function and HIV inhibitory activity in the female genital tract during chronic HIV infection

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the degree of
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Abstract

HIV infection is characterized by generalized high levels of systemic immune activation that plays an important role in accelerating the depletion of CD4⁺ T-cells. Levels of immune activation are higher at mucosal sites like the female genital tract than blood during HIV infection. While initiation of highly active antiretroviral therapy (HAART) during HIV infection reduces plasma viral load and partially restores CD4⁺ T-cell numbers, individuals on HAART retain an activated phenotype in blood that is significantly higher than uninfected individuals. The precise mechanisms leading to recruitment and activation of immune cells into the female genital tract during HIV infection and the influence of HAART on activation are not clear. The aim of this study was to investigate the impact of HAART on the levels of inflammatory cytokines and immune reconstitution (measured by immune activation, proliferation and exhaustion of T-cells) in the female genital tract compared to blood.

Peripheral blood mononuclear cells and cervical cytobrush-derived cells from the genital tract of 26 HIV-infected women were included in this study. Of these, 15/26 had been HAART for a long period of time (~7 years) while 11/26 were enrolled as they were about to start HAART and followed until 1 month after initiating HAART. A 10-colour flow cytometry panel was designed and optimized to measure expression of phenotypic and memory markers on T-cells (CD3, CD4, CD8, CCR7, and CD45RO). In addition, activation (CD38 and HLA-DR), proliferation (Ki67) and senescence (CD57) of T-cells were evaluated. The concentration of IL-1 β , IL-6, IL-8, IP-10, MIP-1 α , MIP-1 β , TNF- α , IL-7, G-CSF and IL-10 in plasma and genital secretions were assessed using Luminex.

The majority of T-cells in the genital tract were found to be effector memory T-cells (TEM), while the majority of T-cells in blood were naïve and effector memory CD4⁺ T-cells and terminally-differentiated CD8⁺ T-cells. HAART did not influence the predominance of TEM cells in the genital tract, although the frequency of the central memory T-cell (TCM) population within the genital tract did increase with initiation of HAART. Higher frequencies of T-cells from the

genital tract were activated (measured by expression of CD38 and HLA-DR) than those in blood from HIV-infected women on HAART. Initiation of HAART resulted in a significant reduction in the level of T-cell activation in blood but not in the genital tract, where activation levels were slightly increased compared to before initiation of HAART. T-cells collected from the genital tract of women on HAART were significantly more exhausted than those in blood (measured by CD57 expression); and 1 month on HAART resulted in an increased population of exhausted T-cells in the genital tract, particularly for TCM and TEM subsets. CD4:CD8 ratios in blood of HIV-infected women on long-term HAART (1:1.4) showed evidence of partial CD4 immune reconstitution compared to other studies, although genital tract CD4:CD8 ratios (1:0.8) were significantly lower. While complete viral suppression in both plasma and in genital secretions was evident as early as 1 month following initiation of HAART, immune reconstitution (measured by CD4:CD8 ratios) was not significantly higher. Lastly, cytokine concentrations in the genital tract were generally higher than those in blood. Viral suppression seen early after HAART initiation was associated with significantly lower IP-10 concentrations in plasma as well as the level of activation of T-cells in blood. In contrast, inflammatory cytokine concentrations in genital secretions were largely unchanged after initiation of HAART although the anti-inflammatory cytokine IL-10 was detected at significantly lower concentrations.

In conclusion, initiation of HAART during HIV infection had a more pronounced impact on T-cell activation and inflammation in blood than in the genital compartment, which remained inflamed and highly activated. Being on HAART for longer periods of time was still accompanied by higher levels of genital T-cell activation and inflammation in genital tract than blood, despite the fact that most women had fully suppressed blood viral loads. Since several studies have shown that women on HAART continue to shed HIV in their genital secretions (despite being fully virologically suppressed in blood), understanding local immunological factors leading to on-going inflammation in the genital tract and local immune activation during HAART is very important as this would potentially place women at increased risk for HIV genital shedding despite full suppression of viraemia systemically.

List of Abbreviations

°C	Degrees Celcius
ABC	Abacavir
ACD	Acid Citrate Dextrose
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin / Antigen-presenting cell
ARV	Antiretroviral
ART	Antiretroviral Therapy
BV	Bacterial vaginosis
CCR5	C-C chemokine receptor type 5
CCR7	C-C chemokine receptor type 7
CD	Cluster of Differentiation
CMC	Cervical mononuclear cells
CMV	Cytomegalovirus
CNAR	CD8 ⁺ cell Noncytotoxic Antiviral Response
CRI	Co-receptor inhibitor
CRP	C-reactive protein
CTL	Cytotoxic T-lymphocyte
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
ddC	Zalcitabine
ddI	Didanosine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
d4T	Stavudine
ECD	Phycoerythrin-Texas Red conjugate
FBS	Fetal Bovine Serum
FI	Cell entry/ Fusion inhibitor
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
FSC-A	Forward Scatter - Area
FSC-H	Forward Scatter - Height
FTC	Emtricitabine
HPV	Human Papillomavirus
HSV-2	Herpes Simplex Virus 2
g	Gravity
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
gp120	Glycoprotein 120
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Disease

HLA	Human Leukocyte Antigen
ICS	Intracellular Cytokine Staining
IFN	Interferon
IL	Interleukin
INI	Integrase Inhibitor
IP-10	Interferon-Gamma Inducible Protein 10kDa
IQR	Interquartile range
IRIS	Immune Reconstitution Inflammatory Syndrome
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitres
NK	Natural Killer
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NSAID	Non-Steroidal Anti-Inflammatory Drug
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death-1
PE	Phycoerythrin
PerCP Cy5.5	Peridininchlorophyll protein Cy5.5
PI	Protease Inhibitor
Qdot	Quantum Dot
RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
RNA	RiboNucleic Acid
SIV	Simian Immunodeficiency Disease
SSC	Side Scatter
STD	Sexually transmitted disease
STI	Sexually transmitted infection
TCM	Central memory T-cell
TCR	T-cell Receptor
TD	Terminally differentiated T-cell
TDF	Tenofovir
TEM	Effector memory T-cell
T_{fh}	Follicular helper T-cell
T_h1	T-helper 1
TNF	Tumor Necrosis Factor
Vivid	Violet Fluorescent Reactive Dye
µl	Microlitres
ZDV	Zidovudine
3TC	Lamivudine

Chapter 1. Literature Review

1.1. Introduction

Thirty years have elapsed since the Human Immunodeficiency Virus (HIV) was first described in 1983 and the heterosexual epidemic in Africa was identified (Merson et al., 2008). According to the 2013 UNAIDS report, it is estimated that there were about 35.3 million adults and children living with HIV worldwide. For the first time since the first identification of HIV, the UNAIDS report details an overall decrease in HIV incidence, with 2.3 million new people getting infected with HIV in 2012 compared to 3.4 million in 2001. Despite this 33% decrease in the number of new HIV cases, the UNAIDS report clearly shows an overall increase in the total number of HIV-infected individuals worldwide, which could be attributed to the fact that more and more HIV-infected people now have access to antiretroviral therapy (ART) and death rates are declining. Of all the countries affected by HIV, sub-Saharan Africa is the most severely affected region of the world, with about 70% of the global total of HIV-infected individuals living in this region (Figure 1.1, UNAIDS, 2013). With an HIV prevalence of 17.9%, South Africa has the highest number of cases of HIV in the world. Internationally, it is also the country with the highest number of women between the ages 15-49 who are infected with HIV (3.4 million women) (www.unaids.org/en/dataanalysis/datatools/aidsinfo/).

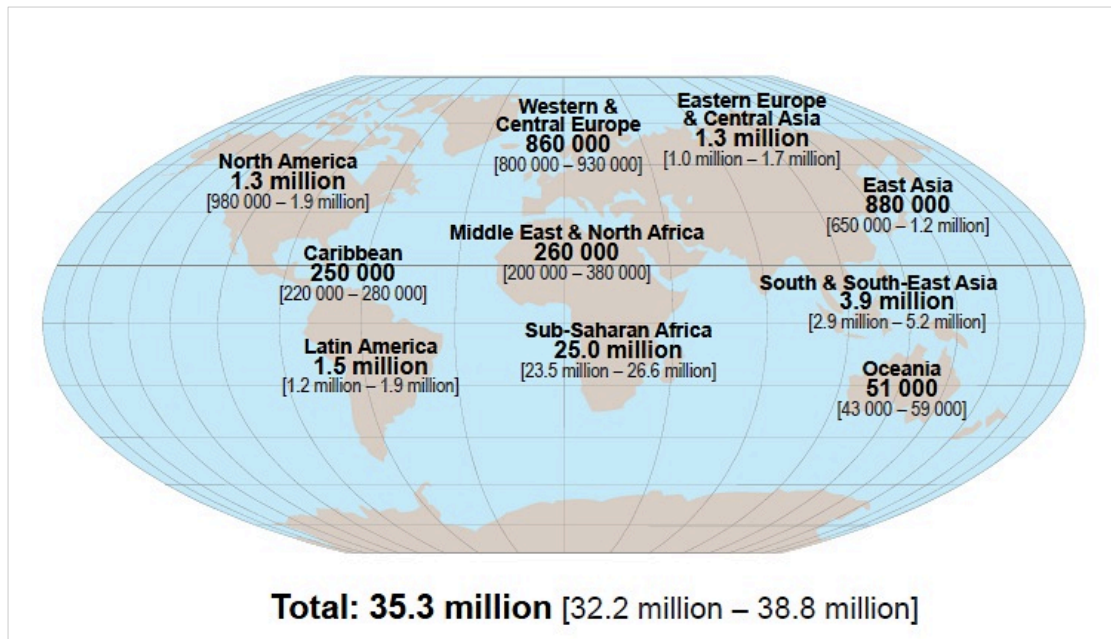


Figure 1.1. Total number of people estimated to be living with HIV in 2012 (taken by [UNAIDS, 2013](#)).

1.2. HIV replication and cell tropism

HIV-1 is a retrovirus of the lentivirus family derived from the simian immunodeficiency virus (SIV) of chimpanzees (SIV_{cpz}) ([Chahroudi et al., 2012](#); [Hahn, 2000](#)). Like other lentiviruses, HIV infection develops into a chronic disease, with a relatively long period of clinical latency and persistent viral replication. HIV infects CD4⁺ immune cells, predominantly of the lymphocyte and mononuclear phagocytic lineages ([Rosenberg & Fauci, 1991](#); reviewed by [Swanstrom et al., 2012](#)). It has an RNA genome, which consists mainly of nine open reading frames that can produce fifteen proteins ([Frankel and Young, 1998](#)). Using reverse transcriptase, it converts its ribonucleic acid (RNA) to double-stranded deoxyribonucleic acid (DNA), which can be integrated into the genome of the infected cell. The virus depends mostly on the three genes, *gag*, *pol* and *env*, for successful replication. *Gag* and *env* genes code for the virus nucleocapsid and the membrane glycoproteins while the *pol* gene codes for reverse transcriptase and other enzymes ([Frankel and Young, 1998](#)). The genome also consists of six smaller genes encoding for various accessory (*vif*, *vpr*, *vpr* and *nef*) and regulatory (*tat* and *rev*) proteins ([Tang et al., 1999](#)). These

genes control processes such transcription, intracellular transport and translation of the viral RNA into the provirus (as reviewed by [Lever, 2005](#)).

The first step in the replication of HIV within CD4⁺ host cells is the interaction between the virus envelope glycoprotein, gp120 and the cell membrane receptor CD4 ([Myszka et al., 2000](#)). However, the virus also needs to bind to an obligatory chemokine receptor for successful entry. Binding of gp120 to CD4 causes a conformational change, enabling the virus to then bind to either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4), two chemokine receptors found on the host cell membrane, leading to membrane fusion ([Myszka et al., 2000](#)). The virus capsid is released into the cell cytoplasm where reverse transcriptase starts the transcription of the viral RNA to double-stranded DNA. Using p17, the integrase enzyme and *vpr*, the genome is then transported to the cell nucleus where it integrates the chromosome of the cell ([Melikyan, 2008](#)). The integrated provirus is able to generate new viral messenger RNA transcripts using the host cellular RNA polymerase II. Transcription produces more copies of the RNA genome, which is then translated to proteins in the cytoplasm. These proteins return to the nucleus where *tat* causes the amplification of viral RNA production and *rev* interacts with the nuclear pore proteins, allowing the RNA molecules to be transported to the cytoplasm (reviewed by [Lever, 2005](#)). In the cytoplasm, the translated envelope glycoprotein and *gag* proteins are transported to the cell surface. A capsid structure forms, which expresses the glycoproteins on its surface and encloses two RNA molecules, the replicative enzymes and the structural and accessory proteins (reviewed by [Lever, 2005](#)).

The chemokine receptors CCR5 and CXCR4 are the two main co-receptors that HIV uses to gain entry into cells (reviewed by [Alkhatib, 2010](#)). Generally, HIV are either T-tropic (which infect mainly blood CD4⁺ T-cells) or M-tropic (which are able to infect CD4⁺ T-cells, monocytes and macrophages) (reviewed by [Alkhatib, 2010](#)). In addition to being dependent of the availability of the CD4 receptor, T-tropic viruses need CXCR4 to enter the cell (X4 viruses) while the M-tropic viruses use CCR5 (R5 viruses). Several studies have shown that HIV that is

transmitted sexually across genital mucosal surfaces are mostly M-tropic and use CCR5 (Deng et al., 1996; Dragic et al., 1996; reviewed by Lever, 2005).

1.3. The natural course of HIV infection

Three main stages of HIV infection in humans have been defined: the acute phase, the asymptomatic chronic phase and the onset of AIDS (reviewed by An & Winkler, 2010) as shown in figure 1.2. The infection starts with a severe symptomatic illness which is associated with a high viraemia, a drastic decrease in blood CD4⁺ T-cell counts, the formation of a reservoir of latently-infected CD4⁺ T-cells, and the development of HIV-1 specific immune responses (Clark et al., 1991; Schacker et al., 1998; Freel et al., 2011). Following this is a 100-1000 fold decrease in viral load, a fractional increase in blood CD4⁺ T-cell counts (although these rarely reach pre-infection numbers or proportions) and then the asymptomatic phase of chronic HIV infection, which is characterised by a slow decrease in CD4⁺ T-cell counts and an incremental increase in viraemia. At the time of seroconversion, the level of HIV-RNA would have decreased to <1% of the original viral load and remains at a relatively constant level for a number of years. This is called the viral set point and several studies have shown that the higher the viral set point, the faster the decrease in CD4⁺ T-cell counts during the chronic phase (reviewed by Fanales-Belasio et al., 2010). Viral set point is dependent on a variety of host-specific and viral factors such as human leukocyte antigen (HLA)-type or chemokine receptor mutations, cytotoxic T-lymphocyte (CTL) responses, amongst others (reviewed by Simon & Ho, 2003). The immune system gradually deteriorates as the numbers of CD4⁺ T-cells (providing help) decline and other cells become dysfunctional. This leads to increased susceptibility to opportunistic diseases and the eventual development of acquired immunodeficiency syndrome (AIDS). If left untreated, an individual infected with HIV will succumb to opportunistic AIDS-defining illnesses as a result of a compromised immune system at a median of 8-10 years after infection. These illnesses, rather than the HIV infection itself, will eventually result in the death of the individual (Hoffman and Rockstroh, 2013).

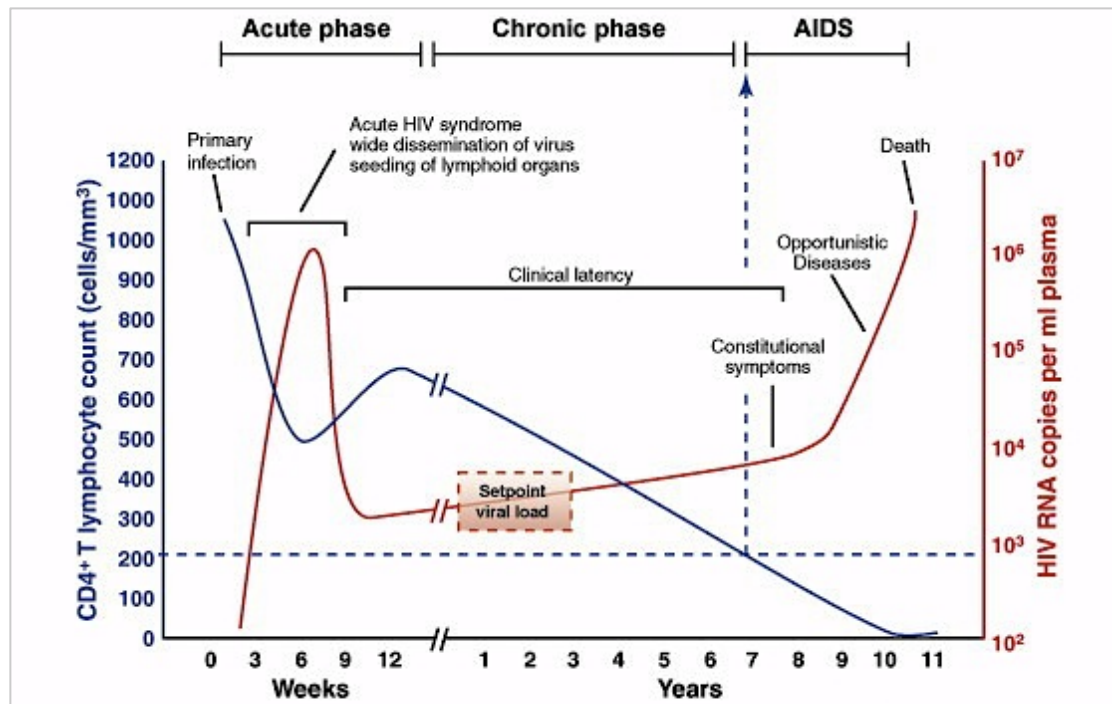


Figure 1.2. The natural history of an HIV infection, according to two clinical markers of disease progression - plasma viraemia (red line) and blood CD4⁺ T-cell counts (blue line). The acute phase of HIV infection is accompanied by a rise in viral load and a sharp drop in CD4⁺ T-cell count and is associated with clinical symptoms. After the first months, plasma viraemia stabilises (set point) and the CD4⁺ T-cell numbers become normalised. The chronic phase is asymptomatic despite the fact that HIV-1 carries on replicating. AIDS is defined by the occurrence of opportunistic infections, the risk of which increase as the CD4 T-cell counts fall below 200 cells/ml (taken from [An & Winkler, 2010](#))

1.3.1. CD4⁺ T-cell depletion in HIV infection

HIV infection is characterised by a gradual decrease in CD4⁺ T-cell numbers, which eventually leads to AIDS ([Mehandru et al., 2004](#)). This CD4⁺ T-cell decline has been described in mucosal tissues, within the first weeks of infection and is more severe than depletion measurable in blood ([Mehandru et al., 2004](#)). More than 60% of the total CD4⁺ T-lymphocyte population are found within the gastrointestinal mucosa and other secondary lymphoid organs, and as a result, a greater depletion of CD4⁺ T-cell happens at these sites compared to peripheral blood ([Brenchley et al., 2004](#)). Early during HIV infection, this destruction of cells is countered by CD4⁺ memory T-cell regeneration which allows the immune system to maintain CD4⁺ T-cell count above the threshold for immunodeficiency ([Gorochov et al., 1998](#)). However, as the chronic phase of infection is established and immune dysregulation becomes more pronounced, memory T-cell

homeostasis fails and the effector CD4⁺ T-cell populations fall below the minimum levels needed to protect against opportunistic diseases (Okoye et al., 2007).

Several mechanisms have been suggested to explain HIV-mediated CD4⁺ T-cell depletion. One proposed mechanism relates to the direct viral elimination of HIV-infected cells, such as the infection-mediated death of progenitor cells or the destruction of the supporting stromal network required for lineage-restricted haematopoiesis (Zeng et al., 2012). Alternatively, CD4⁺ T-cell depletion during HIV infection could be due to immune responses to the infection, for example cytokine dysfunction, HIV-induced apoptosis of bystander cells or opportunistic infections of the bone marrow by cytomegalovirus (CMV), which would end up in the depletion of uninfected cells (reviewed by McCune, 2001 and Okoye & Picker, 2013).

1.3.2. Immune responses to HIV

Both innate and adaptive immune responses are mounted following infection with HIV (figure 1.3). The innate immune response is immediate and non-specific while the adaptive response occurs later in the primary infection via the activation, proliferation and differentiation of B- and T-lymphocytes.

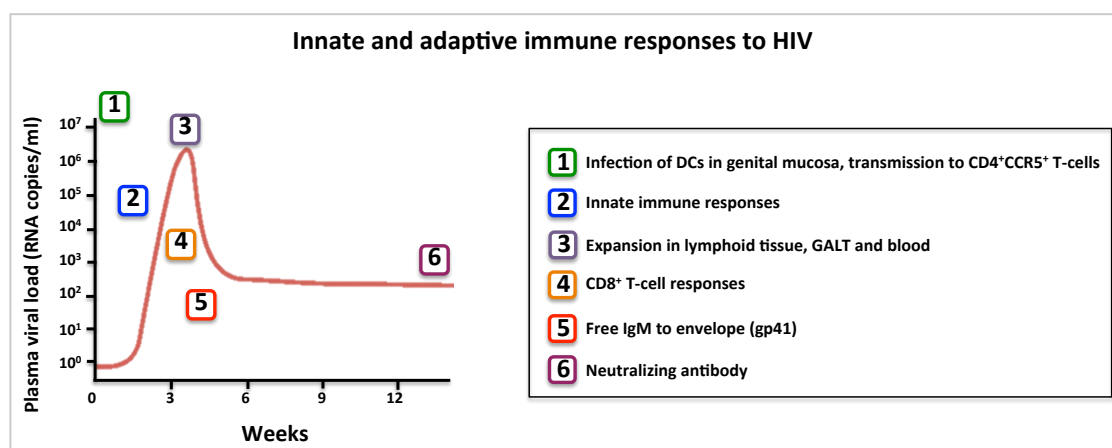


Figure 1.3. Temporal emergence of different arms of the innate and adaptive immune responses to appear following the acute phase of an HIV infection (adapted from McMichael & Dorrell, 2009).

The innate immune system is the body's first line of defence against pathogens and is especially important in fighting off viral infections at the mucosal cell surface. It involves both soluble components (cytokines, complements, defensins, etc) and cells [including macrophages, neutrophils, natural killer (NK) cells, CD8⁺ T-cells with noncytotoxic antiviral activity (CNAR), $\gamma\delta$ T-cells and dendritic cells (DCs)] (figure 1.3; reviewed by [Levy, 2001](#)). Cytokines involved in innate responses determine whether a T-helper (T_h)-1 or a T_h2 immune response is mounted. Tumor-necrosis factor (TNF)- α and interferons (IFNs) also help in controlling the degree of HIV replication. Chemokines are involved in the recruitment of NK cells, T-cells and macrophages to the site of infection. Cytokine production by the innate immune system induces both innate and adaptive cellular responses to HIV infection ([Graziosi et al., 1996](#); [Biron et al., 1999](#); reviewed by [Levy, 2001](#)).

Adaptive immune responses against HIV take longer to mount than innate responses and consists of different phases, including recognition of the antigen, activation of lymphocytes and the effector phase where the antigen is eliminated (figure 1.3). Eventually, memory immune cells are formed, with the B- and T-lymphocytes considered to be the principle effector cells of the antigen-specific immune response. B- and T-cells work in tandem to provide protective immunity; with CD8⁺ T-cells being necessary to control viral replication while antibodies block viral entry into host cells. Since this dissertation focuses on T-cell responses, this review will focus on CD8⁺ and CD4⁺ T-cell immunity to HIV infection.

1.3.2.1. CD8⁺ T-cell responses to HIV

Virus-specific CD8⁺ T-cells are detected relatively early after HIV infection within 2 weeks; and are responsible for the decrease in viral load and the control of viral replication observed during acute infection ([Walker et al., 1987](#)). CD8⁺ T-cell responses occur before the appearance of neutralizing antibodies ([Fiebig et al., 2003](#)). HIV-specific CTL are able to eliminate infected CD4⁺ T-cells and also produce cytokines and chemokines on recognition of HIV antigen fragments

expressed on the target cell surface (peptide: MHC class I complex). The β -chemokines [including macrophage inflammatory proteins (MIP)-1 α , MIP-1 β and regulated-on-activation, normal T-cell-expressed and secreted (RANTES)] produced by the CTLs act to suppress viral replication and entry into target CD4⁺ T-cells (reviewed by [Alfano & Poli, 2005](#)).

The initial decrease in viraemia during acute HIV infection is associated with the emergence of HIV-specific CTLs ([Borrow et al., 1994](#)). They are able to recognise and eliminate HIV-infected cells and are essential for the control of viral replication ([Kaul et al., 2001b](#)). The crucial role played by CD8⁺ T-cells during HIV infection was confirmed in primate studies where CD8⁺ T-cells were depleted using in vivo administration of anti-CD8 monoclonal antibodies in SIV-infected macaques, which resulted in a marked rise in viraemia ([Schmitz et al., 1999](#)). In these experiments, viral RNA levels subsequently decreased as SIV-specific CD8⁺ T-cell numbers were restored, suggesting that these cells were important in controlling the progression of infection ([Jin et al., 1999](#); [Schmitz et al., 1999](#)). Many studies have also shown that CTL escape mutants appear regularly in many HIV-infected individuals, both during acute and chronic stages of infection, indicating that CTLs play an active role in the immune response against HIV and that the CTL-mediated pressure leads to the formation of HIV mutants ([Friedrich et al., 2004](#); [Jones et al., 2004](#)). Although most HIV-specific CD8⁺ T-cells are able to kill infected target cells, they also exhibit other effector functions such as the ability to produce cytokines, including IFN- γ , interleukin (IL)-2, MIP-1 α , MIP-1 β and RANTES ([Betts et al., 2006](#); [Streeck et al., 2008](#)).

The differentiation status of CD8⁺ T-cells has been shown to influence the kind of functions these cells exhibit ([Surh and Sprent, 2008](#)). Naïve CD8⁺ T-cells, which have never been exposed to their cognate antigen before, require several days of antigenic stimulation before becoming activated to produce cytokines. In contrast, memory CD8⁺ T-cells respond faster and produce IFN- γ after only a few hours of antigenic stimulation ([Veiga-Fernandes et al., 2000](#)). Effector memory T-cells are known to release perforin and granzymes within minutes of encountering the antigen ([Lalvani et al., 1997](#)).

1.3.2.2. CD4⁺ T-cell responses to HIV

CD4⁺ T-cells are the known targets for HIV infection but can also recognise and respond to HIV antigens. In fact, of all CD4⁺ T-cell specificities and subsets, HIV-specific CD4⁺ T-cells have been shown to be preferentially infected and depleted by HIV (reviewed by [Okoye & Picker, 2013](#)). The proliferative capacity and ability of these HIV target cells to produce cytokines is lost early during infection but they preserve the capacity to produce IFN- γ during the chronic phase of infection ([Harari et al., 2006](#)). In addition to directly recognizing HIV antigens, cytokine production by CD4⁺ T-cells is known to provide crucial help for the maturation of B- and CD8⁺ T-cell memory ([Mascola and Montefiori, 2010](#)).

During the acute phase of HIV infection, memory CD4⁺ T-cells are drastically depleted from the lymphoid system, especially the gut, both directly by the virus and indirectly via bystander activation-induced cell death. This happens to all memory CD4⁺ T-cells in general but HIV-specific cells are preferentially depleted ([Douek et al., 2009](#); [Mattapallil et al., 2005](#)). HIV-infected individuals who progress slowly to AIDS without treatment (termed slow progressors) have been shown to have stronger CD4⁺ T-cell responses than normal or rapid progressors, and their delayed progression correlated with the rate of disease progression ([Rosenberg and Billingsley, 1997](#)). Furthermore, others have reported a positive relationship between the strength of an infected individuals HIV-specific CD4⁺ T-cell responses and an effective CD8⁺ T-cell response ([Kalams et al., 1999](#)). Two independent studies have shown that IL-21-producing CD4⁺ T-cells have an important role to play in maintain effective CD8⁺ T-cell responses. These cells have been found in higher numbers in elite controllers (individuals with undetectable plasma viral loads and high CD4⁺ T-cell counts in the absence of HAART for longer than 10 years; [Cao et al., 1995](#)) who are able to control their infection ([Chevalier et al., 2011](#); [Williams et al., 2011](#)).

1.4. Memory T-cell subsets

Heterogeneity within the memory T-cells was first characterised using the homing receptor C-C chemokine receptor type 7 (CCR7) ([Sallusto et al., 1999](#)).

CCR7 interacts with its ligand CCL21 expressed by endothelial cells (Gunn et al., 1998) and this interaction causes the adhesion and transmigration of T-cells into the lymph node (Campbell et al., 1998). On the other hand, cells lacking CCR7 migrate mostly through peripheral tissues and are controlled by a different set of integrins and chemokine receptors (Mackay et al., 1990). CCR7⁺ memory T-cells were considered to be TCM because of their capacity to home to the secondary lymphoid tissues. CCR7⁻ memory T-cells are considered as TEM due to their potential to home to peripheral lymphoid tissue and also due to their rapid effector function *ex-vivo*. TEM have downregulated CCR7, allowing them to circulate through the periphery, and are able to immediately produce cytokines after antigen recognition (Unsoeld et al., 2002; reviewed by Mahnke et al., 2013).

Immune memory leads to a faster and more sensitive secondary immune response than naïve cells (reviewed by Mahnke et al., 2013 & Jameson & Masopust, 2009). The CD45RO⁺ memory T-cell population can be further subdivided into three subsets, including effector memory (TEM), central memory (TCM) and tissue-resident memory T-cells (Shin and Iwasaki, 2013). For the purpose of this study, the circulating memory T-cell populations - TEM and TCM - will be discussed in detail.

T-cells can be classified into four idealised basic subsets based on markers of their respective longevity and proliferative capacity: naïve, TCM, TEM and terminally differentiated (TD) T-cells (Jameson and Masopust, 2009). Naïve T-cells (CD45RO⁻CCR7⁺) are long-lived and have not encountered any antigen yet. Their longevity viability relies on certain exogenous signals such as the presence of the cytokine IL-7 or contact with self-peptide/MHC ligands (Appay & Rowland-Jones, 2004; Ma et al., 2006). Memory T-cells respond more rapidly to antigens. TCM cells (CD45RO⁺CCR7⁺) are long-lived cells that have little effector function. They respond to antigen exposure by dividing and differentiating into TD/effector cells. On the other hand, TEM (CD45RO⁺CCR7⁻) are short-lived and respond to antigen with more rapid effector functions than TCM. They also have a lower ability to proliferate and express pro-apoptotic genes (Day and Walker, 2003; Kassiotis and Stockinger, 2004; Riou et al., 2007). It has been suggested

that TCM can maintain longer telomeres (which means they have a longer lifetime and can go through more cycles of cell division and differentiation; [Roth et al., 2003](#)) than TEM cells and are able to generate TEM cells, but not vice-versa ([Sallusto et al., 1999](#)). TD (CD45RO⁺CCR7⁻) are the most differentiated subset of T-cells and most of them die upon restimulation ([Jameson and Masopust, 2009](#)).

The pathway followed during differentiation of memory T-cells is still not clearly understood. Chang et al. (2007) showed that naïve T-cells follow a divergent pathway where progeny of a single naïve T-cell could develop into either effector or memory T-cells (figure 1.4A). This means that memory T-cells are formed directly from naïve T-cells without first becoming effector T-cells ([Chang et al., 2007](#)). An alternative model explaining the formation of memory T-cells was proposed by Hu et al. (2001) (figure 1.4B). Hu et al. (2001) showed, in an adoptive transfer experiment in mice, that effector T-cells have the potential to become memory T-cells directly, without further division, thus suggesting that the development of memory T-cells follows a linear pathway from naïve to memory cells. Other studies have shown that memory T-cells are formed in a non-linear fashion and this depends on certain priming conditions such as the duration of antigenic stimulation or the type of cytokines encountered (reviewed by [Wakim & Bevan, 2010](#); [Srinivasula et al., 2013](#); figure 1.4C). For example, a short period of stimulation results in the formation of TCM while a longer duration of stimulation promotes the differentiation of effector cells into TEM ([Badovinac and Harty, 2007](#); [Sallusto et al., 1999](#)).

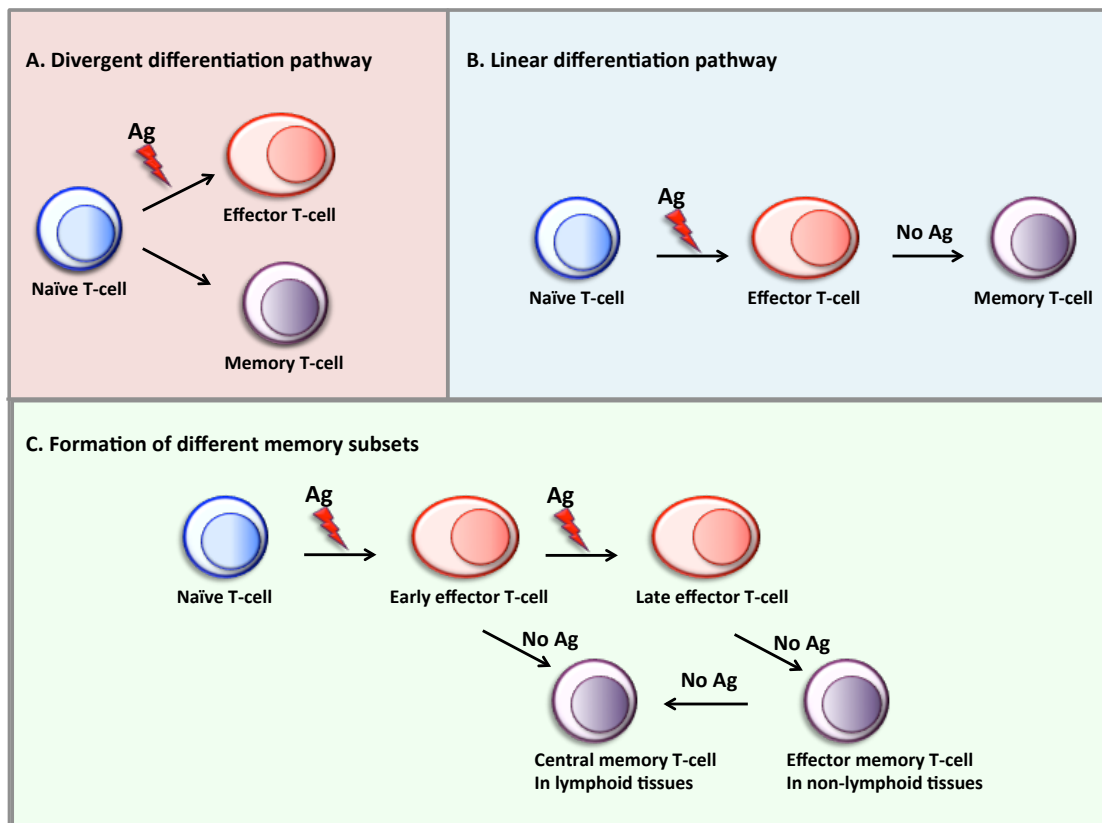


Figure 1.4. Proposed mechanisms for the development of memory T-cells. **A.** Divergent differentiation pathway where daughter cells from a naïve T-cell can develop directly into effector or memory T-cells. **B.** Linear development of effector and memory T-cells where memory T-cells are directly formed from effector cells. **C.** Differential development pathway where the type of memory T-cell formed depends on the duration of antigenic stimulation. (adapted from [Chang et al., 2007](#); [Hu et al., 2001](#); [Badovinac & Harty, 2007](#); [Kaech, Wherry & Ahmed, 2002](#); [Wakim & Bevan, 2010](#))

1.4.1. Role of cytokines in T-cell differentiation

Cytokines belonging to the γ -chain family, for example IL-2, IL-4, IL-7, and IL-15, have a crucial role in the maintenance of T-cells and homeostasis. Being a strong activation, growth and survival factor for T-cells, the haematopoietic cytokine IL-7 is especially important in the survival of naïve T-cells. The mechanism behind this occurs via an intracellular pathway which involves the induction of the anti-apoptotic protein Bcl-2 ([von Freuden-Jeffry et al., 1997](#); [Ma et al., 2006](#)).

T-cells can proliferate in response to cytokines in a T-cell receptor (TCR)-independent manner ([Geginat et al., 2003](#); reviewed by [Seder et al., 2008](#)). IL-2, TNF- α and IFN- γ are the main effector cytokines involved in T-cell differentiation and the formation of T-cell responses (reviewed by [Mahnke et al., 2013](#); figure

1.5). These specific cytokines are also used to define vaccine-generated responses against infections that require T-cells for effective protection (reviewed by [Seder et al., 2008](#)). The amount of IL-2 produced has an impact on the proliferation, differentiation and survival of antigen-specific T-cells and determines whether T-cells will preferentially become terminally differentiated effector cells or memory cells ([Kalia et al., 2010](#)). Strong, sustained IL-2 production by different lymphocyte subsets (including CD4⁺ and CD8⁺ T-cells) have a paracrine effect where it causes other T-cells to undergo chromatin remodelling and display effector functions such as perforin and granzyme expression ([Pipkin et al., 2010](#); figure 1.5A). On the other hand, short-term exposure to IL-2 results in an effector differentiation to a lesser extent and promotes the generation of long-lived memory T-cells (figure 1.5B; [Kalia et al., 2010](#); [Pipkin et al., 2010](#)). TNF- α is one of the first effector cytokines produced by activated CD4⁺ T-cells under conditions that favour T_h1 cellular differentiation ([Darrach et al., 2007](#)). The ability of cells to produce TNF- α is lost as the cells become more differentiated, with terminally-differentiated effector cells lacking the ability to produce TNF- α ([Seder et al., 2008](#)). IFN- γ is only produced by differentiated cells, which have undergone several rounds of proliferation ([Fritsch et al., 2005](#)). Non-lymphoid tissue-resident TEM and TD have been shown to produce more IFN- γ than TCM, suggesting that it is part of a mechanism for more rapid responses to re-infections (figure 1.5C; [Fritsch et al., 2005](#); [Hamann et al., 1997](#)).

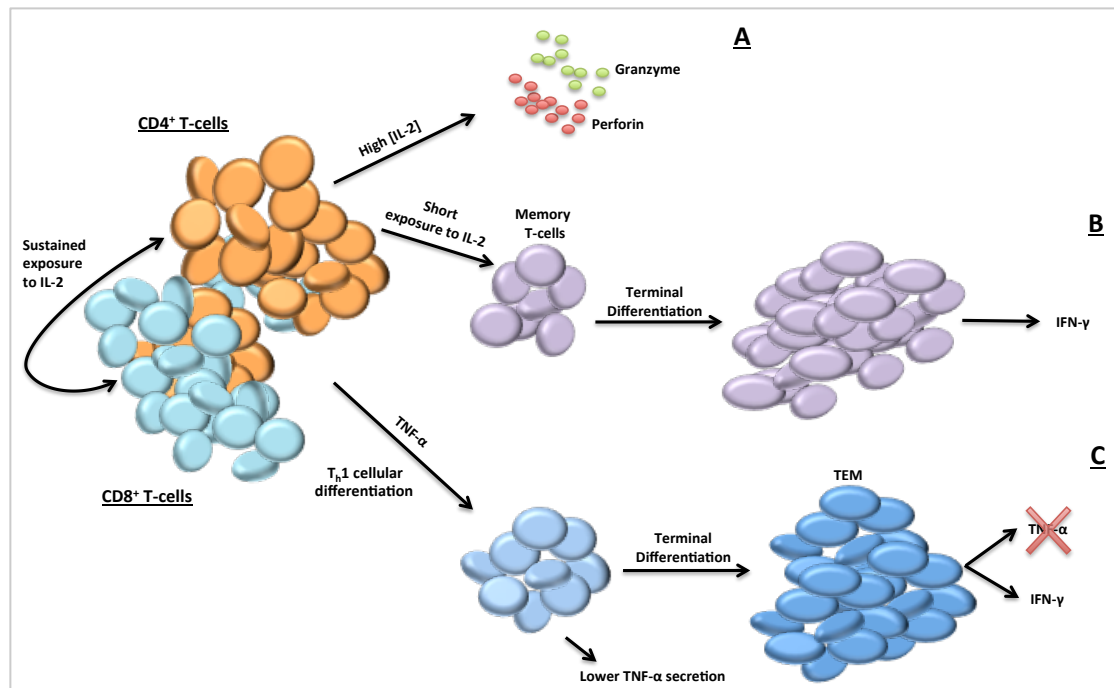


Figure 1.5. Role of cytokines in T-cell differentiation and the generation of immune responses. **A.** Strong, persistent IL-2 production causes the activated T-cells to secrete effector molecules such as granzyme and perforin. **B.** Short-term exposure to IL-2 results in the differentiation of T-cells into long-lived memory T-cells. **C.** TNF- α production by the less differentiated cells favours T_h1 cellular differentiation while IFN- γ is only produced by differentiated cells.

1.4.2. HIV preferentially infects memory T-cell subsets

Several studies have reported that HIV preferentially targets specific CD4⁺ T-cell subsets based on factors like the level of activation and expression of HIV co-receptors CCR5 and CXCR4 (reviewed by Grossman et al., 2006 & Picker, 2006). Memory CD4⁺ T-cells are the main targets of HIV (Schnittman et al., 1990) and a massive depletion of this cell population occurs during primary infection, both systemically and at mucosal sites (Mattapallil et al., 2005). TEM express higher levels of CCR5 than other memory T-cell subsets, while CXCR4 is predominantly expressed on TCM and naïve cells (Sallusto et al., 1998; Geginat et al., 2001). Since HIV first infects activated CD4⁺ T-cells and then relies on them for dissemination following transmission across mucosal surfaces, the differentiation status of mucosal CD4⁺ T-cells and their level of expression of CCR5 co-receptor have been proposed to determine risk of HIV transmission. Saba et al. (2010) showed that CD4⁺ T-cells in mucosal tissues are predominantly

CCR5-expressing TEM. TCM have been identified as one of the major cellular compartments of the latent HIV reservoir ([Chomont et al., 2009](#)).

During an HIV infection, the proportion of the different CD4⁺ T-cell subsets can be used to indicate the extent of disease progression (reviewed by [McCune, 2001](#)). As disease progresses, there is an accumulation of short-lived, highly differentiated T-cells, resulting in immunosenescence and immune dysfunction. Thus, having increased frequencies of TD cells can be considered to be a sign of clinical immunodeficiency (reviewed by [Appay & Sauce, 2008](#)).

It is worth noting that naïve T-cells undergo distinct differentiation patterns to generate effector and memory T-cells (reviewed by [Seder & Ahmed, 2003](#); [Sallusto et al., 2004](#)). It has been proposed that CD4⁺ T-cells follow a linear differentiation pathway by which they gain their effector functions ([Wu et al., 2002](#)): The more the CD4⁺ T-cells that are stimulated, the lower the potential of forming memory T-cells (figure 1.6a). In contrast, CD8⁺ T-cells fully differentiate into activated effector cells upon stimulation that can follow different development pathways to form either memory T-cells or terminally differentiated cells (figure 1.6b; reviewed by [Seder & Ahmed, 2003](#)). It has been suggested that naïve CD8⁺ T-cells are able to differentiate into effector cells more rapidly than CD4⁺ T-cells and that CD8⁺ T-cells have greater proliferative capacity upon encountering an antigen, and are thus able to establish a stable population of memory T-cells after the contraction phase than CD4⁺ T-cells ([Homann et al., 2001](#)). On the other hand, naive CD4⁺ T-cells proliferate to a lower degree and a higher percentage of the activated cells die after the contraction phase ([Homann et al., 2001](#); [Foulds et al., 2002](#); reviewed by [Seder & Ahmed, 2003](#)).

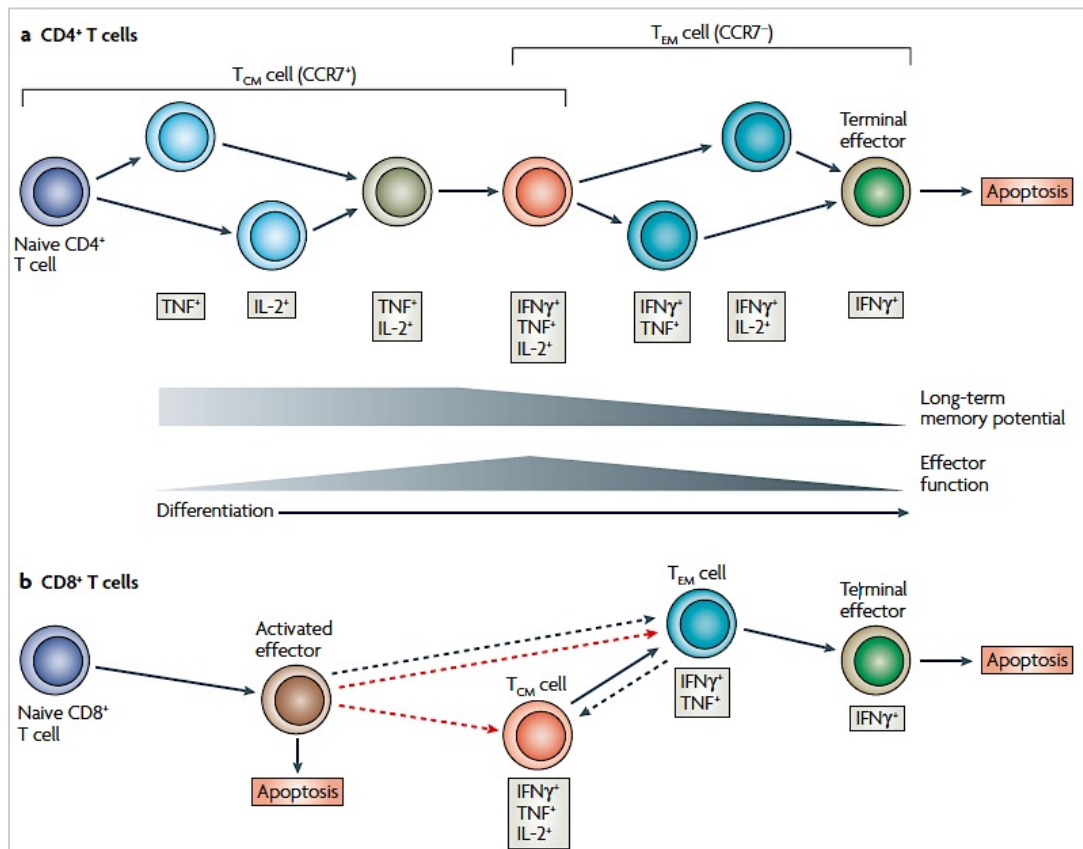


Figure 1.6. Models for effector and memory T-cell differentiation for CD4⁺ and CD8⁺ T-cells. **a.** Naïve CD4⁺ T-cells follow a linear development pathway where they progressively gain functionality with further differentiation until they are optimised for their effector function. Persistent antigenic stimulation leads to the formation of a higher proportion of terminally differentiated T-cells and a lower frequency of memory T-cells. **b.** When stimulated, naïve CD8⁺ T-cells directly form activated effector CD8⁺ T-cells, most with cytolytic activity. These cells can follow two different pathways to form either central or effector memory T-cells. Persistent antigenic stimulation then leads to the formation of terminally differentiated cells followed by apoptosis (taken from [Seder et al., 2008](#)).

1.4.3. Exhaustion of HIV-specific T-cells

It has been suggested that exhausted T-cells represent a separate and distinct state of T-cell differentiation (reviewed by [Wherry, 2011](#)). T-cell exhaustion as a result of chronic HIV infection is characterised by a state of T-cell dysfunction, reduced effector function and an altered transcriptional state compared to functional effector cells or memory T-cells. High levels of persistent cellular activation has been proposed as a major trigger of proliferation and T-cell differentiation which results in the formation of cells with increased expression of CD57 (reviewed by [Appay & Sauce, 2008](#)). CD57 is an epitope expressed on T-lymphocytes and is considered to be a marker of replicative senescence. It

defines cells with a high susceptibility to activation-induced cell death and low proliferative capacity even though they retain the ability to rapidly induce the production of cytokines in the presence of antigens (Brenchley et al., 2003; Burgers et al., 2009; Lopez-Vergès et al., 2010; Papagno et al., 2004). Several studies have shown the surface marker CD57 to be a reliable marker of replicative senescence in T-cells and NK cells (Brenchley et al., 2003; Ibegbu et al., 2005; Lopez-Vergès et al., 2010). During HIV infection, T-cell exhaustion can be caused by several factors including high viral load, the lack of CD4⁺ helper T-cells, inflammation and antigen-presenting function (Barber et al., 2006; Papagno et al., 2004; Wherry et al., 2007). Effros et al. (1996) suggested that T-cells expressing CD57 have reached the irreversible shortening of telomeres that restricts cell division (also known as the Hayflick limit), suggesting that HIV induces more replication cycles in T-cells from infected-individuals compared to T-cells from seronegative individuals, leading to exhaustion of T-cell responses. Palmer et al. (2005) reported that CD57 was expressed at significantly higher levels in the TD compared to TCM or TEM subsets of CD4⁺ T-cells during HIV infection, suggesting that the most terminally differentiated CD4⁺ T-cell subset was expressing the highest levels of CD57. In CD8⁺ T-cells, increased CD57 expression has been associated with chronic immune activation during HIV infection and frequencies of CD8⁺CD57⁺ T-cells correlate with the stage of HIV disease progression (Betts et al., 2005; Papagno et al., 2004; Wood et al., 2005). Some controversy has emerged about the proliferative capacity of CD8⁺CD57⁺ T-cells: Chong et al. (2008) showed that CD8⁺CD57⁺ T-cells were capable of rapidly expanding in numbers and this was accompanied by the production of high levels of IL-15 while others have shown that they have limited proliferative ability (Wherry et al., 2005; Barber et al., 2006; reviewed by Focosi et al., 2010).

The function of CD8⁺CD57⁺ T-cells during HIV infection is unclear. EBV- and CMV-infected (but HIV-negative) individuals exhibit a coordinated up-regulation of CD57 with down-regulation of CD27 [a T-cell co-stimulatory receptor necessary for the generation and maintenance of T-cell memory (Hendriks et al., 2000)] by memory CD8⁺ T-cells. In contrast, HIV-infected individuals accumulate CD27^{high}CD57^{low} HIV-specific CD8⁺ T-cells, which correlate with plasma viraemia

(Hoji et al., 2007). Hoji et al. (2007) argued that this suggests that HIV-specific CD8⁺ T-cells maintain impaired effector differentiation during the course of HIV infection and this lack of normal differentiation could have a role to play in the failure of the immune system to control HIV infection.

1.5. Immune activation

It is now widely accepted that generalised immune activation plays a major role to play in HIV pathogenesis. The immune system is crucial in controlling HIV infection and replication but long-term control has been associated with chronic immune activation and inflammation, subsequently leading to the exhaustion of the host immune system and the onset of non-AIDS-defining illnesses (Deeks, 2011; Hunt, 2012). Immune activation in HIV infection encompasses several related events including cellular activation, proliferation, and ultimately cellular death and the production of soluble immunomodulatory molecules. During chronic immune activation, T-cell activation has been shown to better predict CD4⁺ T-cell loss and disease progression than plasma viral loads (Deeks et al., 2004). Expression of CD38 and HLA-DR by memory T-cells indicate the response of the immune system against HIV antigens and is strongly associated with the degree of systemic immune activation (Appay and Sauce, 2008).

1.5.1. Causes of immune activation during HIV infection

1.5.1.1. Direct activation as a result of HIV replication

The primary cause of T-cell activation is thought to be through the direct stimulation of T-cells by HIV antigens (Rieckmann et al., 1991). During acute HIV infection, HIV induces T-cell responses (especially by CD8⁺ T-cells), which persist throughout the infection. Binding of gp120 to the CD4 receptor is able to trigger/enhance T-cell activation via the production of TNF- α (Lee et al., 2003; Rieckmann et al., 1991). HIV *Nef* is also involved in the activation of lymphocytes through the down-regulation of the TCR-CD3 complex on infected cells (Wang et al., 2000). The degree of CD8⁺ T-cell activation is also dependent on whether these cells are stimulated by the dominant or subdominant HIV epitopes (Kaul et

al., 2001a; Kiepiela et al., 2007). CD8⁺ T-cells specific for the more dominant epitopes, such as p24 Gag, have a more diverse V β repertoire than those which recognise the less dominant epitopes like *Env*, and the degree of activation and clonal expansion may therefore be greater for the dominant epitopes than for the sub-dominant ones (Kiepiela et al., 2007). In addition, the frequencies of multifunctional CD8⁺ T-cells are higher for the immunodominant epitopes than for the subdominant ones (Betts et al., 2006).

In HIV-infected individuals on HAART, one of the triggers of immune activation could be ongoing HIV replication below the detection limit of clinically available assays (Maldarelli et al., 2007). In addition, intensification of the treatment does not seem to reduce the level of immune activation, except for only one clinical trial with raltegravir which showed a significant reduction in T-cell activation after 48 weeks (Llibre et al., 2012).

1.5.1.2. Bystander activation of T-cells

HIV has also been proposed to cause immune activation via indirect mechanisms (reviewed by Moir et al., 2011 & Hunt, 2012). It is a well-documented fact that the direct killing of HIV-infected cells is not the predominant mechanism for the massive depletion of CD4⁺ T-cells that happens during the chronic phase of infection (reviewed by Douek et al., 2003; Doitsh et al., 2014). Several studies have also shown that HIV-specific cellular responses can induce activation of surrounding immune cells, an effect termed as bystander effect (Li et al., 2005; reviewed by Brenchley & Douek, 2008 & Moir et al., 2011). This includes activation of cells through membrane-bound molecules that bind to receptors other than TCRs or through soluble factors. (reviewed by Lawn & Butera, 2001; Bangs et al., 2006). The depletion mainly occurs due to the activation-induced cell death of uninfected cells (Silvestri and Feinberg, 2003). HIV infection has been associated with changes in the inflammatory milieu and this alone could lead to bystander activation. Certain cytokines (including IL-2, IL-6, IL-7 and IL-15) are capable of making resting cells more susceptible to HIV infection (Clerici and Shearer, 1993; Unutmaz et al., 1999). Type I IFNs and lipopolysaccharide

(LPS) have also been shown to be potent cell activators of naïve and memory T-cells in a murine models and in humans (Tough et al., 1996; Kamath et al., 2005; De et al., 2005).

1.5.1.3. Microbial translocation induces immune activation

HIV infection has been linked to gut epithelial cell apoptosis and barrier dysfunction, as a result of the massive CD4⁺ T-cell depletion that occurs in the gut-associated lymphoid tissue (Jiang et al., 2009; reviewed by Klatt et al., 2013). This phenomenon, in conjunction with the preferential loss of T_h17 cells, is termed microbial translocation. Because of this damage to the gut epithelial barrier, there is an increased absorption of microbial products systemically (such as LPS) across the damaged mucosal barrier, and increased concentration of these microbial products in blood has been implicated in chronic T-cell activation in blood, and the eventual loss of CD4⁺ T-cell-mediated immunity (Brenchley et al., 2006). Subsequent studies have confirmed that levels of microbial products in blood decrease in HIV-infected individuals who have initiated HAART, but these do not normalise to pre-HIV-infection levels and continue to induce systemic monocyte and T-cell activation as well as poorer T-cell recovery (Jiang et al., 2009; Marchetti et al., 2008).

1.5.1.4. Co-infections leading to sustained immune activation

HIV-infected individuals often have other chronic viral infections which get worse during untreated HIV infection, and these may exacerbate the level of systemic immune activation. The most common co-infection evident during untreated HIV infection is chronic CMV infection, which accounts for ~10% of circulating memory T-cell specificities in HIV uninfected individuals (Sylwester et al., 2005). In HIV-positive individuals, even if they have been on HAART for a long period of time, the CMV virus generates higher frequencies of CMV-specific T-cell responses, contributing to the inflammatory environment (Naeger et al., 2010).

1.5.2. Role of mucosal antigens in chronic immune activation

Depletion of mucosal cells is rapidly followed by a state of generalised immune activation ([Brenchley et al., 2004](#); [Mattapallil et al., 2005](#); reviewed by [Xu et al., 2013](#)). This is categorised by increased numbers of T-cells expressing activation markers, higher frequencies of memory T-cells, a faster rate of B- and T-cell turnover and increased levels of pro-inflammatory cytokines (reviewed by [Grossman et al., 2006](#)). In mucosal tissues, all T-cells - not just HIV-specific T-cells - become activated during HIV infection, although the exact reasons for this are not clear. Grossman et al. (2006) suggested that the increased turnover of mucosal T-cells may be a result of recurrent antigenic stimulation and not a homeostatic response to the killing of infected or uninfected cells. They also propose that chronic activation may induce bursts of lymphocyte proliferation, differentiation and death ([Grossman et al., 2006](#)). These overlapping bursts may regenerate the pool of resting memory T-cells and thereby generate a variety of short-lived but differentiated effector T-cells which can migrate to mucosal effector sites ([Picker et al., 2004](#)).

1.5.3. Immune activation and inflammation

In addition to HIV antigens, the production of type I IFNs, mainly produced by plasmacytoid DCs, has been proposed to be a major trigger of immune activation (reviewed by [Reizis et al., 2011](#)). Activated plasmacytoid DCs and IFN- α have been shown to significantly contribute to chronic immune activation ([Martinson et al., 2010](#)). High levels of pro-inflammatory cytokines (such as TNF- α , IL-6, IL-1 β) as well as chemokines (such as MIP-1 α , MIP-1 β and RANTES) are found both in blood and mucosal tissues during the early stages of HIV infection ([Freer & Matteucci, 2009](#); reviewed by [Shey et al., 2013](#)).

1.5.4. T-cell turnover during HIV infection

The intracellular nuclear protein Ki67 is necessary for cellular proliferation and is a useful marker for T-cell proliferation ([Scholzen and Gerdes, 2000](#)). In the context of HIV infection, Ki67 expression has also been used as a marker of cellular activation ([Sachsenberg et al., 1998](#)). In a longitudinal study carried out

by Al-Harathi et al. (2007) comparing CD4⁺ T-cell dynamics during acute to early-treated HIV infection, they reported a significant reduction in the expression of Ki67 by CD4⁺ memory and naïve T-cells among the treated compared to untreated individuals. These findings suggested that the increase in Ki67 in CD4⁺ memory T-cells is mainly driven by the individual's viral load (Al-Harathi et al., 2007). In CD8⁺ T-cells, Hazenberg et al. (2000) reported that a decrease in plasma viral load induced by HAART was associated with a decrease in the frequencies of dividing CD8⁺ T-cells. They concluded that the T-cell proliferation seen during a chronic HIV infection was a consequence of generalised immune activation (Hazenberg et al., 2000).

1.5.5. Evidence for the role of immune activation in HIV/SIV pathogenesis

1.5.5.1. Evidence from natural hosts of SIV infection

Studies in non-human primates showed that natural hosts of SIV infections (such as sooty mangabeys and African green monkeys) often had lower levels of immune activation despite having higher viral loads than non-natural hosts such as rhesus macaques (Broussard et al., 2001). In these natural hosts, chronic SIV infection was accompanied by low levels of CD4⁺ and CD8⁺ T-cell activation and proliferation (Chakrabarti et al., 2000; Silvestri et al., 2003), reduced levels of T-cell apoptosis (Kim et al., 2007), and conserved bone marrow, thymus and lymph node T-cell regenerative capacity (Muthukumar et al., 2005; Silvestri et al., 2003). In addition, the overall levels of T-cell activation and proliferation in acute SIV infections in sooty mangabeys were much lower than those seen during the acute phase of pathogenic SIV infections in rhesus macaques. Furthermore, Pandrea et al. (2008) showed that immune activation could be induced in African green monkeys using microbial products to mimic microbial translocation. This induced immune activation in African green monkeys resulted in a significant level of mucosal CD4⁺ T-cell loss and a rise in viral loads, confirming that the levels of immune activation and proliferation of target cells have a marked impact on the pathogenicity of SIV/HIV infections. Pandrea et al. (2008) suggested that therapeutic strategies aimed at reducing immune activation, used

in conjunction with HAART, might be effective in halting the progression to AIDS in chronically infected people.

1.5.5.2. HIV-2 infection

Compared to infections with HIV-1, HIV-2 infection is characterised by significantly slower rates of progression and HIV-2-infected individuals are able to maintain higher CD4 counts compared to HIV-1-infected individuals (Rowland-Jones and Whittle, 2007). Studies have shown that the lower pathogenicity of HIV-2 was due to lower levels of lymphocyte activation (Michel et al., 2000; Jaffar et al., 2005). Michel et al. (2000) reported that markers of immune activation (β_2 -microglobulin and HLA-DR), which are associated with disease progression in both HIV-1 and HIV-2 infected individuals, were lower in HIV-2 than HIV-1 infected individuals.

1.5.5.3. Methods to modulate immune activation

Statins are anti-cholesterol drugs, which have been found to have *in-vitro* anti-inflammatory and potential anti-viral effects (reviewed by Jain & Ridker, 2005). Ganesan et al. (2011) evaluated the effect of atorvastatin on the level of HIV-1 RNA and T-cell activation markers in the blood of HIV-infected individuals. Despite finding no change in the plasma HIV-1 RNA levels, they reported a significant decrease in T-cell activation, especially in the CD8⁺ T-cell population (Ganesan et al., 2011). The antimalarial drug hydroxychloroquine is known to have immunomodulatory properties and is able to decrease inflammatory cytokine production (Karres et al., 1998). Murray et al. (2010) showed that chloroquine-treated HIV-infected individuals had decreased levels of memory CD8⁺ T-cells activation (measured by CD38 and HLA-DR expression), decreased CD4⁺ and CD8⁺ T-cell proliferation and lower levels of LPS in plasma compared to those not being treated with chloroquine. They reported no change in the level of plasma HIV-1 RNA (Murray et al., 2010). Similarly, another drug called Leflunomide, an anti-rheumatoid arthritis drug that inhibits pyrimidine synthesis, has been tested for its ability to reduce immune activation during HIV infection (Read et al., 2010). HIV-infected individuals treated with Leflunomide

had reduced numbers of proliferating T-cells, lower frequencies of activation marker (HLA-DR/CD38) expression by CD8⁺ T-cells and lower levels of co-receptor (CCR5 and CXCR4) expression on both CD4⁺ and CD8⁺ T-cells than untreated individuals (Read et al., 2010). Therapy with prednisolone, a glucocorticoid used to treat various inflammatory and auto-immune conditions, during HIV infection was found to reduce activation of CD8⁺ T-cells (measured by CD38 expression) and reduce the concentration of molecules known to drive immune activation (including soluble CD14, LPS-binding protein, soluble urokinase plasminogen activator receptor, and soluble CD40L; Kasang et al., 2012).

1.6. HIV infection and the female genital tract

1.6.1. Anatomy of the female genital tract

The female genital tract consists of two main types of mucosal tissues – type I and type II mucosa (reviewed by Xu et al., 2013). The upper genital tract (the endocervix and endometrium) is made up of the type I mucosal surface, which is covered with a single layer of columnar epithelial cells with tight junctions and high levels of IgA (reviewed by Xu et al., 2013; Figure 1.7). In contrast, the lower genital tract (comprising the vagina and ectocervix) is made up of type II mucosal tissues, with stratified squamous epithelia that lack luminal IgA and mucosa-associated lymphoid tissues (reviewed by Xu et al., 2013).

While the mucosa of the gastrointestinal tract and the lungs are lined with a single layer of epithelial cells (necessary for food absorption and air exchange respectively), the cervicovaginal mucosa of the female reproductive tract differs in that it is covered with multi-layered stratified squamous epithelia. In addition, the mucus composition within the female genital tract, commensal microbiome and local immune responses all differ from the other mucosal tissues (reviewed by Iwasaki, 2010). The cervical transformation zone, which marks the transition between the ectocervix and the endocervix, is the part of the female genital tract most densely populated with T-cells and APCs (reviewed by Iwasaki, 2010; Figure 1.7). The rest of the vaginal mucosa contains much lower

frequencies of these immune cells. The density of immune and HIV target cells at the cervical transformation zone has led many to suggest that this site may be the major site for both cell-mediated immune responses and vulnerability to HIV infection ([Pudney et al., 2005](#); [Kaushic, 2009](#); reviewed by [Iwasaki, 2010](#)). The distribution of epithelial cells in the genital tract is shown in figure 1.7.

Within the female genital tract, the relative susceptibility of the vaginal, ectocervical and endocervical mucosa to HIV infection is unclear although some have suggested that transmission can potentially occur in all three areas ([Hladik and Hope, 2009](#)). HIV infection could occur via translocation of HIV particles across the columnar epithelium of the endocervical canal, which can then infect sub-epithelial mononuclear cells ([Mestecky et al., 2009](#)). The single layer of polarised epithelium of the endocervix may be more susceptible to HIV transmission than the non-polarised multilayer ectocervix and vagina but factors like micro-ulcerations, breaks in the epithelium or co-infections with sexually transmitted diseases may increase the risk of transmission at these multi-layered sites ([Broliden et al., 2008](#); [Pope and Haase, 2003](#)). However, the vaginal wall and the ectocervix have a much larger surface area than the endocervix and breaches in their epithelium would also allow the penetration of HIV into the mucosa ([Hladik and Hope, 2009](#)). Stromal DCs have also been implicated in HIV transmission: in addition to expressing CD4, they express CCR5 (so can be productively infected; [Prakash et al., 2004](#)) and also express dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN; which can bind and internalise HIV particles into DCs without causing a productive infection; [Jameson et al., 2002](#)).

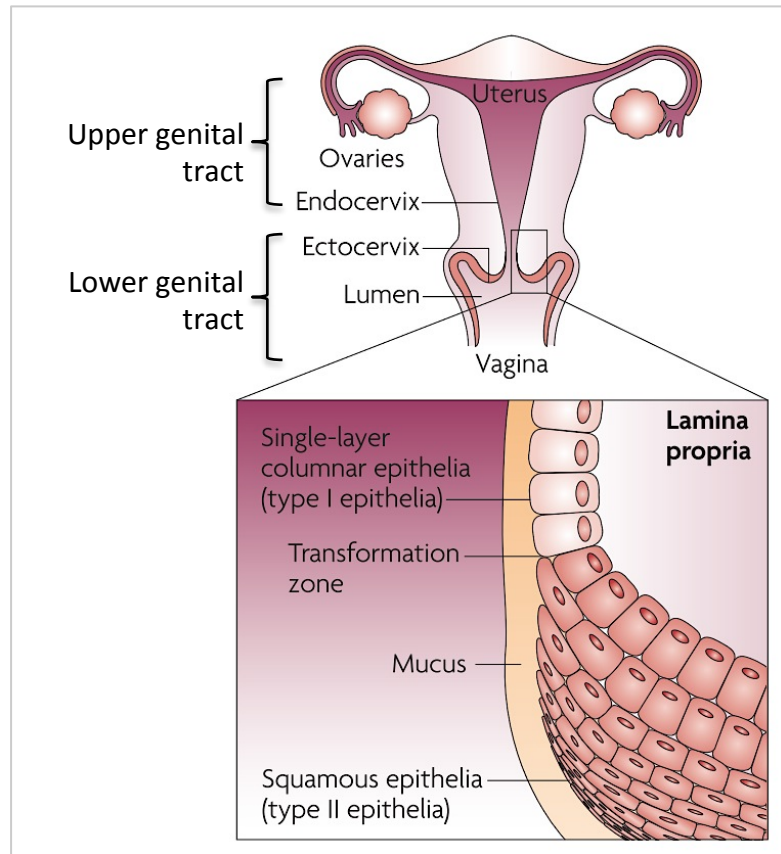


Figure 1.7. Structural characteristics of the female genital tract mucosa. The female genital tract consists of the upper genital tract (uterus and endocervix) which is made up of type I epithelia and the lower genital tract (vagina and ectocervix), made up of type II epithelia. The transformation zone forms the boundary joining the columnar epithelial cells and the stratified squamous epithelium (adapted from [Iwasaki, 2010](#)).

1.6.2. Determinates in the genital mucosa on rate of HIV transmission

The vast majority of new HIV infections occur via sexual transmission, involving either the cervicovaginal or rectal mucosa in adults (reviewed by [Hladik & Hope, 2009](#)). Studies have proposed that rates of male-to-female transmission of HIV are higher than female-to-male rates ([Boily et al., 2009](#); [Hughes et al., 2012](#)). Despite these estimates, others have argued that transmission across the female cervicovaginal mucosa is relatively inefficient because of the multi-layered squamous epithelium that protects the vaginal vault in healthy women with no ulcerative or inflammatory sexually transmitted infections ([Hughes et al., 2012](#)). A study in HIV-1 serodiscordant couples from Africa estimated that as few as 1/900 unprotected sexual exposures actually resulted in HIV transmission ([Hughes et al., 2012](#)). The rate of HIV transmission was shown to depend on viral load of the partner (a major determinate of semen viral load) ([Pope and Haase,](#)

2003). In women, cervicovaginal mucus acts as a physical barrier to HIV, blocking the movement of viral particles and preventing contact of infectious particles with the female cervicovaginal mucosa (Shukair et al., 2013).

Although an HIV-infected individual remains “infectious” to their sexual partners throughout the course of their disease, several studies have suggested that rates of transmission are higher during the acute (primary) and late stages of an HIV infection because of the increased viral loads during these stages of disease (Anderson and May, 1988; Longini et al., 1989). A landmark study by Wawer et al. (2005) in Uganda showed that the probability of HIV transmission to uninfected partners during the first five months of infection was about 8-10 times higher than during the chronic phase and that the probability of transmission also increased by 4-8 times during the two years before death (Wawer et al., 2005).

Use of long-acting injectable hormonal contraception methods in women has been shown to almost double the risk of vaginal HIV transmission (Heffron et al., 2012). Studies conducted in primates infected vaginally with SIV have suggested that this most likely happens due to the thinning of the vaginal or the ectocervical epithelium (Veazey et al., 2012).

1.6.3. Anti-viral immunity in the female genital tract

Because HIV transmission is estimated to be relatively inefficient, with <1% of unprotected sexual exposures resulting in a productive HIV infection, some have suggested that HIV must be quite vulnerable during the new infection of a host (Kaul et al., 2008). The virus must encounter several barriers before transmission can occur. Together, the vaginal or cervical epithelial layer, mucus in the genital tract, acidic pH and innate immune responses generated by commensals (via the production of anti-microbial components) participate to protect against HIV infection in the genital tract (Fredricks et al., 2005; Shukair et al., 2013).

In response to HIV infection, the squamous and columnar epithelial cells of the genital mucosa (shown in figure 1.8) are known to produce pro-inflammatory cytokines (such as IL-6, IL-8, and IFN- β) and anti-microbial peptides (such as defensins; [Kayisli et al., 2002](#); [Valore et al., 1998](#)). Tissue-resident macrophages, dendritic cells and $\gamma\delta$ T-cells (figure 1.8) also secrete as cytokines and type I IFNs (reviewed by [Iwasaki, 2010](#)). $\gamma\delta$ T-cells within the mucosa have also been shown to be able to lyse HIV-infected target cells and produce suppressor factors such as RANTES, MIP-1 α and MIP-1 β , which bind to CCR5 and reduce SIV/HIV infection ([Lehner et al., 2000](#)).

DCs within the genital mucosa have been proposed to be the link between innate and adaptive immune responses as they most efficiently present the foreign antigens to naïve T-cells ([Cunningham et al., 2008](#)). The female genital tract contains several subsets of DCs, including submucosal plasmacytoid, myeloid DCs and Langerhans cells (figure 1.8; [Cunningham et al., 2008](#)). Instead of just priming T-cell responses during HIV infection, DC subsets, which express DC-SIGN, can also cause infection because they can internalise HIV without being infected themselves and then transport the endocytosed viral particles across the epithelium and transmit them to CD4⁺ T-cells ([Ballweber et al., 2011](#)). DC-SIGN is a membrane protein that binds to the envelope of HIV and causes adhesion to DCs and the DC-SIGN⁺ DCs are mainly found in the sub-epithelial lamina propria ([Geijtenbeek et al., 2000](#); [Jameson et al., 2002](#)).

At mucosal surfaces such as the genital tract, induction of T-cell responses is essential for protection against infections, with CD8⁺ T-cells mainly participating in viral clearance ([Reynolds et al., 2005](#); [Veazey et al., 2003](#)) and CD4⁺ T-cells mainly involved in host protection ([Mascola & Montefiori, 2010](#); [Schoenberger et al., 1998](#); reviewed by [Iwasaki, 2010](#)). Synergy between CD4⁺ and CD8⁺ T-cell responses are essential during HIV infection as CD8⁺ T-cells play a major role in controlling infection, although they are dependent on the functionality of CD4⁺ T-cells for help during their priming and differentiation phases and for the formation of memory T-cells, and (reviewed by [Iwasaki, 2010](#)). Figure 1.8

summarises the various innate and adaptive immune responses involved in eliminating an infection in the genital tract.

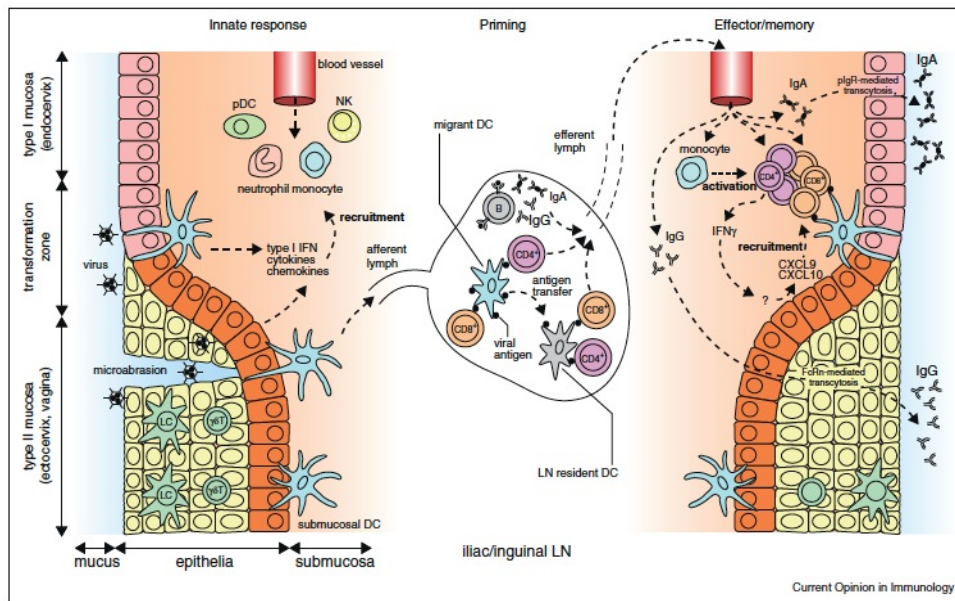


Figure 1.8. Anti-viral immune responses in the female genital tract during HIV infection. The transformation zone between the columnar epithelium of the endocervical canal and endocervix and the squamous epithelium of the ectocervix form the boundary between these two tissues. During infection, epithelial cells and innate leukocytes secrete type I IFNs, cytokines and chemokines to recruit DCs, neutrophils, monocytes and NK cells. Viral particles are endocytosed by DCs and presented to naïve T-cells. Activated T-cells traffic to the site of infection. Virus-specific IgG is transcytosed to the vaginal lumen for additional protection (taken from [Kumamoto & Iwasaki, 2012](#)).

1.6.4. Mucosal CD4⁺ T-cell depletion during HIV infection

Within the genital mucosa, HIV has been proposed to be able to infect various CD4⁺ immune subsets expressing CCR5 (or CXCR4), including lymphocytes, DCs, and other cells of the macrophages/monocyte lineage throughout the lamina propria and epithelia (reviewed by [Hladik & McElrath, 2009](#)). Although the other CD4 expressing immune subsets have been suggested to be infectable with HIV, it is widely accepted that CD4⁺ T-cells are likely to be the initial and main target for HIV infection ([McKinnon and Kaul, 2012](#)). CD4⁺ T-cells in the female genital tract are dispersed through the sub-mucosa of the vagina, ectocervix and endocervix (reviewed by [Shacklett, 2009](#)). They are predominantly activated memory T-cells that express high levels of CCR5 compared to T-cells in the peripheral blood ([Prakash et al., 2001](#); [Rancez et al., 2012](#)). In vitro studies using

human cervical explant tissue have shown that HIV efficiently targets CD4⁺ T-cells in the genital mucosa and this occurs even in the absence of Langerhans cells (Gupta et al., 2002). Since higher frequencies of activated memory CD4⁺ T-cells reside at mucosal surfaces than in blood, it is important to study mucosal CD4⁺ T-cells in the context of HIV infection. Studies in SIV-infected macaques have shown that ~60% of mucosal memory CD4⁺ T-cells are infected in the first days of infection, in contrast to only ~ 1% of mucosal CD4⁺ T-cells being infected during the chronic phase (Mattapallil et al., 2005). Less is known about proportion of CD4⁺ T-cells in the female genital tract that are infected by HIV although previous studies have shown that genital tract CD4⁺ T-cells are significantly more activated (expressing CD38, HLA-DR and CCR5) than those circulating in blood (Cohen et al., 2010; Prakash et al., 2001).

Because of active HIV infection and death, it has been postulated that mucosal CD4⁺ T-cell counts remain low throughout the course of HIV infection (purple line in figure 1.9) despite the fact that activated cells consistently traffic to the mucosa to replenish CD4⁺ T-cell numbers (Brenchley et al., 2004; Mehandru et al., 2004). Activation-induced and bystander cell death has been proposed as the predominant mechanisms for this although a smaller proportion of the T-cells are directly killed by the virus (Grossman et al., 2002; Hellerstein et al., 2003). Studies in rhesus macaques infected with SIV have shown that memory CD4⁺ T-cells within mucosal tissues have a half-life of ~2 weeks (Picker et al., 2004). Effector memory T-cells are generated by long-lived cells that form only a small fraction of the T-cells activated during infection (Mehandru et al., 2004). As a result, studies have shown that reconstitution of long-lived CD4⁺ T-cells will inevitably be very slow once the mucosal effector CD4⁺ T-cells have been depleted (Guadalupe et al., 2003; Mehandru et al., 2004).

In HIV-infected individuals on HAART, studies have shown that recovery of mucosal CD4⁺ T-cells is slower than that observed in blood (reviewed by Grossman et al., 2006). One of the reasons proposed was that the regeneration of TEM happens through the slow accumulation of long-lived T-cells, which are only a small proportion of the recently activated cells (Guadalupe et al., 2003).

The incomplete mucosal CD4⁺ T-cell reconstitution in individuals on HAART has also been attributed to several other factors such as low levels of viral replication happening below detectable level, the persistent highly inflammatory environment in the genital tract or the low bioavailability of some antiretroviral drugs in the genital mucosa (Kwara et al., 2008; Mkhize et al., 2010; Okoye and Picker, 2013).

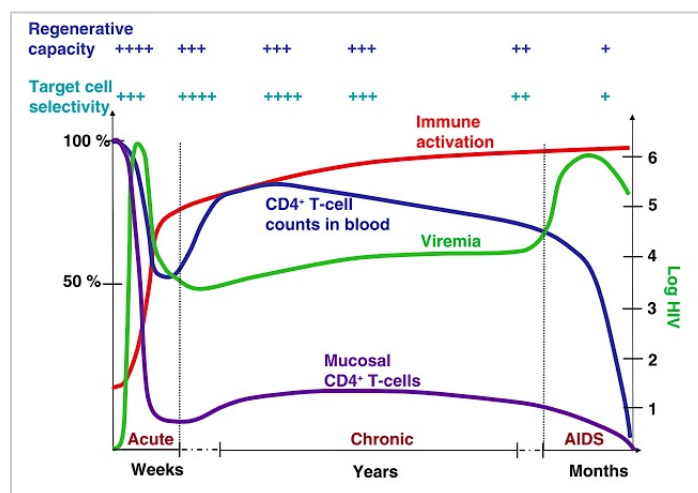


Figure 1.9. Qualitative measures of disease progression in blood and the mucosa. In the acute phase, as viral loads go up, CD4⁺ T-cell numbers go down, to a much higher degree in mucosal tissues. In the chronic phase, mucosal CD4⁺ T-cell numbers remain low. At the onset of AIDS, changes seen in the chronic phase accelerate, the cellular regenerative capacity decreases and mucosal CD4⁺ T-cells reach very small frequencies (taken from Grossman et al., 2006).

1.6.5. Role of genital tract inflammation during an HIV infection

During acute infection, the cytokine cascade that is mounted as a result of infection has been suggested to have contrasting effects (Li et al., 2009). While these cytokines may be important to up-regulate anti-viral responses in the host to control viral replication, they have also been proposed to be detrimental as they increase the frequency of target cells being recruited to the site of infection and cause the activation of CD4⁺ T-cells which are the main target cells for the virus (Li et al., 2009; reviewed by Haase et al., 2010; Katsikis et al., 2011). Cytokines such as TNF- α can increase anti-viral immunity but it has also been directly linked to enhanced viral replication through the induction of NF- κ B (Osborn et al., 1989; Nkwanyana et al., 2009). Li et al. (2009) showed that

dampening inflammation in the genital mucosa could block infection in macaques exposed vaginally to SIV.

Changes in genital cytokine levels during HIV infection may influence immune responses in this site and may impact on the degree of viral shedding into genital secretions. McGowan et al. (2004) reported that individuals with elevated concentrations of TNF- α , RANTES, IL-1 β , IFN- γ , IL-10, and IL-12 in their genital secretions also had higher viral loads in their genital tracts. Similarly, Mukura et al. (2012) reported significantly higher levels of TNF- α and RANTES, but also IL-1 α , eotaxin, fractalkine, IL-6, MCP-1, MIP-1 α , MIP-1 β , and granulocyte-macrophage colony-stimulating factor (GM-CSF) in cervicovaginal lavage samples collected from women who had detectable genital viral loads compared to those with undetectable viral loads (Mukura et al., 2012).

Pro-inflammatory cytokines play an important role in the recruitment, activation and differentiation of immune cells in the genital tract (Lajoie et al., 2008; Li et al., 2009). Women chronically infected with HIV generally have higher levels of pro-inflammatory cytokine responses in their genital tracts than uninfected women, which in turn contribute to cellular recruitment (reviewed by Lajoie et al., 2012). Li et al (2009) showed that the endocervical epithelium of macaques exposed to SIV produces MIP-3 α to recruit plasmacytoid DCs, which in turn secrete IFN- α , IFN- β , MIP-1 α and MIP-1 β that promote inflammation and T-cell recruitment (Li et al., 2009). HIV-infected women have been found to have higher levels of the pro-inflammatory cytokines IL-1 β , IL-6 and IL-8 compared to healthy individuals (Nkwanyana et al., 2009). HIV-infected commercial sex workers (CSW) have significantly elevated levels of MCP-1, MIP-1 β , RANTES, MCP-3, MIG, TNF- α and IFN- γ compared to HIV-uninfected CSW and low risk women (Lajoie et al., 2008). These cytokines and chemokines are secreted by several types of epithelial, stromal and immune cells in the genital tract (reviewed by Rancez et al., 2012). MCP-1, MIP-1 β , RANTES, MCP-3 are known to recruit neutrophils, monocytes, macrophages DCs, T-cells and NK cells. MIG, in contrast, attracts T-cells and NK cells (reviewed by Rancez et al., 2012).

1.6.6. Sexually transmitted infections that impact on HIV shedding

A strong association has consistently been demonstrated between the presence of sexually transmitted infections (STIs) or bacterial vaginosis (BV) and increased risk of HIV acquisition (reviewed by [Ward & Rönn, 2010](#)). While STIs are caused by infections with pathogens, bacterial vaginosis (BV) does not involve a pathogenic infection but rather an imbalance in the composition of the normal commensal vaginal flora, characterised by the depletion of hydrogen peroxide-producing lactobacilli and the proliferation of anaerobic bacteria ([Hillier, 1998](#); reviewed by [Atashili et al., 2008](#)). Of all the STIs, the strongest risk for HIV transmission has been associated with genital ulcer disease, gonorrhoea, syphilis and *Trichomonas vaginalis* (reviewed by [Galvin & Cohen, 2004](#) & [Ward & Rönn, 2010](#)). STIs like HSV-2 and gonorrhoea increase the risk of HIV infection by causing microabrasions in the genital epithelial barrier, inflammation, recruitment of activated HIV target cells into the mucosa, and by increasing viral shedding in genital secretions (reviewed by [Fox & Fidler, 2010](#)).

HSV-2 is known to cause chronic infections, especially in immunocompromised individuals, that manifest as symptomatic ulcerative disease at the genital mucosa or as asymptomatic sub-clinical disease during the latent phase of infection ([Celum et al., 2010](#)). Studies have shown that both symptomatic and asymptomatic HSV-2 can increase an individual's risk of becoming infected with HIV, but also increases the risk of an HIV-infected individuals in discordant relationships transmitting HIV to their uninfected partners ([Johnson and Lewis, 2008](#)). HSV-2 reactivation is highly inflammatory and cause ulcerative lesions that increase plasma and genital tract HIV viral loads, independent of the level of immunodeficiency ([LeGoff et al., 2007](#)). However, a large phase 3 clinical trial aimed at treating HIV-infected individuals co-infected with HSV-2 with acyclovir (to suppress HSV-2 reactivation) did not reduce HIV transmission rates despite significant reductions in the plasma HIV viral load, showing that the lesions may only be one of the factors causing the shedding of HIV ([Celum et al., 2010](#)).

Mycoplasma genitalium is a STI of the genitourinary tract, associated with a 2-fold increase in the risk of HIV acquisition ([Mavedzenge et al., 2012](#); [Vandepitte](#)

et al., 2012). Findings reported by Mavedzenge et al. (2012) suggested that *M. genitalium* causes tissue damage as a result of host cell inflammatory responses, which resulted in recruitment of activated HIV target cells to the endocervix. In addition to increasing risk of HIV infection in uninfected women, *M. genitalium* is also common in HIV-infected individuals, especially in sub-Saharan Africa where it has a prevalence of 11-33% (Mavedzenge and Weiss, 2009).

Bacterial vaginosis (BV), although not caused by a pathogen, is the most common abnormal vaginal condition that results in a vaginal pH increasing to >4.5 (normal pH ranges between 4.0-4.5). BV reduces the vaginal defence capacity against HIV by influencing vaginal pH. A lower vaginal pH inhibits CD4⁺ T-cell activation and proliferation so, the rise in vaginal pH resulting from the absence of lactobacilli leads to an increased availability of activated CD4⁺ T-lymphocytes (Schmid et al., 2000). BV has been linked to a reduction in the level of secretory leukocyte protease inhibitor in vaginal fluid, a component that has been shown to block HIV infection *in-vitro* (Atashili et al., 2008; Draper et al., 1998). Genital mucous has a protective effect against HIV because it traps HIV and prevents contact with the genital mucosal barrier. BV has been shown to degrade mucous via anaerobic bacteria secreting glycosidases and proteases (Olmsted et al., 2003; Atashili et al., 2008).

1.7. Highly Active Anti-Retroviral Therapy (HAART)

Sustained highly active anti-retroviral drug combination therapy, better known as HAART, has been shown to significantly improve the quality of life and reduce the mortality of HIV-infected individuals (Johnson et al., 2013). The number of HIV-infected people receiving HAART has tripled over the last five years (UNAIDS, 2013). Importantly, there has been a significant rise in the number of people receiving HAART in low- and middle-income countries, with an estimated 9.7 million HIV-infected people in 2012 being treated with HAART compared to only 1.6 million in 2011 (UNAIDS, 2013). Although this gain in absolute numbers of HIV-infected individuals being treated is important, it is also important to

consider that only 61% of the individuals eligible for treatment in these countries currently have access to HAART ([UNAIDS, 2013](#)).

Initiation of HAART is accompanied by a rapid decrease in plasma viraemia in HIV-infected individuals, accompanied by a rise in blood CD4 counts (reviewed by [Swanstrom et al., 2012](#)). HAART leads to the rapid elimination of cell-free virus with a half-life of less than six hours and the loss of productively infected cells with a half-life of 1.6 days, causing the concentration of HIV-1 in blood to drop by ~99% in the first two weeks of treatment ([Perelson et al., 1997](#)). The current combination treatment strategies available through use of HAART are able to suppress HIV-RNA to levels below the detection limit of diagnostic assays. However, HAART does not completely eliminate the HIV infection and a rapid viral rebound occurs as soon as treatment is stopped ([Chun et al., 1997](#); [Ho and Zhang, 2000](#)).

The antiretroviral therapy era started in 1986 with the licensing of the first anti-HIV drug, a nucleoside reverse transcriptase inhibitor (NRTI) called zidovudine (ZDV, previously called AZT) ([Wright, 1986](#)). However, the benefits of using just AZT as monotherapy were short-lived and did not delay the onset of AIDS or death, despite a significant initial reduction in the symptoms, because of rapid emergence of AZT drug resistance mutations in HIV ([St Clair et al., 1991](#)). This led to the introduction of combination nucleoside/nucleotide reverse transcriptase inhibitor therapy where AZT was used in conjunction with zalcitabine (ddC) or didanosine (ddI) in the early 1990's ([Delta Coordinating Committee, 1996](#)). Because of intolerable side-effects of AZT, lamivudine (3TC) and stavudine started to be prescribed along with ZDV in the mid-1990's to improve both the durability and tolerability of anti-retroviral treatments ([Kuritzkes et al., 1999](#)). Currently, fixed-dose once-daily combination tablets are most commonly used and are more efficacious, particularly because they are easy to use, better tolerated and as a result there are higher rates of adherence to the treatment.

1.7.1. Types of anti-retroviral drugs and mechanism of action

HAART predominantly targets viral replication and reduces viral loads in plasma by 99% within the first 2 weeks of treatment (Perelson et al., 1997) and to undetectable levels within 6 months of HAART initiation (Chaisson et al., 2000). All licensed antiretroviral drugs can be classified into seven different classes: (1) nucleoside reverse transcriptase inhibitors (NRTIs), (2) nucleotide reverse transcriptase inhibitors (NtRTIs), (3) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (4) protease inhibitors (PIs), (5) cell entry/ fusion inhibitors (FIs), (6) co-receptor inhibitors (CRIs), and (7) integrase inhibitors (INIs). The combination approach encompassed in HAART generally consists of a nucleoside/nucleotide backbone (two drugs among the NRTIs and NtRTIs) and another drug class (selected from the different NNRTIs, PIs, INIs or CRIs). These drugs target different stages of the HIV life cycle (figure 1.10).

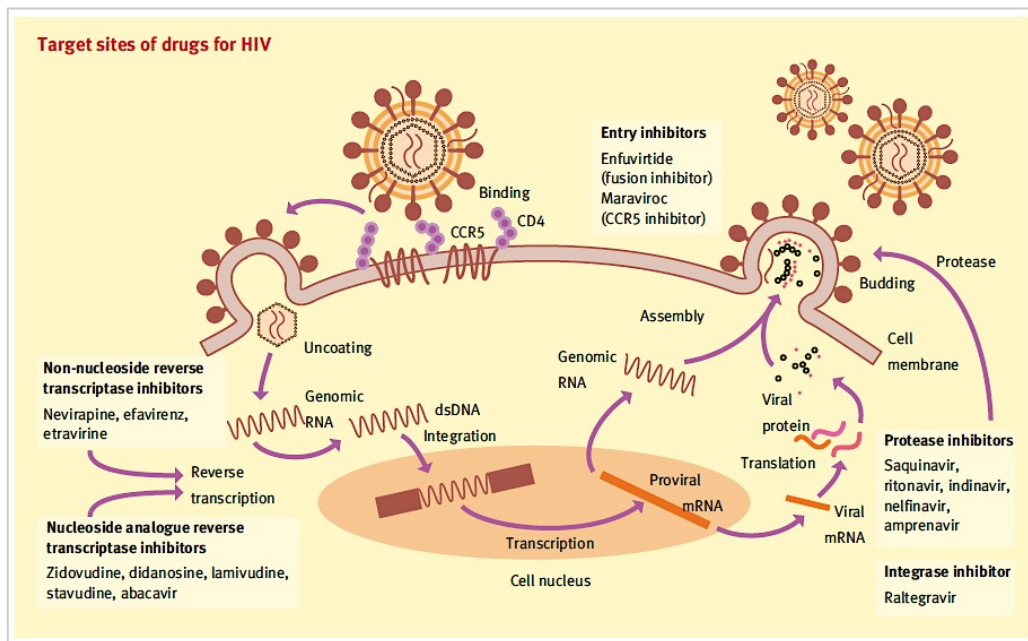


Figure 1.10. Diverse mechanisms of action of the different antiretroviral agents at the various stages of the HIV life cycle (taken from Breckenridge, 2009)

1.7.1.1. Mechanism of action of NRTIs, NtRTIs, and NNRTIs

NRTIs, NtRTIs and NNRTIs all act on HIV reverse transcriptase, which transcribes the HIV single-stranded RNA genome into double-stranded pro-viral DNA (figure 1.10). They work by substituting a chemically modified nucleoside

into the transcribing DNA strand, stopping further transcription from occurring (reviewed by [Lichterfeld & Zachary, 2011](#)). The NRTIs and the NtRTIs bind to the catalytic site of the enzyme while the NNRTIs interact with an allosteric site ([Tantillo et al., 1994](#)). NtRTIs are different from NRTIs in that they have an additional phosphonate group that makes them more resistant to cleavage after they have bound to the 3'-end of the DNA strand (reviewed by [De Clercq, 2009](#)). Unlike NRTIs and NtRTIs, NNRTIs are allosteric, noncompetitive inhibitors which act on a binding site close to the catalytic site of HIV-1 reverse transcriptase (reviewed by [De Clercq, 2009](#)). This binding restricts with the substrate-binding site and disrupts the enzyme's normal function.

NRTIs are the most commonly used anti-HIV drugs in first line regimens in South Africa, and at present, the seven licensed NRTIs are ZDV, ddi, ddC, stavudine (d4T), lamivudine (3TC), abacavir (ABC) and emtricitabine (FTC) ([WHO, 2013](#)). The NtRTI, tenofovir (namely tenofovir disoproxil fumarate (TDF)) is now one of the most prescribed drugs for the treatment of HIV in the world (reviewed by [De Clercq, 2009](#)). The drugs that fall into the NNRTI category are nevirapine, delavirdine, efavirenz and etravirine ([WHO, 2013](#)). Worldwide, Efavirenz is the most commonly prescribed drug from this class of anti-HIV drugs. Etravirine is used in second-line regimens and is very effective in acting against HIV variants which have escape mutations to the first line NNRTIs such as efavirenz and nevirapine ([Andries et al., 2004](#)).

1.7.1.2. Mechanism of action of PIs

PIs target HIV's assembly stage by inhibiting the viral protease (figure 1.10). PIs use the 'peptidomimetic' principle where they use a scaffold that resembles the normal peptide linkage but cannot be cleaved (reviewed by [De Clercq, 2009](#)). This prevents the protease from working as intended, and blocks the production of mature viral proteins from precursor molecules. There are 10 PIs currently available for HIV treatment: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir and darunavir ([WHO, 2013](#)). Lopinavir is the most prescribed PI worldwide.

1.7.1.3. Mechanisms of action of co-receptor inhibitors (CRIs)

The only licensed CRI is maraviroc, which interacts with the co-receptor CCR5 used by R5 viruses to enter host cells (figure 1.10). Since maraviroc is effective only against R5-tropic HIV variants, there is the danger of it selecting for X4 strains and a viral tropism test is usually done before prescribing this drug (reviewed by [De Clercq, 2009](#)).

1.7.1.4. Mechanisms of action of integrase inhibitors (INIs)

INIs act by hindering the function of HIV integrase, which control the insertion of the HIV-1 pro-viral DNA into the host cell genome (figure 1.10; [Hare et al., 2011](#)). The two INIs currently approved for use internationally are the twice-daily raltegravir and dolutegravir, a once-daily pill ([Raffi et al., 2013](#)). Raltegravir has been on the market since 2007 and dolutegravir was just approved by the US FDA in August 2013. Dolutegravir was shown to be efficient in cases where HIV had developed resistance to raltegravir and it is reported to be as well tolerated as the raltegravir ([Eron et al., 2013](#)). In the future, the plan is to make a fixed-dose combination of abacavir, lamivudine and dolutegravir available, to further simplify treatment ([Waters and Barber, 2013](#)).

1.7.1.5. Mechanisms of action of cell entry/fusion inhibitors (FIs)

The only FI commercially available is enfuvirtide (figure 1.10). It is a polypeptide which binds in a coil-coil interaction with gp41 and restricts fusion of the virus with the outer membrane of the host cell ([Matthews et al., 2004](#)). It is however not orally bioavailable and has to be given subcutaneously. As a result, it is mostly used as salvage therapy when HIV-infected individuals develop multi-drug resistant viruses ([Xu et al., 2005](#)).

1.7.2. Debate on best time to start HAART

While the current WHO guidelines for initiation of HAART stipulates that an HIV-infected individual should initiate HAART if their CD4⁺ T-cell counts are lower

than 500 cells/ml (WHO, 2013), the standardised South African national guidelines are yet to adopt this policy and currently require that HIV-infected individuals start HAART when their CD4⁺ T-cell counts reach <350 cells/ml (irrespective of their WHO clinical stage). In some specific cases in South Africa, HAART should be started immediately, as outlined in table 1.1 It is currently recommended that eligible HIV-infected adults be initiated on the first-line regimen, which constitutes of tenofovir, efavirenz and emtricitabine or lamivudine in South Africa. It is recommended that HIV-infected adolescents, on the other hand, be initiated on a different treatment combination, which comprises abacavir, lamivudine and efavirenz (2 NRTIs and an NNRTI, respectively) as tenofovir has been associated with a higher rate of bone mineral density loss (Stellbrink et al., 2010).

In the case of virological failure despite being on HAART, it is recommended that HIV-infected individuals be switched to the recommended second-line regimen. In South Africa, this consists of zidovudine, lamivudine and lopinavir/ritonavir or a combination of tenofovir, lamivudine or emtricitabine and lopinavir/ritonavir (2 NRTIs and protease inhibitors respectively) (*The South African Antiretroviral Treatment Guidelines, 2013*).

Table 1.1. South African national guidelines for HAART

Eligibility to start lifelong HAART:
• CD4 count ≤350 cells/ml irrespective of WHO clinical stage
• All types of TB (in patients with TB drug resistant or sensitive, including extra pulmonary TB) irrespective of CD4 count
• WHO stage 3 or 4 irrespective of CD4 count
HAART initiation within 7 days of being eligible:
• HIV positive women who are pregnant or breast feeding
• Patients with low CD4 <200 cells/ml
• Patients with Stage 4, irrespective of CD4 counts
• Patients with TB/HIV co-morbidity with CD4 counts <50 cells/ml

1.7.3. WHO new guidelines and current system

The WHO issued new guidelines in June 2013, requiring HIV-infected individuals to be initiated on HAART as soon as their CD4 counts fell below 500 cells/ml, immediately if they were pregnant, if they had HIV serodiscordant partners, if they had TB or Hepatitis B, or if they were children ≤ 5 years old. This decision was motivated by several recent studies which have shown the advantages of starting HAART earlier than CD4 counts ≤ 350 cells/ml (Cohen et al., 2011; Severe et al., 2010; Walensky et al., 2009). These new treatment guidelines will demand a significant scale-up in the current treatment program to ensure that every HIV-infected individual who needs HAART receives it. With the current South African treatment guidelines, ~61% of eligible HIV-infected individuals have access to HAART (UNAIDS, 2013). In low- and middle-income countries (including countries in sub-Saharan Africa), a higher proportion of HIV-infected women than men were on HAART, with 73% of eligible women being treated in 2012 compared to only 57% of men (UNAIDS, 2013). The impact of HAART roll-out and scale-up in South Africa is clearly evident, with the life expectancy of South African HIV-infected individuals being an average of 11.3 years longer in 2011 than it was in 2003 (Bor et al., 2013). If the new WHO guidelines are implemented in South Africa, an even bigger scale-up of treatment will be necessary as the current numbers on HAART represent only 34% of the 28.3 million individuals with CD4 counts ≤ 500 cells/ml who will be eligible for therapy (UNAIDS, 2013). Intensifying the treatment program will help limit the circulation of HIV within the population and thus, reduce the number of new infections.

1.7.4. Bioavailability of HAART drugs and toxicity

South African HAART guidelines require that all new HIV-infected individuals who are eligible to start HAART be put on the first line regimen consisting of one NNRTI (efavirenz) and two NRTIs (tenofovir and emtricitabine/lamivudine) (*The South African Antiretroviral Treatment Guidelines, 2013*). NRTIs and NNRTIs are usually given together during treatment as they have a complementary effect (Breckenridge, 2009; King et al., 2002). These ARVs have very wide tissue

bioavailability ranges, with a capacity to penetrate tissues of 25%, 50-60%, 86% and 93% for tenofovir, efavirenz, lamivudine and emtricitabine, respectively (Breckenridge, 2009). The NNRTIs have a higher plasma half-life than the other drug classes, with efavirenz being active for ~30-40 hours compared to tenofovir being active for only 17 hours, lamivudine for 5-7 hours and emtricitabine for 1-2 hours (Anderson and Rower, 2010; Breckenridge, 2009). NRTIs cause side effects, including nausea, appetite loss and fever, while NNRTIs may cause disorientation and gastrointestinal complications (Breckenridge, 2009).

1.7.5. Impact of HAART on genital shedding and inflammation

Several studies have reported that plasma viral loads are the best predictor of genital HIV loads in infected individuals (Anderson and Cu-Uvin, 2008; Kovacs et al., 2001). Although plasma HIV load is a recognised predictor of genital HIV shedding, concomitant genital tract infections with *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Candida albicans* may further influence genital tract HIV loads, through recruitment of HIV-infected cells to the genital tract in response to the infection or as a result of the increased production of inflammatory cytokines, which facilitate HIV replication (Johnson and Lewis, 2008). Mukura et al. (2012) showed that elevated concentrations of chemotactic and inflammatory cytokines (including RANTES, eotaxin, fractalkine, IL-1 α , IL-6, MCP-1, MIP-1 β , MIP-1 α , TNF- α and GM-CSF) were associated with higher genital viral loads. In support of this, Mitchell et al. (2011) reported that IL-1 β and IL-8 were strongly associated with cervicovaginal HIV-RNA levels, after adjusting for plasma viral load and vaginal co-infections.

Just as HAART causes a decrease in plasma viral loads in HIV-infected individuals, it has also been strongly associated with a suppression of genital tract viral loads (Cu-Uvin et al., 2006; Fiore et al., 2003). However, HIV can be detected in mucosal secretions in some HIV-infected individuals with fully suppressed plasma viral loads (Chun et al., 2008; Mehandru et al., 2006). The effect that HAART has on HIV shedding in the female genital tract has been shown to be dependent on several factors, including STIs, hormonal

contraception use and pharmacokinetics of the drugs used (reviewed by [Else et al., 2011](#)). Cohen et al. (2007) showed that highly protein-bound drugs have a lower bioavailability in the genital tract. The standard South African first line HAART regimen consists of 2 NRTIs and 1 NNRTI or PI (*The South African Antiretroviral Treatment Guidelines, 2013*). Min et al. (2004) showed that there were lower concentrations of NNRTIs and PIs available in genital tract tissues compared to blood. Kwara et al. (2008), in contrast, showed that NNRTIs had the highest penetrative capacity to cervicovaginal sites ([Kwara et al., 2008](#); [Min et al., 2004](#)). A study conducted by Graham et al. (2007) showed that HIV-RNA levels decreased significantly in plasma within 2 days of initiating HAART [which consisted of 2 NRTIs (stavudine and lamivudine) and one NNRTI (nevirapine)], while viral loads in vaginal secretions decreased only after 4 days. Importantly, they reported that 35% of the women in their study failed to fully suppress HIV in genital secretions, indicating an ongoing risk of HIV transmission ([Graham et al., 2007](#)).

1.7.6. Effect of HAART on immune activation

Given the role of immune activation in disease progression during untreated HIV infection, several studies were conducted to explore changes in activation during treatment with HAART. HAART has been very effective in reducing the overall mortality associated with HIV although excessive immune activation still persists in certain HIV-infected individuals with low CD4⁺ T-cell recovery ([Anthony et al., 2003](#); [Hunt et al., 2003b](#)). In HIV-infected individuals on HAART, elevated levels of T-cell activation have been associated with lower CD4⁺ T-cell restoration, cardiovascular diseases and increased mortality (reviewed by [Hunt, 2012](#) & [Klatt et al., 2013](#)). In individuals who have been on HAART for several years, there is an overall decline in the level of T-cell activation although it still remains higher than observed in HIV negative individuals ([Hunt et al., 2003b](#); [Valdez et al., 2002](#)). In a recent study by Vigneault et al. (2011) in which the transcriptome of people on HAART compared to HIV-uninfected individuals was compared, >500 gene transcripts involved in immune activation are significantly overexpressed in those on HAART. This compelling study serves to emphasise the failure of

HAART-treated individuals, despite full viral suppression, to lower activation levels to those observed in HIV negative people (Vigneault et al., 2011). A study comparing the degree of T-cell activation before HAART treatment, and after one year on HAART, showed that CD8⁺ T-cell activation predicted slower CD4⁺ T-cell reconstitution even on HAART (Hunt et al., 2011a). Ongoing CD8⁺ T-cell activation, even on HAART, could predict higher risk of mortality due to HIV infection (Hunt et al., 2011a). HAART-mediated viral suppression reduces both CD4⁺ and CD8⁺ T-cell activation although it remains higher than in uninfected individuals (Neuhaus et al., 2010). Other markers of immune activation systemically, (such as IP-10, IFN- α , IL-6 or D-dimer) remain higher in HIV-infected individuals on HAART compared to uninfected individuals (reviewed by Hunt, 2012). The level of immune activation observed in HIV-infected individuals on HAART also depends on how early HAART was initiated (Jain et al., 2013). Some studies have reported that markers of monocytes activation (such as CD163) decrease to almost normal levels if HAART was started during the acute phase of HIV infection but remain elevated if HAART was started during the chronic phase of infection (Burdo et al., 2011). Similarly, Jain et al. (2013) showed that individuals initiating HAART within the first six months of HIV infection had lower levels of CD4⁺/CD8⁺ T-cell activation and a smaller HIV reservoir during long-term therapy compared to patients who started HAART at least two years after infection.

1.7.7. Impact of HAART on activation and inflammation

HAART can, to some extent, dampen the increased immune activation and inflammation that occurs during HIV infection (reviewed by Deeks, 2011). Keating et al. (2011) showed that being on HAART was associated with a decrease in plasma IP-10 and TNF- α concentrations and an increase in IL-12 (p40) and IL-15 (necessary for the maintenance of long-lived memory T-cells and to induce cellular proliferation of NK cells; Villinger & Ansari, 2010; Yu et al., 2011) concentrations compared to untreated HIV-infected women. They further reported that HAART-treated individuals had a plasma cytokine profile similar to that of the HIV-uninfected women. Similarly, Amirayan-Chevillard et al. (2000)

reported that individuals on HAART had decreased concentrations of TNF- α , but also IL-1 β , IL-6 and IL-10 compared to HIV-infected individuals not taking HAART. Concentrations of these cytokine increased again once treatment was interrupted, indicating that the changes in cytokine production seen was directly due to HAART ([Amirayan-Chevillard et al., 2000](#)). In another study that used proteomics to compare plasma cytokines from HAART treated and untreated individuals confirmed that IP-10 but also MIG was significantly lower following 24 weeks on treatment ([Relucio et al., 2005](#)).

Even though HAART does suppress HIV replication, the levels of immune activation and inflammation in HAART-treated individuals do not return to levels typically observed before infection ([French et al., 2009](#); [Hunt et al., 2003b](#); [Marchetti et al., 2008](#); [Neuhaus et al., 2010](#)). As a result, these individuals are still vulnerable to other chronic diseases, such as cardiovascular, renal and liver disease, due to the persistently high levels of inflammation ([Hunt et al., 2011a](#); [Kuller et al., 2008](#); [Rodger et al., 2009](#)). Relative to uninfected individuals, HIV-infected individuals on HAART have higher levels of C-reactive protein, D-dimer, IL-6 and other markers of activation ([Boulware et al., 2011b](#); [Neuhaus et al., 2010](#)). As a result, some studies have explored anti-inflammatory drugs, such as statins, in an attempt to counteract the high levels of T-cell activation and chronic immune dysfunction that cause faster disease progression ([Ganesan et al., 2011](#); reviewed by [Card, Ball & Fowke, 2013](#)). In addition to statins, other drugs that has been tested to reduce persistent immune activation during HAART include chloroquine, type I interferon blockers and cyclooxygenase type 2 inhibitors (reviewed by [Card et al., 2013](#)). Alternatively, treating co-infections may reduce systemic immune activation. Hunt et al. (2011) tested the effect that valganciclovir (an anti-viral agent against CMV infections) had on HAART-treated patients who were failing to reconstitute CD4⁺ T-cell counts despite having fully suppressed plasma viral loads. They found, after eight weeks of treatment with valganciclovir, that CD38 and HLA-DR expression by CD8⁺ T-cells and the level of CRP in plasma decreased significantly compared to the control group, suggesting that better management of CMV may improve health outcomes in HAART-treated individuals ([Hunt et al., 2011b](#)).

An alternative approach that has been proposed is the use of a new class of anti-retroviral drugs called Anti-Viral-HyperActivation Limiting Therapeutics (AV-HALTS; [Lori et al., 2012](#)). One of these, called VS411, consists of a combination of didanosine and hydroxycarbamide. [Lori et al. \(2012\)](#) reported that VS411 treatment of HIV-infected individuals resulted in a significant decrease in the level of plasma HIV-1 RNA, a rise in CD4⁺ T-cell counts, reduced proliferation (Ki67 expression) reduced programmed cell death (PD)-1 receptor expression (a marker of exhaustion), and reduced expression of activation markers CD38 and HLA-DR. This class of drugs has been designed to act both as an anti-retroviral agent and an immune-modulatory agent, thereby limiting both viral replication and excessive immune activation ([Lori et al., 2012](#)).

Unlike these observations in blood plasma, a different trend was seen at the mucosa following initiation of HAART where immune activation and inflammation does not return to levels observed before infection: unlike in blood, no immune reconstitution was evident in the gut mucosa in HIV-infected individuals who had been on HAART for ~7 years, who had ~50-60% lower numbers of lamina propria T-cells than normal pre-infection levels, and persistent depletion of memory T-cells ([Mehandru et al., 2006](#)). In a study comparing colon mucosal levels of cytokines before HAART and 9 months on treatment, [Schulbin et al. \(2008\)](#) reported that tissue levels of TNF- α , IFN- γ , IL-4, IL-6 and IL-10 did not change pre- versus post-treatment ([Schulbin et al., 2008](#)). [Nkwanyana et al. \(2009\)](#) showed that there was no difference in the levels of inflammatory cytokines in genital fluid collected from chronically HIV-infected women on and off HAART.

1.7.8. Latent HIV reservoirs and HAART

One of the major limitations of HAART is that latent HIV reservoirs continue to exist in HIV-infected but resting CD4⁺ T-cells, that have HIV integrated in their host DNA ([Coiras et al., 2009](#)). Because of these latent reservoirs, viral load will rapidly rebound to detectable levels once HAART is discontinued, to levels

similar to those experienced before HAART was initiated (Hatano et al., 2010; Trono et al., 2010). Even if fully suppressive HAART is maintained long-term, mathematical estimates on the latent reservoir half-life suggest that it would take ~70 years to completely eliminate the latent reservoirs (Blankson et al., 2002; Finzi et al., 1999; Siliciano et al., 2003).

1.7.9. HAART and immune reconstitutions inflammatory syndrome (IRIS)

IRIS is an immune reconstitution disease which occurs in a significant proportion of people who initiate HAART worldwide, including about 10-27% of South Africans (Haddow et al., 2012). IRIS is characterised by an excessive and unregulated immune response to previously sub-clinical infections (reviewed by Chahroudi & Silvestri, 2012 & Dhasmana et al., 2008). The pathogenic effect of IRIS is still unclear although immune suppression as a result of HIV infection seems to cause IRIS (Miller et al., 2004; reviewed by French, 2009). IRIS was found to be common in HIV-infected individuals on HAART who experience a drastic decrease in HIV-RNA levels and a rapid increase in CD4⁺ T-cell counts compared to baseline. The most common reported causes of IRIS are TB (*Mycobacterium avium* complex and *Mycobacterium tuberculosis*), CMV, cryptococcal meningitis (*Cryptococcus neoformans*) and hepatitis virus infections (Cheng et al., 2000; Elliott et al., 2009; Race et al., 1998). At the genital mucosa, HIV-related IRIS was found to cause dermatological problems due to genital herpes simplex (HSV)-2 and human papilloma virus (HPV) (Ratnam et al., 2006). In a study conducted in South African women, profiling of blood cytokines showed a positive relationship between concentrations of IFN- γ and IL-6 and risk of developing IRIS, mostly in individuals with TB, lip zoster Kaposi's sarcoma and cryptococcal meningitis (Worsley et al., 2010). Treatment with HAART results in a shift from a T_h2 to a T_h1 cytokine profile, which may result in an increase in IFN- γ levels (Shankar et al., 2007). This increase may be due to the higher number of activated IFN- γ -producing T-cells present in the circulation after stimulation with pathogen-specific antigens (French et al., 2009). IL-6 is a marker of persistent immune activation and it was suggested that it plays a role in the development of IRIS (Stone et al., 2002), although it is unclear whether it is

a cause or a consequence of IRIS ([Worsley et al., 2010](#)). Trials have been carried out where the treatment of IRIS with corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) have been evaluated ([Meintjes et al., 2012a](#)). The glucocorticoid prednisolone had marked anti-inflammatory effects and caused a significant decrease in the serum concentrations of IL-6, IL-10, IL12 (p40), TNF- α , IFN- γ and IP-10 ([Meintjes et al., 2012b](#)). NSAIDs prevent the formation of prostaglandin mediators of inflammation and have been recommended as a first-line treatment for mild IRIS ([Meintjes et al., 2012a](#)). This suggests that some of the immediate gains in immune function during HAART, especially in individuals starting with low CD4⁺ T-cell counts, are potentially counter-balanced by the detrimental effects of IRIS, where a reconstituting immune system responds inappropriately to previously sub-clinical infections. While the focus of many IRIS studies has been on systemic diseases such as tuberculosis and cryptococcal meningitis, the potential effects of IRIS in the female genital tract in response to STIs is important to consider.

1.8. Aims and objectives

The overall aim of this study was to investigate the impact of HAART on the levels of inflammatory cytokines and immune activation, proliferation and exhaustion of T-cells collected from the female genital tract and blood of chronically HIV-infected women about to initiate or already taking HAART.

Rationale

HIV infection is marked by the progressive loss of CD4⁺ T-cells ([Anthony et al., 2003](#); [Sousa et al., 2002](#)) and immune activation has been described as one of the major causes of both HIV pathogenesis and associated CD4⁺ T-cell loss (reviewed by [Hunt, 2012](#); [Papagno et al., 2004](#)). Initiating HAART has been shown to significantly improve the lifestyle and raise the life expectancy of HIV-positive individuals ([Johnson et al., 2013](#)). The current HAART regimens recommend simultaneous administration of three drugs, which enable the rapid suppression of blood and genital viral replication to undetectable levels in most individuals (reviewed by [Lichterfeld & Zachary, 2011](#)). In blood, HAART has been shown to play a crucial role in suppressing the viral load, reconstituting CD4⁺ T-cell counts and in dampening HIV-induced immune activation (reviewed by [Hunt, 2012](#)). However, individuals who have underlying opportunistic infections have a higher chance of developing a series of inflammatory symptoms termed immune reconstitution inflammatory syndrome (IRIS) when starting HAART ([Chahroudi and Silvestri, 2012](#)). IRIS is characterised by a rapid phase of restoration of pathogen-specific immunity to subclinical infections and an overwhelming inflammatory response, leading to a paradoxical clinical deterioration ([Dhasmana et al., 2008](#)). The risk factors and biomarkers of IRIS in blood have been relatively well researched, especially in patients with opportunistic infections such as *Mycobacterium avium* (mycobacterial disease), *Cryptococcus neoformans* (cryptococcal meningitis) and CMV ([Mahnke et al., 2012](#)). Fewer studies have documented the impact of HAART in the genital tract and how the T-cell distribution and differentiation profiles in the genital mucosa differ from that of blood. The genital mucosa is an effector tissue site, constantly exposed to organisms (including commensal and other opportunistic infections with pathogenic viruses, bacteria, parasites and fungi) as well as antigens from

the outside environment. Immune suppression during HIV infection and subsequent immune reconstitution following initiation of HAART may therefore influence immune responses to this overwhelming multitude of antigen and pathogens typically encountered at this mucosal barrier. The effect of HAART on immune responses and reconstitution in the genital mucosa may be influenced by local factors in the genital microenvironment, including genital tract inflammation or presence of common cytokine receptor γ -chain family cytokines, such as IL-2, IL-7 or IL-15, which are crucial for the formation and survival of memory T-cells). Recruitment, activation and differentiation of immune cells in the genital tract have been linked to elevated levels of pro-inflammatory cytokines (such as IL-1 β and IL-6) and have also been shown to be involved in the differentiation of helper T-cells via the T_h1 pathway (Crowley-Nowick et al., 2000; Nkwanyana et al., 2009) but the role of HAART in reducing genital inflammation has not been fully elucidated. Also, the influence of other underlying infections in the genital mucosa on immune reconstitution and the inflammatory response during the initiation of HAART is still unclear (Couppié et al., 2006; Posavad et al., 2004). Overall, while several studies have detailed the longitudinal effects of HAART on the systemic immune activation and inflammation, there are very few studies describing its impact in the genital tract immune reconstitution, especially those describing the initial changes happening at the very start of therapy in chronic HIV infected individuals.

Specific Objective 1:

To compare the level of inflammatory cytokines, and concomitant differentiation, activation and exhaustion of T-cells isolated from the genital tract and blood in a cross-sectional study of chronically HIV-infected women on HAART.

Hypothesis: HIV-infected women on HAART will have reconstituted CD4⁺ T-cell numbers in both blood and the genital compartment, that correlate with length of time on HAART. A higher level of inflammation in the female genital tract compared to blood will result in a higher degree of T-cell activation at the mucosa compared to in blood. Higher degrees of T-cell activation may, in turn, cause an imbalance in the quality of T-cells present in the genital tract, with a higher

proportion of effector memory T-cells and a smaller proportion of central memory T-cells.

Specific Objective 2: To investigate the temporal association between initiation of HAART on local genital versus blood T-cell reconstitution, differentiation, activation, exhaustion and inflammation in a longitudinal study of chronically HIV-infected women immediately before and 1 month after starting HAART.

Hypothesis: Initiation of HAART will suppress both plasma viraemia and HIV shedding in the genital tract and reduce the level of T-cell activation in both compartments as well as improving local genital tract and blood T-cell numbers. HIV-infected women initiating HAART may exhibit symptoms of “genital IRIS” to opportunistic infections upon reconstituting immune responses in their genital tracts.

Chapter 2. Materials and Methods

2.1 Description of women enrolled in the study

A total of 26 HIV-infected women were recruited from the Nyanga East Day Clinic (Cape Town, South Africa) for this study to compare the impact of HAART on CD4⁺ T-cell immune reconstitution and inflammation in the female genital tract and blood. Of these 26 HIV-infected women, 15/26 (57.7%) were on antiretroviral therapy at the time of study and were included in a cross-sectional study (Chapter 3). The remaining 11/26 (42.3%) women were recruited immediately prior to starting HAART and 1 month after starting HAART (Chapter 4). Ethics approval was obtained for the study from the Faculty of Health Sciences Research Ethics Committee (HREC Ref 206/2002 for the cross-sectional study and HREC Ref 154/2012 for the longitudinal study). Written informed consent was obtained from all women.

2.2 Clinical samples collection

At each visit, whole anti-coagulated blood (~30 ml) was collected from each woman into acid citrate dextrose (ACD) vacutainers for isolation of peripheral blood mononuclear cells (PBMCs). A cervical cytobrush sample was also collected using a Digene cervical sampler (Qiagen, Hilden, Germany) according to the method described by Gumbi et al. (2008). Briefly, the cytobrush was inserted into the endocervical os under speculum examination, rotated 360° to obtain cervical cells and then placed in 3ml of transport medium (10% FBS in RPMI supplemented with penicillin, streptomycin, fungin and L-glutamine, also known as R10). Immediately after sampling, the cytobrush was kept at 4°C in a bench top cooler and transported to the laboratory for further processing within 4-6 hours of collection.

2.3 Processing cervical mononuclear cells (CMCs)

The cervical cytobrush was rotated against the side of the 15ml transport centrifuge tube to dislodge the cells from the cytobrush. Using a sterile Pasteur pipette, transport medium was used to flush the cytobrush bristles ~30 times. The cytobrush bristles were then gently scraped with the tip of the Pasteur pipette to remove any remaining cells and the cytobrush removed from the tube and discarded. The tube containing the cell suspension was centrifuged at 320g for 10 minutes to pellet the cervical cells. The supernatant fraction from the cytobrush was transferred to two cryovials: (1) for measurement of genital shedding of HIV-RNA; and (2) for measurement of secreted cytokine concentrations by luminex. The cell pellet was retained for cellular assays of the CMC phenotype. The pelleted cells were resuspended in 3ml of R10 and washed again by centrifuging at 320g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 100µl plain PBS for cell counting (section 2.5) and then used immediately for flow cytometry.

2.4 PBMC isolation

PBMC isolation was carried out according to the protocol described previously ([Gumbi et al, 2008](#)) using Ficoll-Hypaque density gradient centrifugation and Leucosep tubes (Greiner Bio One, Frickenhausen, Germany). Red blood cells and granulocytes have a higher density than Ficoll and so, after centrifugation, they collect below the Ficoll layer (underneath the porous polyethylene barrier) while the mononuclear cells collect just above the barrier.

All blood samples were processed within four hours of collection. Ficoll-Hypaque (15ml) was added to each of two 50ml Leucosep® tubes (Greiner Bio-one; Frickenhausen, Germany) and the tubes were centrifuged at 1257g for 1 minute for the Ficoll to cross the porous barrier. Equal amounts of the whole blood were then poured into the two tubes, which were then centrifuged at the same speed for 15 minutes.

A sterile Pasteur pipette was used to transfer the upper plasma layer to four cryovials (for subsequent cytokine concentration measurement by Luminex). The rest of the plasma layer and most of the platelets were discarded. Using a fresh sterile pipette, the 'buffy' layer of PBMCs (just above the porous barrier) was carefully transferred from both Leucosep tubes to a sterile 15ml tube and 1% wash buffer was used to top up the volume to 15ml. The tube was centrifuged at 320g for 10 minutes and then the supernatant was discarded and the cell pellet was loosened. The volume was made up to 15ml using R10, the cell suspension was mixed and the tube was centrifuged again at 320g for 10 minutes. The supernatant was discarded and the cell pellet was dislodged. The PBMC were resuspended in 2ml of R10 for cell counting (section 2.5) and subsequently cryopreserved in liquid nitrogen (section 2.6).

2.5 Trypan Blue cell counting and viability measurement

The concentration and viability of PBMCs and CMCs isolated from each women were determined using Trypan Blue counting on a light microscope. Trypan blue cell counting is a dye-exclusion assay based on the principle that live viable cells are able to actively transport the dye particles out of the cells (appearing clear under a light microscope) while dead cells do not (appearing blue under a light microscope). The cell count was done using an improved Neubauer chamber, which gave both the total lymphocyte count and the percentage viability of the sample. Briefly, a 10 μ l aliquot of the PBMCs or CMCs was mixed with 10 μ l of Trypan Blue and the mixture was carefully loaded onto the Neubauer haemocytometer. The cell count was done at 400x magnification. First, the total number of cells (including all cells irrespective of whether they were stained with dye or not) in two squares (A and B as shown in the figure 2.1) were counted. Second, the numbers of dead cells (only those stained with trypan) in two squares were counted. The total number of viable cells as well as the percentage viability was determined using the following formulae:

$$1. \text{ Number of live cells} = \left(\frac{A \text{ (live)} + B \text{ (live)}}{2} \times \text{Dilution factor} \times 10^4 \times \text{Original volume} \right)$$

$$2. \% \text{ viability} = \left(100 - \frac{A(\text{dead}) + B(\text{dead})}{A(\text{live}) + B(\text{live})} \right) \times 100$$

After determining PBMC numbers, cells were centrifuged at 320g for 10 minutes to pellet cells and the supernatant was discarded before adding the freezing medium for cryopreservation (section 2.6).

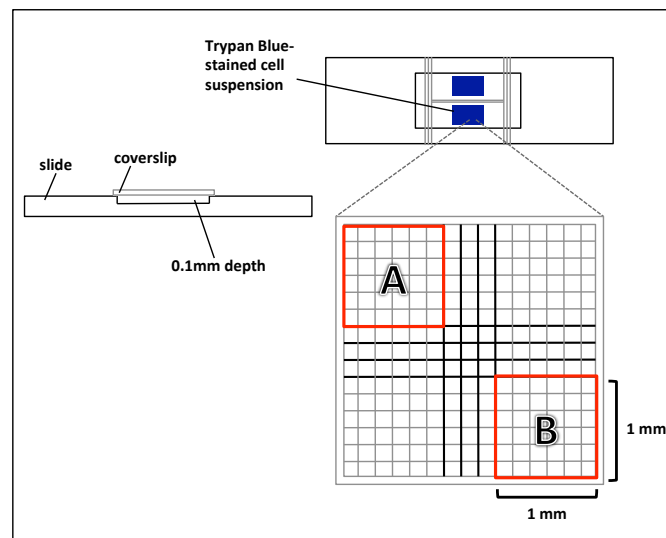


Figure 2.1. Summary of method used for Trypan Blue cell counting. A 10 μ l aliquot of sample mixed with 10 μ l of Trypan Blue dye was loaded onto the slide (as shown in blue in the diagram). Using a microscope at 400x magnification, the number of cells appearing blue or clear were counted in 2 squares (labelled A and B) and the equations described above were used to determine the number of viable cells in the 10 μ l of sample.

2.6 Cryopreservation of PBMCs

PBMCs were cryopreserved in liquid nitrogen immediately after isolation from whole blood. To prepare PBMCs for cryopreservation, freezing medium (FBS containing 10% DMSO) was used. The presence of 10% DMSO in the freezing medium served to reduce both the size and the number of ice crystals formed during the freezing step by lowering the solute concentration, resulting in lower ionic stress. All reagents were kept on ice during the process to slow down or prevent oxidative stress and maintain viability of the PBMCs.

PBMCs pellets (section 2.5) were resuspended in 1ml of FBS, followed by 1ml of 20% DMSO in FBS drop wise while mixing continuously. The cell suspension was then transferred to two cryovials (1ml in each) and these were placed in pre-cooled 'Mr Frosty®' (Nalgene; Rochester, NY, USA) freezing containers. These were then placed at -80°C overnight. In the "Mr Frosty" container, the cells were surrounded by a layer of isopropanol, allowing the temperature to decrease from -4°C to -80°C by 1°C increments to limit the extent of cell death. The next day, the cryopreservation tubes were transferred to liquid nitrogen.

2.7 Thawing of PBMCs

PBMCs were thawed the day before each experiment to allow cells to "rest" and to allow any apoptotic cells that were dying as a result of freeze/thawing to die before the assay was carried out. Thawing was done quickly while dilution of the freezing medium, containing DMSO, with R1 (1% FBS in RPMI) was done slowly to avoid cell death and clumping. For this study, PBMCs were thawed by placing cryovials in a 37°C waterbath until only a small ice crystal remained in the vial. Each vial was then wiped with 70% alcohol and transferred to the biosafety hood. R1 was added to the cryovial drop wise while mixing continuously and the cell suspension was transferred to a sterile 15ml tube. The cryoprotectant, DMSO, has to be diluted at least 30-fold to limit the extent of cell death and therefore, the volume was made up to 15ml with more R1. The tube was centrifuged at 320g for 10 minutes, the supernatant was discarded and the pelleted cells were resuspended in 500µl of diluted DNase to prevent clumping of dead cells. The cell suspension was allowed to stand for 2 minutes at room temperature. The volume was made up to 15ml with R10 and the tube was centrifuged at 320g for 10 minutes. The supernatant was discarded, the pellet was loosened and the cells were resuspended in 5ml of R20. The cells were left to incubate in a 5% CO₂ and 37°C incubator overnight before carrying out the experiment the next day.

2.8 Intracellular staining (ICS) and flow cytometry

A ten-colour polychromatic flow cytometry panel was designed and optimised as part of this study (described in detail in appendix II) to measure the memory phenotype (CD45RO and CCR7), level of activation (CD38 and HLA-DR), proliferation (Ki67) and exhaustion (CD57) of CD4⁺ and CD8⁺ T-cells isolated from PBMCs and CMCs. A dump channel was also added in the panel to exclude dead cells, B-cells and monocytes (with VIVID, CD14 and CD19, respectively).

2.9 Staining the PBMC and CMC samples

CMC samples were stained and assessed in real time (fresh) while PBMC samples were cryopreserved, thawed and assessed in batches. For PBMCs, cells were adjusted to 1x10⁶ cells/ml with PBS. An aliquot of 1ml was transferred to a sterile tube. For CMCs, the whole sample was used in a single tube for a single staining reaction because cell numbers from a single cytobrush are generally low.

Before adding antibodies to each tube, PBMCs and CMCs were centrifuged at 320g for 10 minutes and the supernatant was discarded. The cell pellet was loosened, resuspended in 100µl of PBS and transferred to one of the wells in a 96-well V-bottomed plate (Corning; Tewksbury MA). The plate was centrifuged at 1215g at 4°C for 3 minutes, the supernatant was discarded and the pellet was loosened. The cells were washed once more with 150µl PBS to ensure that all the protein in solution was removed. The supernatant was discarded and the cell pellet was resuspended in 50µl of pre-titrated Vivid (live/dead cell marker). The plate was allowed to incubate in the dark at room temperature for 20 minutes. The cells were washed twice with PBS (with 100µl for the first wash and 150µl for the second wash) and the plate was centrifuged at 1215g at 4°C for 3 minutes. The cells were first stained for the extracellular markers (CD8, CD4, CD14, CD19, CCR7, HLA-DR, CD45RO, CD38 and CD57) using an extracellular staining cocktail of pre-titrated antibody volumes for in PBS (procedure described in appendix II, section A2.3). For this, 50µl of the antibody mix was added, the cell suspension was carefully mixed and the plate was incubated on ice for 30min before washing twice with PBS, as described earlier. The cell pellet

was loosened and resuspended in 100µl of Cytofix/Cytoperm (BD Biosciences) to fix and permeabilise the cells and the plate was placed on ice to incubate for 20 minutes. After this, the cells were washed twice with PermWash (with 100µl and 150µl respectively) and the plate was centrifuged at 1215g at 4°C for 3 minutes both times. Next, 50µl of the intracellular staining cocktail containing pre-titrated volumes of antibodies for CD3 and Ki67 in PBS was added, the cell suspension was carefully mixed and the plate was placed on ice for 30min. Staining for CD3 was done following permeabilisation (intracellularly) as stimulation of cells can sometimes cause the internalization of the CD3 receptor, which is usually expressed on the surface of T-cells. The cells were again washed twice with PermWash. The supernatant was discarded and the pellet was dislodged. The cells were resuspended with 100µl Cellfix to fix them and the cells suspension was transferred to a 5ml FACS tube. The well was rinsed again with 150µl Cellfix, which was also transferred to the FACS tube. The tube was then wrapped in aluminium foil (to keep it in the dark) and stored in the fridge, at 4°C, until acquisition of flow cytometry data using the BD LSRFortessa™. All the samples were acquired within 48 hours of staining. For PBMCs, 1x10⁶ events were acquired while the whole sample was acquired for CMCs. The data obtained was then analysed using FlowJo software (version 9.6.2; Treestar; Ashland, OR)

2.9.1 Preparation of compensation tubes

Compensation tubes are prepared every time a sample was acquired. Samples have to be compensated to avoid spectral overlap when several fluorochromes are simultaneously used (described in appendix II). For compensation, a control tube (no antibodies) and a series of singly-stained tubes for each antibody in the panel were prepared. A total of 11 compensation tubes were used in this study, one for each of the ten fluorochromes and one for the unstained control. Both negative and positive BD CompBeads™ (BD Biosciences) were used for the compensation tubes. The positive CompBeads are used to bind to the Kappa light chain of immunoglobulins raised in mice, rats or hamsters and emit a strong fluorescence signal irrespective of the attached antibody. The unstained control tube was included to determine the background noise produced from the

scattered light falling on the detector and the singly-stained tubes are used to determine the range of each of the fluorochromes in the panel (Seddon et al., 2003).

To the eleven 5ml FACS tubes to be used for compensation, 100µl of PBS was added, followed by one drop each of the positive and the negative CompBeads™ beads. For this study, except for the anti-CCR7-PECy7 compensation tube (where anti-rat beads were used because this monoclonal was raised in rats), anti-mouse beads were used for all other compensation tubes (because all other monoclonal antibodies included in the panel were from mice). The CompBeads vials were vortexed thoroughly for about 1 minute to avoid clumping of the beads. The pre-titrated volume of each antibody (detailed in Appendix II, section A2.3) was added to each of the respective compensation tube. For the dump channel, either anti-CD14-PacificBlue or anti-CD19-PacificBlue was used. The tubes were incubated on ice for 20min before washing the beads by adding 1ml of PBS and centrifuging at 1215g at 4°C for 3 minutes. The supernatant was discarded and the beads were fixed in 250µl of Cellfix. The tubes were then wrapped into aluminium foil and kept at 4°C in the fridge until acquisition on the BD LSRFortessa.

2.9.2 Fluorescence Minus One (FMO) controls

FMO controls, in flow cytometry, consist of cells treated with all the antibodies of the panel being investigated except one (described in detail in appendix II, section A2.6). In addition to showing the amount of non-specific binding (since the cells have been labelled with all the fluorochromes except one), FMO controls help to define the position of the positive population as well as the location of double positives on the scatter plots.

To prepare the FMO controls, 50µl Vivid was added to aliquots of 1×10^6 cells in a 96-well V-bottomed plate and the plate incubated in the dark at room temperature. After washing the cells with PBS, extracellular and intracellular staining (as previously described in Section 2.8) was carried out while making

sure, for each particular tube, that the antibody for which the FMO was being carried out was not included. The cells were resuspended with 100µl Cellfix to fix them and the cells suspension was transferred to a 5ml FACS tubes. Each well was rinsed again with 150µl Cellfix, which was also transferred to the respective FACS tubes. The tubes were then wrapped in aluminium foil (to keep it in the dark) and stored in the fridge, at 4°C, until acquisition of using the BD LSRFortessa™ and at least 100,000 events were acquired for each tube

2.9.3 Quantitative determination of cytokine concentrations

Multiplex luminex flow cytometry was used to measure cytokine concentrations in blood plasma and cervical secretions (collected and stored as described in sections 2.3 and 2.4) obtained from 8/15 of the women from the cross-sectional part of the study (Chapter 3) and for all (11/11) women from the longitudinal study (Chapter 4). A Human Cytokine Group I 10-plex kit (Bio-Rad Laboratories Inc[®]) was used to determine the concentration of seven inflammatory cytokines (IL-1β, IL-6, IL-8, IP-10, MIP-1α, MIP-1β and TNF- α), two hematopoietic cytokines (IL-7 and G-CSF) and a regulatory cytokine (IL-10).

Bio-Plex luminex kits (Bio-Rad Laboratories Inc[®]) are composed of microscopic magnetic polystyrene beads ~5.6µm in diameter, which have distinct spectral properties to allow discrimination between the different assays (Tighe et al., 2013). The beads are dyed with specific proportions of red and near-infrared fluorophores, which define the different spectral capabilities for each bead population. The luminex apparatus used to read the plates in this study had a three-colour signal detection system. Two of the colours are used for the microsphere classification and the third colour to measure the fluorescence intensity from the different wells. In principle, the assay is similar to a sandwich ELISA. The magnetic beads were coupled with capture antibodies specific for the cytokine of interest. When the samples are added to the wells, these coupled beads bind to the biomarker of interest. The unbound protein is removed using a series of wash steps and then a biotinylated detection antibody is added, forming the sandwich complex. Finally, streptavidin-phycoerythrin (SA-PE) conjugate is

added, where the phycoerythrin works as the fluorescent signal recorded by the Luminex instrument.

2.9.4 Preparation of the samples

Plasma and cervical supernatant samples were gently thawed by placing them on ice at 4°C overnight. Immediately before starting the assay, the samples were centrifuged at 1950g for 10 minutes at 4°C in SPIN-X® centrifuge tube filters (0.22µm cellulose acetate filters; Corning Inc, NY) to exclude any particulate debris.

2.9.5 Preparation of the standards

Samples for the standard curves were prepared by 1:4 serial dilution according to the manufacturers guidelines, with separate standard curves being prepared for plasma and genital secretions. Briefly, the individual lyophilised standards for each cytokine provided in the kit were reconstituted using 500µl R10. The vial was vortexed gently for 1-3 seconds and then placed on ice for 30 minutes. The dilution series for plasma samples was made up using the provided serum-based standard diluent while the one for the genital samples was made up using more R10 as diluent. For the plasma standard curve, the standard diluent was used as the blank, while R10 was used for the blank for the genital sample standard curve.

2.9.6 Preparation of coupled beads, detection antibodies and streptavidin-PE

Each of the 96 wells required 5µl of coupled beads adjusted to a final volume of 50µl. To prepare the coupled beads, 4.6ml of assay buffer was added to a 15ml tube, followed by 575µl of the stock coupled beads (10X) for each of the cytokines to obtain a final volume of 5.75ml of 1X coupled beads. The tube was wrapped in foil and allowed to equilibrate for 20 minutes before use. Each well of the assay required 2.5µl of detection antibody adjusted to a final volume of 25µl. To achieve this, 2.4ml of assay buffer was added to a 15ml tube, followed

by 300µl of the stock detection antibodies (10X) for each of the cytokines to obtain a final volume of 3ml of 1X detection antibodies. Each well in the assay required 0.5µl of streptavidin-PE adjusted to a final volume of 50µl. To achieve this, 5.94ml of assay buffer was added to a 15ml tube, followed by 60µl of the stock streptavidin-PE (100X) for each of the cytokines to obtain a final volume of 6ml of a 1X solution of streptavidin-PE. This solution was only made up 10 minutes before it was needed. The tube was again wrapped in foil to protect it from light until needed.

2.9.7 Detailed method for the Luminex assay

The diluted coupled beads were vortexed for 30 seconds at medium speed and poured into a petri dish. Using a multichannel pipette, 50µl was added to all the 96 wells. The plate was covered with a layer of sealing tape, wrapped in foil and allowed to incubate on a shaker for 30 minutes. The plate was then washed twice as follows: 100µl of wash buffer was added to each well, the plate was placed on the shaker for 30 seconds and, using a hand-held magnetic plate washer (Milliplex®), the plate was inverted and all the liquid was discarded. The plate was then blotted on a clean piece of tissue to remove any excess liquid. After this, 50µl of the standards, blanks and samples were added to the respective wells, the plate was covered with sealing tape, wrapped in foil and was allowed to incubate at room temperature on a shaker for 30 minutes. The standards were added in duplicates and two wells were used as blanks for each standard curve. The samples were not diluted before being added to the wells. The sealing tape was removed and the plate was then washed three times as described above. Using a multichannel pipette, 25µl of diluted detection antibodies was added to each well. The plate was covered with a new sheet of sealing tape, wrapped in foil and incubated on the shaker for 30 minutes. After incubation, the plate was washed three times again and 125µl of assay buffer was added to each well. The plate was covered with a clean sheet of sealing tape and placed on the shaker at 1100rpm for 30 minutes. The sealing tape was then removed and the plate was placed on the reader to be read and analysed using the Bio-Plex Manager software™ 6.0 at a low PMT setting. All incubation periods were closely

monitored and kept consistent for optimal assay performance and reproducibility.

The sensitivity of the kit ranged from 0.3pg/ml to 2.6pg/ml for the ten cytokines measured. The fluorescence data was acquired using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®) and a 5 PL regression formula was used to derive the cytokine concentrations from the standard curves. Cytokine concentrations that were read as 'below detectable level' were reported as half the value of the lowest concentration measured for that particular cytokine (Roberts et al., 2010). Figure 2.2 gives a summary of the steps described above.

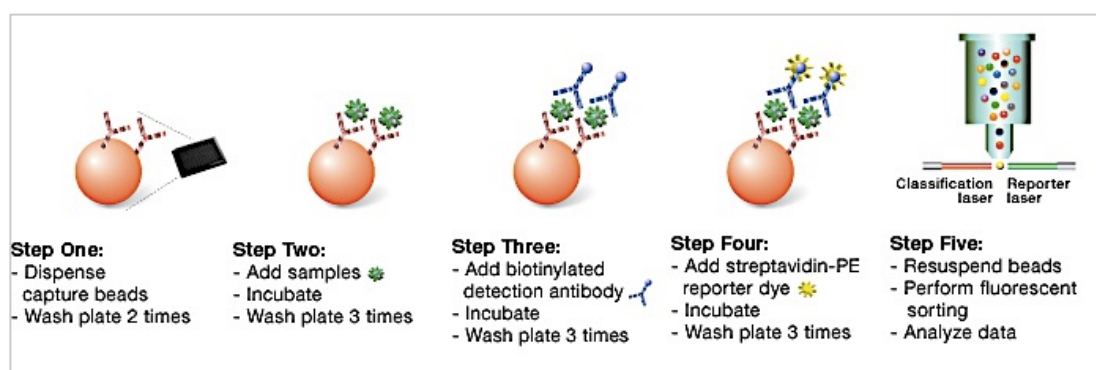


Figure 2.2. Steps involved in preparing the plate for analysis by the multiplex Luminex platform (Geng and Ma, 2012).

2.10 Measurement of HIV loads

Viral loads in the genital secretions and plasma samples (collected as described in sections 2.3 and 2.8) from 8/15 of the women from the cross-sectional study and all (11/11) of the women from the longitudinal study were determined using the Abbott® m2000 RealTime HIV-1 assay by the NHLS Diagnostic Virology Laboratory (Groote Schuur Hospital). This assay consists of an *in-vitro* reverse transcription-polymerase chain reaction (RT-PCR) assay for the quantitation of HIV-1 over the range of 40 to 10,000,000 copies/ml. Using a partially double stranded probe, it targets the integrase region of the polymerase gene in the virus (<http://www.abbottmolecular.com/products/infectious-diseasesrealtime-pcr/hiv-1-assay.html>). The detection limit of the assay was 40 HIV-RNA copies/ml (Abbott Laboratories, 2013).

2.11 Screening for sexually transmitted infections (STIs)

All 11 women involved in the longitudinal study (Chapter 4) were screened for STIs, including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium* at each of visit by nucleic acid amplification using a real-time multiplex PCR assay (Lewis et al., 2013). STI testing was not done on the 15 women enrolled in the cross-sectional study (Chapter 3). Testing for STIs was performed at the Centre for HIV and STIs, National Institute for Communicable Diseases in the laboratory of Prof David Lewis. A MG Real-TM kit (Sacace Biotechnologies) was used for *Mycoplasma genitalium* and the target region of the kit was DNA gyrase subunit B. Similar kits using the general Sacace protocol was used for the three other STIs with a specific target region for each of them. Genomic DNA extracted from *M. genitalium* (ATCC 33530), *N. gonorrhoeae* (ATCC 700825 or ATCC 49226 or WHO Control strains), *T. vaginalis* (ATCC 30001) and *C. trachomatis* (VR-885) were used as positive controls in this assay.

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6.0; San Diego). The Shapiro-Wilk test was used to determine the distribution of variables. The Mann-Whitney U test was used to compare unmatched samples while the Wilcoxon Signed Rank test was used for paired samples. Correlations between the different groups were determined by the non-parametric Spearman rank correlation test. Linear regression was used to determine the relationship between variables. A p-value of ≤ 0.05 was considered to be statistically significant.

Chapter 3. Impact of long-term HAART in chronic HIV-infected women

3.1 Introduction

Chronic HIV infection is marked by the progressive loss of CD4⁺ T-cells, excessive immune activation, inflammation, exhaustion and an overall immune dysfunction. (reviewed by [Hunt, 2012](#)). HAART has a differential effect on T-cell activation and phenotype in blood and in the genital tract. HAART given to individuals infected with HIV has consistently been shown to improve quality of life, prevent AIDS and reduce the rate of mortality and morbidity. In blood, HAART leads to durable viral suppression, in association with significant reconstitution in CD4⁺ T-cell numbers (reviewed by [Okoye & Picker, 2013](#)). CD4⁺ T-cell reconstitution was in turn associated with decreased immune activation, inflammation, T-cell exhaustion (reviewed by [Sauce et al., 2013](#)), and correction of the imbalance in the distribution of the different memory T-cell subsets ([Hellerstein et al., 2003](#)). Compared to HIV-negative individuals, HIV-infected individuals have been shown to have high levels of immune activation and inflammatory cytokine concentrations systemically, which do not return to pre-infection levels even after several years on HAART. Several recent studies have also shown that CD4⁺ T-cell counts continue to slowly decrease, even in the presence of HAART (reviewed by [Okoye & Picker, 2013](#)), in combination with a continued accumulation of exhausted T-cells, (reviewed by [Sauce et al., 2013](#)).

There are fewer studies on the impact of HAART on immune activation, inflammation, memory marker distribution and exhaustion in T-cells circulating to the genital tract, the site where most of the HIV infections occur and how this compares to blood ([Coombs et al., 2001](#)). Typically, immunity in the female genital tract is not contained within organised lymphoid tissues such as lymph nodes and Peyer's patches found in the gut associated lymphoid tissue. Instead, DCs, B- and T-cells present in or beneath the epithelial barrier in lamina propria

of the vagina and cervix may be involved in the response to HIV infection (reviewed by [Iwasaki, 2010](#)). Exposure of the genital mucosa to HIV has been shown to lead to the upregulation of cytokines/chemokine and their receptors, resulting in the activation and recruitment of more T-cells (reviewed by [Xu et al., 2013](#)). Cytokines such as interferons, TNF- α MIP-1 α , MIP-1 β and RANTES have been found to be overexpressed in the genital tract ([Lajoie et al., 2008](#); reviewed by [Xu et al., 2013](#)). In the mucosa, as in blood, HIV infection is accompanied by a drastic depletion of the activated, memory CD4⁺ T-cells ([Gumbi et al., 2011](#); [Mehandru et al., 2004](#)).

[Nkwanyana et al. \(2009\)](#) evaluated the effect of HIV infection and HAART on genital tract inflammation and mucosal memory cell distribution in women. They found that T-cells isolated from the female genital tract were predominantly effector memory T-cells. They also showed that T-cells from the female genital tract were predominantly CD8⁺ in HIV-positive women (CD4:CD8 ratio of 0.4) as opposed being predominantly CD4⁺ T-cells in HIV-negative women (CD4:CD8 ratio of 2.7). HIV-infected women had higher concentrations of the pro-inflammatory cytokines IL-1 β , IL-6 and IL-8 in their genital secretions than HIV-negative women. Concentrations of these cytokines correlated broadly with the number of immune cells recovered from the cervical cytobrush samples, suggesting that they played a role in recruitment of immune cells to the female genital tract ([Nkwanyana et al., 2009](#)). [Jaspan et al. \(2011\)](#) subsequently showed that T-cells derived from the genital mucosa of HIV-infected women expressed higher frequencies of the activation markers CD38 and HLA-DR and that the extent of activation detected in genital tract T-cells significantly reflected the degree of activation of both CD4⁺ and CD8⁺ T-cells in blood. [Liebenberg et al. \(2010\)](#) showed that, in terms of T-cell exhaustion in a chronic HIV infection, there were comparable levels of senescence between cervical and blood T-cells and that the levels of the exhaustion marker CD57 did not differ from the genital levels in uninfected women. Also, similarly to blood, there was an accumulation of terminally differentiated cells in the genital tract of chronic HIV-infected individuals on HAART, in addition to the fact that the extent of CD4⁺ T-cell

depletion that occurred in the cervix was linked to that in blood ([Gumbi et al., 2011](#)).

The aim of this chapter was to determine whether HAART impacted on the level of activation, proliferation, exhaustion or maturation of T-cells in women chronically infected with HIV. Furthermore, the impact of secreted genital tract inflammatory and homeostatic cytokines on the degree of differentiation and activation of genital T-cells in HIV-infected women on HAART was evaluated. It was hypothesised that higher levels of genital tract inflammatory cytokine concentrations would be associated with a greater number of activated T-cells being detected in cervical cytobrush samples, accompanied by an increase in T-cell differentiation and a higher proportion of effector memory T-cells.

3.2 Methods and Materials

For this chapter, 15 chronically HIV-infected women on HAART were enrolled (materials and methods section 2.1). All women were pre-menopausal, were not pregnant and had not undergone a hysterectomy. At the time of the visit, all chronically HIV-infected women were on HAART. Blood and a cervical cytobrush sample were collected from each woman for the extraction of PBMC and CMC, respectively (materials and methods sections 2.3 and 2.4). Flow cytometry was carried out to characterise the blood and cervical T-cells (materials and methods section 2.8 and appendix II). Plasma and genital cytokine concentrations were determined using multiplex Luminex flow cytometry (materials and methods section 2.9).

3.3 Results

3.3.1 Clinical description of cohort

The median age of the women included in the study was 39 years (IQR: 36-41). Women who were menstruating at the time of sampling, who were post-menopausal or who had undergone a hysterectomy were excluded. Of these, 14/15 (93.33%) women were on a first line HAART regimen (Table 3.1) and

1/15 (6.66%) was on a second line HAART regimen (Table 3.1). The median CD4⁺ count of the women was 518 cells/ml (IQR: 377-626) and the median time on therapy for the 15 HAART-treated individuals was 85 months (IQR: 31-102). Table 3.1 summarises the clinical characteristics of this cohort of women.

Table 3.1. Clinical description of the women enrolled in the study

Patient ID	Age (years)	CD4 count (cells/ml)	ART regimen	Months on HAART
NY029	35	508	1 st line: Nevirapine, Combivir ^a	102
NY162	39	535	1 st line: Efavirenz, additional data not available	42
NY186	39	559	1 st line: 3TC ^b , AZT ^c , Nevirapine	99
NY254	40	626	1 st line: Efavirenz, Combivir	43
NY219	37	693	1 st line: 3TC, Tenofovir, Efavirenz	19
NY226	35	151	1 st line: 3TC, AZT, Stavudine, Nevirapine	85
NY233	53	146	1 st line: Lamivudine, Stavudine, Nevirapine	31
NY006	34	518	1 st line: 3TC, Tenofovir, Nevirapine	101
NY110	42	342	1 st line: 3TC, Stavudine, Nevirapine	38
NY149	38	1014	1 st line: 3TC, AZT, Efavirenz	94
NY167	36	502	1 st line: 3TC, Tenofovir, Nevirapine	26
NY191	41	486	1 st line: Nevirapine, Combivir	119
NY221	40	377	1 st line: 3TC, Tenofovir, Efavirenz	15
NY61	41	551	1 st line: 3TC, Tenofovir, Efavirenz	109
NY248	39	944	2 nd line: AZT, Didanosine, Lopinavir, Ritonavir	103
Median	39	518		85

^aCombivir- Single dose combination of Zidovudine and Lamivudine; ^b3TC – Lamivudine; ^cAZT – Zidovudine

3.3.2 Comparison of T-cell distribution between genital tract and blood

Figure 3.1 shows the representative gating strategy used to define the CD4⁺ and the CD8⁺ T-cell populations isolated from cervical cytobrushes (genital tract) and blood of HIV-infected women on HAART (detailed further in Appendix II).

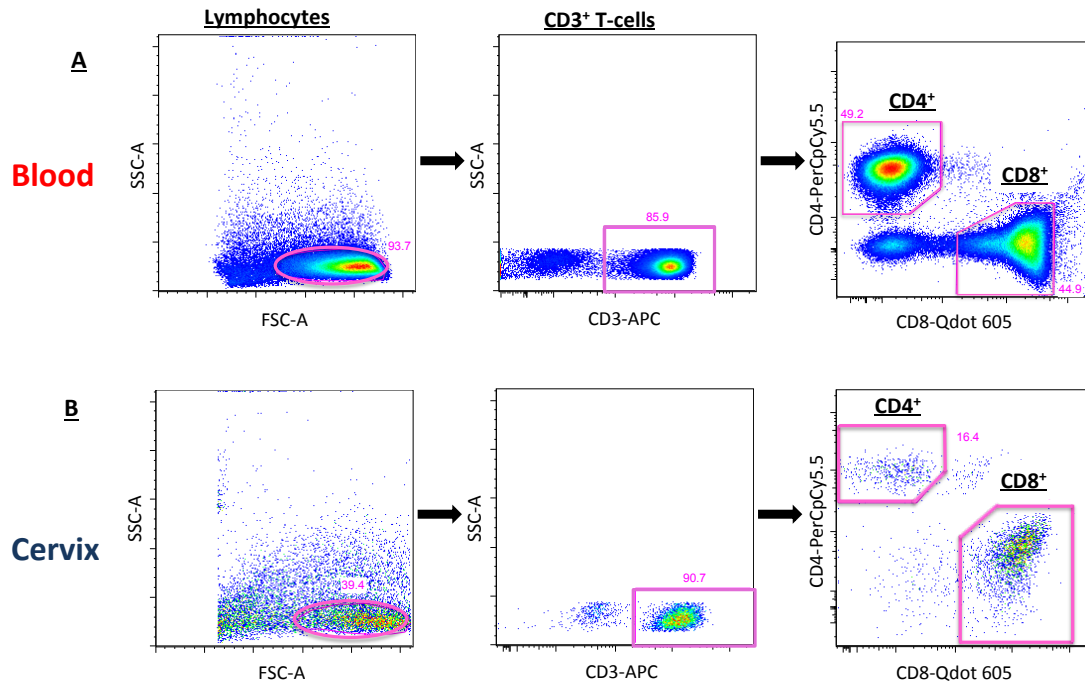


Figure 3.1. Representative flow cytometry plots showing the level of expression of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells from blood (A) and from the genital tract (cervical cytobrush) (B). The parameters SSC (side scatter) and FSC (forward scatter) were used to sort the cells according to granularity and size to identify the lymphocyte population. These cells were then gated on CD3⁺ T-cells and then further on CD4⁺ and CD8⁺ T-cells.

In blood, HIV-infected women on HAART had a median CD4:CD8 ratio of 0.7 (figure 3.2), indicating that these women had fewer CD4⁺ T-cells compared to CD8⁺ T-cells. In contrast, the median CD4:CD8 ratio for cervical mononuclear cells was 1.2, indicating the CD4⁺ T-cells were the dominant T-cell population. There was no significant difference and no correlation between blood and genital tract CD4:CD8 ratios. Barrett et al. (2012) proposed that CD4:CD8 ratios can be used as a surrogate to characterise an “immune risk phenotype”, similarly to the use of total CD4 counts, with CD4:CD8 <1 reflecting immune dysfunction predisposing to several co-morbidities. Previous studies in healthy seronegative individuals have reported that blood CD4:CD8 ratios were typically >1.6 (Margolick et al., 2006) and that cervical CD4:CD8 ratios were 2.7 (Nkwanyana et al., 2009), suggesting that the women enrolled in this study, despite being on HAART for a few years, had not reconstituted to pre-HAART CD4:CD8 ratios.

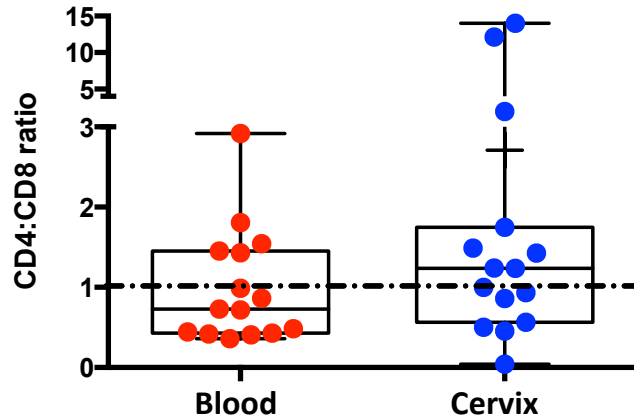


Figure 3.2. CD4:CD8 ratios in blood (●) and cervical (●) compartments. Each dot represents an individual woman's CD4:CD8 ratio in either blood or from cervical cytobrushes (cervix). The box and whisker plots represent the median (centre line), and interquartile range, while the error bars represent the range. The horizontal dotted line shows the threshold (CD4:CD8 ratio=1), below which the ratio becomes inverted.

Previous studies have shown that CD4⁺ T-cell reconstitution was dependent on the length of time HIV-infected women had been on HAART and that, although the CD4⁺ T-cell count increased the most in the first 2-3 months, this reconstitution persisted for up to 4 years in individuals on therapy (Guihot et al., 2011; Hunt et al., 2003a). Therefore, the impact of length of time on HAART on CD4⁺ T-cell reconstitution in this study was investigated (figure 3.3). There was no significant relationship between the length of time for which HIV-infected women had been on HAART and the CD4:CD8 ratios in either compartment.

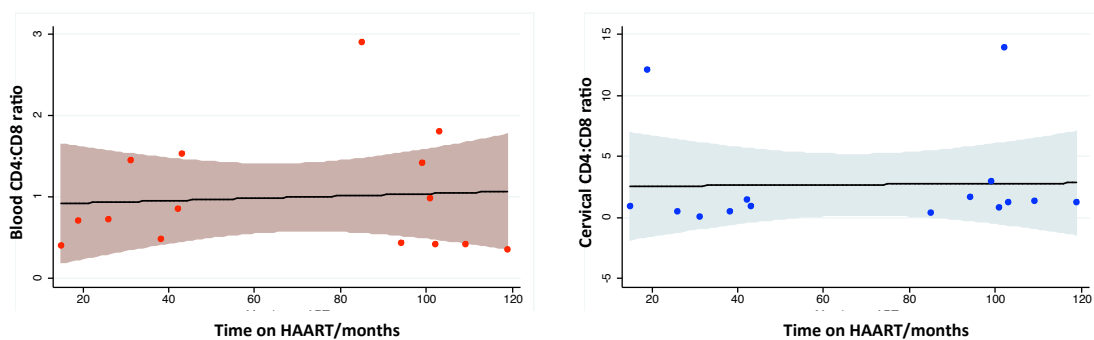


Figure 3.3. Relationship between time on HAART and changes in CD4:CD8 ratios in blood (A) and the female genital tract (B). The black line shows the linear regression line and the shaded area around the line represents the 95% confidence interval for the mean. Each dot represents an individual woman's ratio relative to the length of time she had been on therapy.

Next, CD4⁺ and the CD8⁺ T-cell subsets were further divided into the four memory subsets (naïve, TCM, TEM and TD), based on the surface expression of CD45RO and CCR7 (figure 3.4). CD45RO has been proposed to be useful in defining T-cell memory because it is a glycoprotein preferentially expressed on memory cells and has been reported to enhance receptor-mediated activation of T-cells (reviewed by [Mahnke et al., 2013](#)). CCR7, a chemokine receptor which controls homing of cells to secondary lymphoid organs, has been reported to distinguish naïve and central memory T-cells ([Sallusto et al., 1999](#)). Naïve T-cells have been suggested to be CD45RO⁻ but CCR7⁺, central memory T-cells to be double positive (CD45RO⁺CCR7⁺), effector memory to express CD45RO⁺CCR7⁻ and terminally differentiated (CD45RO⁻CCR7⁻) T-cells ([Sallusto et al., 1999](#); reviewed by [Mahnke et al., 2013](#)).

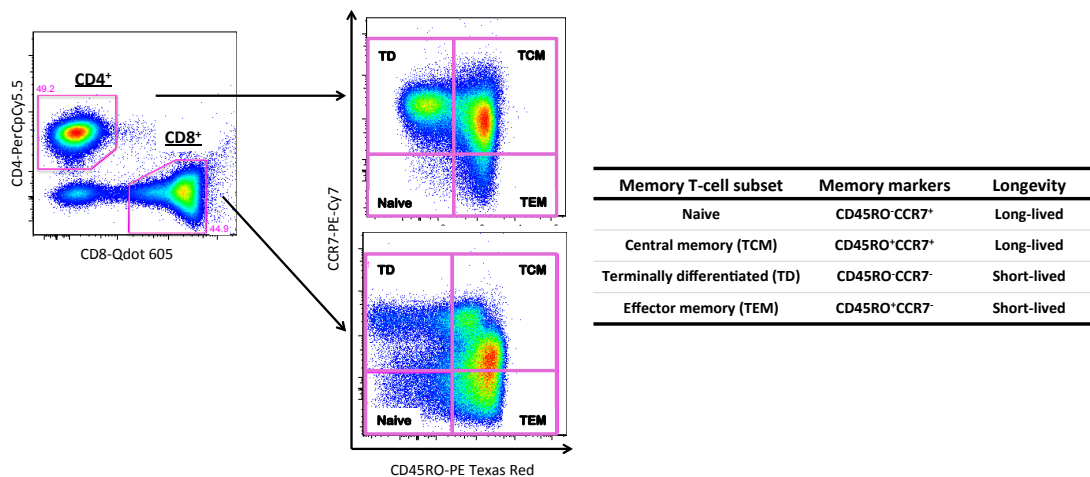


Figure 3.4. Representative plots showing how CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells were subdivided into the different memory subsets using the maturational markers CD45RO and CCR7.

To determine whether the distribution of the T-cell memory subsets differed between blood and the genital tract, the proportion of the CD4⁺ and the CD8⁺ T-cell subsets expressing memory markers were compared between compartments (figure 3.5). TEM were the predominant memory subtype in both blood and the genital tract. In blood, CD4⁺ TEM cells made up 36.0% and CD8⁺ TEM cells made up 40.3% of all CD3⁺ T-cells. In the genital tract, CD4⁺ TEM cells made up 66.7% and CD8⁺ TEM cells made up 74.0% of all CD3⁺ T-cells.

In both compartments, differentiated CD8⁺ T-cell subsets formed the majority of all T-cells, with the TEM and TD being present at higher frequencies than naïve and TCM (Figure 3.5A and 3.5B). In the CD4⁺ T-cell population, memory (CD45RO⁺) T-cells outnumbered naïve (CD45RO⁻) T-cells, with CD4⁺ TEM cells being the most prevalent followed by TCM cells (Figure 3.5C and 3.5D). Overall, when comparing memory subset distribution between blood and the genital tract, the genital compartment had a significantly lower percentage of naïve T-cells and TD and a higher percentage of TEM compared to blood (figure 3.5B and D).

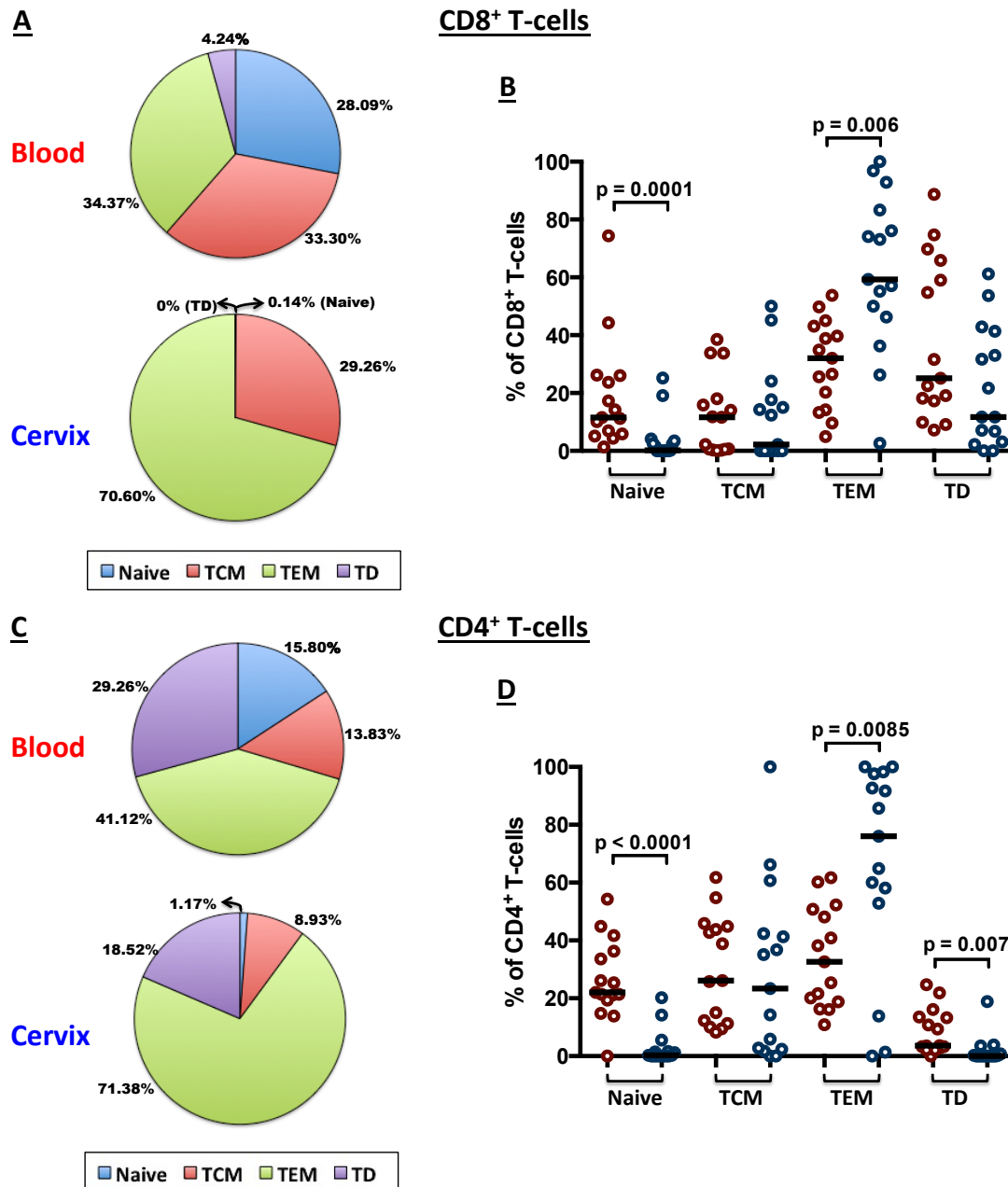


Figure 3.5. Comparison between the percentage of memory subsets in blood and the genital tract for CD8⁺ (A and B) and CD4⁺ T-cells (C and D). The blue, red, green and purple slices of the pie charts (A and C) represent the percentage of naïve, central memory, effector memory and terminally differentiated T-cells respectively. In B and D, PBMC are represented by the red circles (●) and the CMC by the blue circles (●). A p value of ≤ 0.05 was considered to be significant.

3.3.3 Comparison of T-cell activation in blood and the genital tract

Using CD38 and HLA-DR as markers of T-cell activation, the frequencies of T-cells expressing any combination of these markers were measured for blood and cervical cytobrush cells (figure 3.6). Generally, higher frequencies of genital T-cells had an activated phenotype compared to blood T-cells (Figure 3.6B and C),

significantly so for CD4⁺ T-cells expressing CD38⁺ (p=0.02) and HLA-DR⁺ (p=0.03).

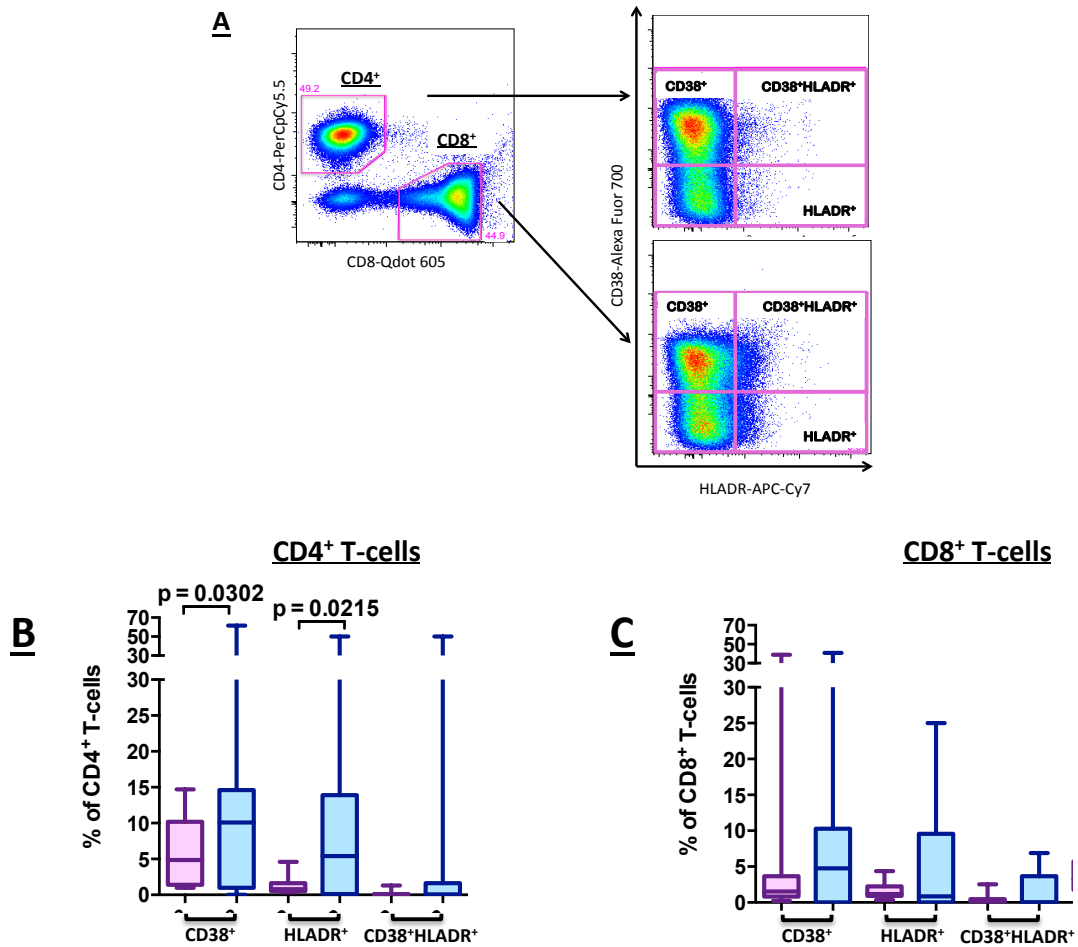


Figure 3.6. Comparison between T-cell activation in blood and the female genital tract of HIV-infected women on HAART. **A.** Representative plots showing how CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells were distinguished based on surface expression of the activation markers CD38 and HLA-DR. CD38⁺HLA-DR⁺ T-cells are those that express both activation markers. **B.** Differences in the percentages of CD38⁺, HLA-DR⁺ and CD38⁺HLA-DR⁺ in CD4⁺ T-cells. **C.** Differences in the percentage of the different activation markers for CD8⁺ T-cells. The Wilcoxon matched-pairs signed rank test was used to compare groups and a p value of ≤ 0.05 was considered significant. The purple box and whisker plots represent the percentage expression of the markers in blood and the blue box and whisker plots represent the percentage expression in the genital tract.

T-cells were subdivided according to their memory marker expression profiles and assessed for their respective levels of activation (figure 3.7). CD4⁺ TEM from the genital tract generally were more activated than those from blood, and this difference was significant for CD4⁺ TEM cells expressing both CD38 and HLA-DR (p=0.02). The level of activation in TCM tended to be higher in CD4⁺ cervical T-

cells compared to blood, although not significantly. In contrast, CD4⁺ TD in blood had significantly higher frequencies of CD38⁺, HLA-DR⁺ and doubly activated cells compared to T-cells derived from the genital tract ($p < 0.0001$ for CD38 and HLA-DR individually and $p = 0.0002$ for expression of both markers). Similarly, significantly higher frequencies of naïve CD4⁺ T-cells from blood expressed CD38 ($p = 0.0002$), HLA-DR ($p = 0.0034$) or both markers ($p < 0.0001$) compared to T-cells in the genital tract, although naïve cells were generally detected at very low frequencies in the genital tract compared to blood.

Similarly to CD4⁺ T-cells, a significantly higher percentage of naïve CD8⁺ T-cells from blood expressed CD38⁺, HLA-DR⁺ and CD38⁺HLA-DR⁺ compared to naïve T-cells from the female genital tract ($p = 0.0005$, $p = 0.0002$ and $p < 0.0001$, respectively). Higher frequencies of CD8⁺ TD cells in blood expressed CD38, HLA-DR or a combination of both markers ($p = 0.0181$, $p = 0.0123$ and $p = 0.0022$, respectively) compared to those in the female genital tract. In contrast, significantly higher frequencies of genital CD8⁺ TCM cells expressed HLA-DR and CD38⁺HLA-DR⁺ compared to blood ($p = 0.0181$ and $p = 0.0071$, respectively).

CD4⁺ T-cells

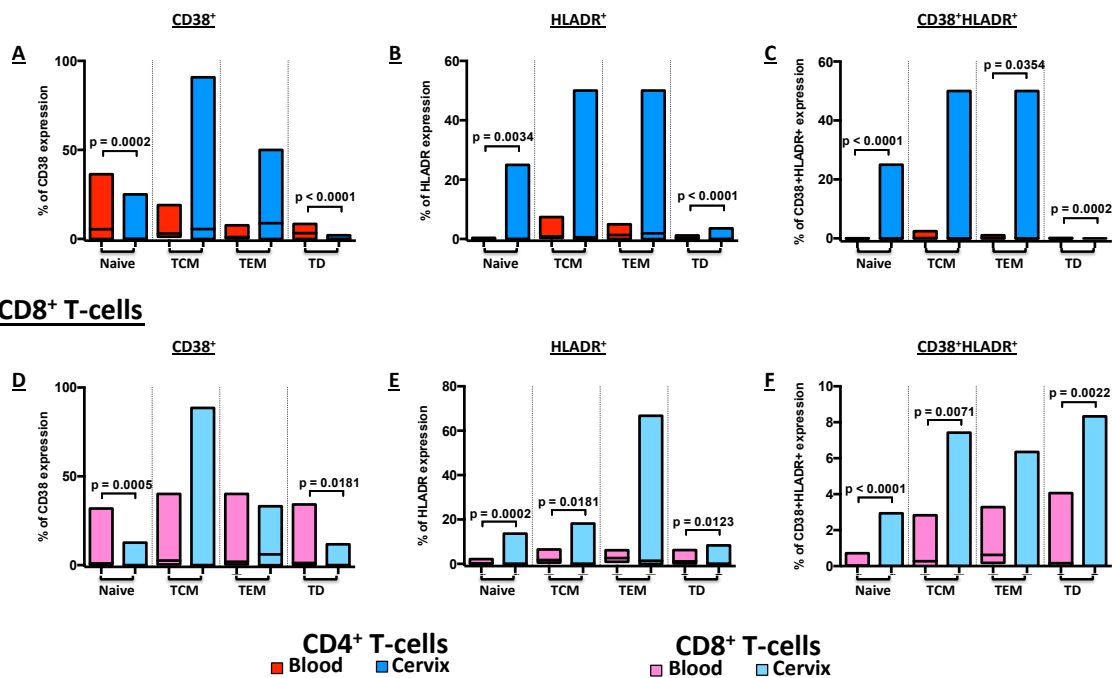
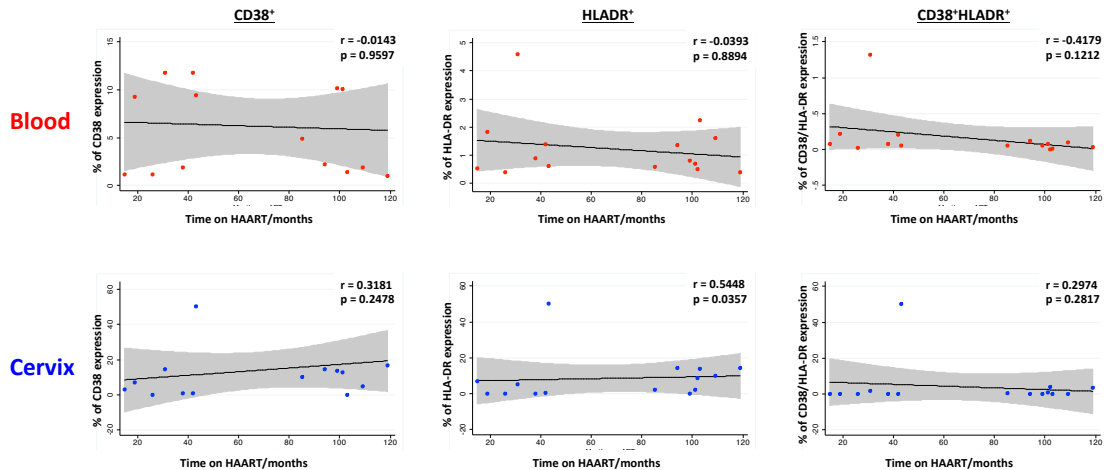


Figure 3.7. Comparison between activation status of memory T-cell subsets in blood and the genital tract. **A-C.** Percentage of CD38⁺, HLA-DR⁺ and CD38⁺HLA-DR⁺ expression in the CD4⁺ T-cell subsets. **D-F.** Percentage of CD38⁺, HLA-DR⁺ and CD38⁺HLA-DR⁺ expression in the CD8⁺ T-cell subsets. Wilcoxon matched-pairs signed rank test was used for statistical comparisons and p values of ≤ 0.05 was considered significant. Blood CD4⁺ and CD8⁺ T-cells are represented by the red and pink bars respectively while the genital CD4⁺ and CD8⁺ T-cells are shown in dark blue and pale blue respectively.

Previous studies have suggested that length of time an HIV-infected individual has been on HAART impacts significantly on the level of T-cell activation (Hunt et al., 2003b; Robbins et al., 2009). To investigate this, the degree of activation for each HIV-infected women included in this study was compared to the length of time she had been on therapy (figure 3.8). No significant relationship was found between the percentage of activation marker expression and length of time on HAART in either compartment.

CD4⁺ T-cells



CD8⁺ T-cells

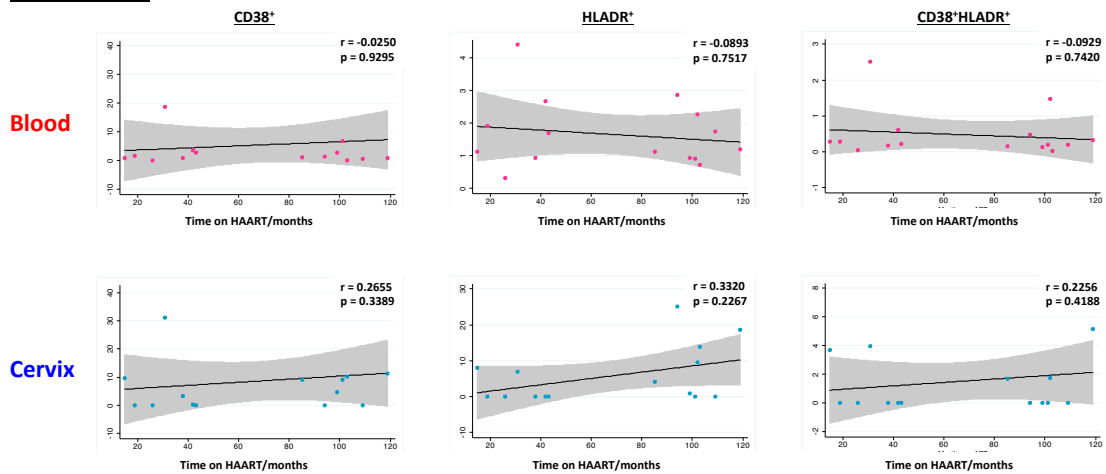


Figure 3.8. Relationship between time on HAART and activation marker expression levels in blood and the genital tract for CD4⁺ (A and B) and CD8⁺ (C and D) T-cells. The black line shows the regression line and the shaded area around the line represents the 95% confidence interval for the mean. The red and dark blue dots represent CD4⁺ T-cells in blood and the genital tract respectively while the pink and light blue dots represent CD8⁺ T-cells in the two compartments.

Jaspan et al. (2011) previously reported that the extent of T-cell activation in the genital tract of HIV-infected women (naïve to HAART) correlated with that present in blood. To determine whether the level of T-cell activation in the genital tract was related to level of activation in blood in this study, linear regression models were used to compare the different activation markers between the two compartments (figure 3.9). The frequency of CD4⁺ T-cells expressing CD38⁺ in blood significantly predicted the frequency in the genital compartment (β coefficient=0.279; $p=0.03$). Similarly, the level of CD8⁺ T-cells in blood expressing CD38⁺ strongly predicted the level in the genital tract (β

coefficient=0.989; $p < 0.0001$). None of the other markers correlated between compartments.

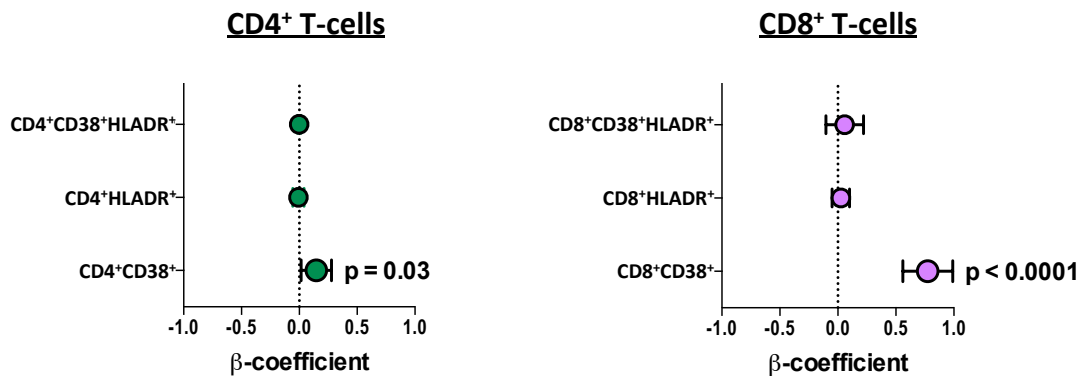


Figure 3.9. Relationship between levels of T-cell activation in blood and the genital compartment. T-cells expressing the activation markers CD38 and HLA-DR, or co-expression of both were compared between compartments. The CD4⁺ T-cells are represented by green circles (●) and the CD8⁺ T-cells by purple circles (●). Simple linear regression models were used and β -coefficients represent the percentage by which the median frequency of each immune activation marker expression in the genital tract will increase with every percentage increase of the corresponding marker in the blood. p values of ≤ 0.05 were considered to be significant.

3.3.4 Comparison of proliferation and exhaustion markers on T-cells from blood and the genital compartment during HIV infection

T-cell turnover (characterised by the presence of the proliferation marker Ki67) and exhaustion (characterised by the presence of the senescence marker CD57) are useful clinical disease progression markers, and their expression has been shown to be down-regulated by consistent suppressive HAART (Mohri et al., 2001; Palmer et al., 2005). The frequencies of T-cells expressing these markers were compared in blood and the genital tract (figure 3.10).

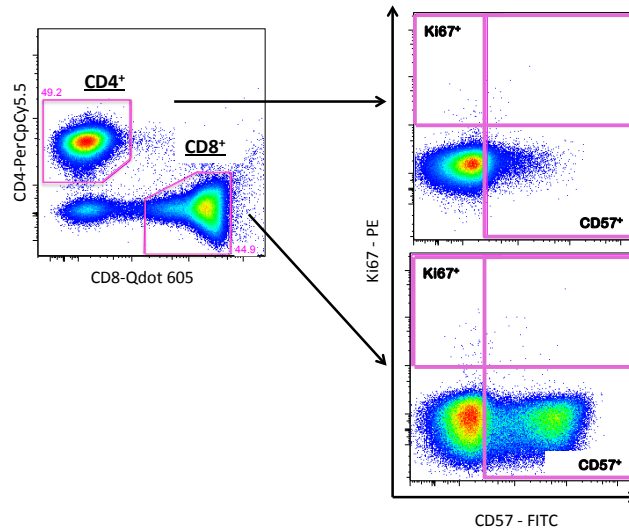


Figure 3.10. Representative plots showing the gating strategy used to determine levels of proliferation (Ki67⁺ T-cells) and senescence (CD57⁺ T-cells) from CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells.

To determine whether there was a difference between the proliferative capacity and cell turnover between CD4⁺ and CD8⁺ T-cells within compartments, the percentage of T-cells expressing Ki67 was compared in blood and the genital tract (figure 3.11A). Comparable frequencies of CD4⁺ and CD8⁺ T-cells in blood expressed Ki67, indicating that CD4⁺ T-cells were proliferating as much as CD8⁺ T-cells. In contrast, significantly higher frequencies of CD4⁺ T-cells expressed Ki67 compared to the CD8⁺ T-cells in the female genital tract ($p=0.0001$; figure 3.11B), suggesting that CD4⁺ T-cells in the genital tract were proliferating more than CD8⁺ T-cells. For both CD4⁺ and the CD8⁺ T-cells, genital cells were more actively proliferating than those in blood (20-fold difference for CD4⁺T-cells: $p=0.0151$; 7.3-fold difference for CD8⁺ T-cells: $p=0.0004$; figures 3.11C and D).

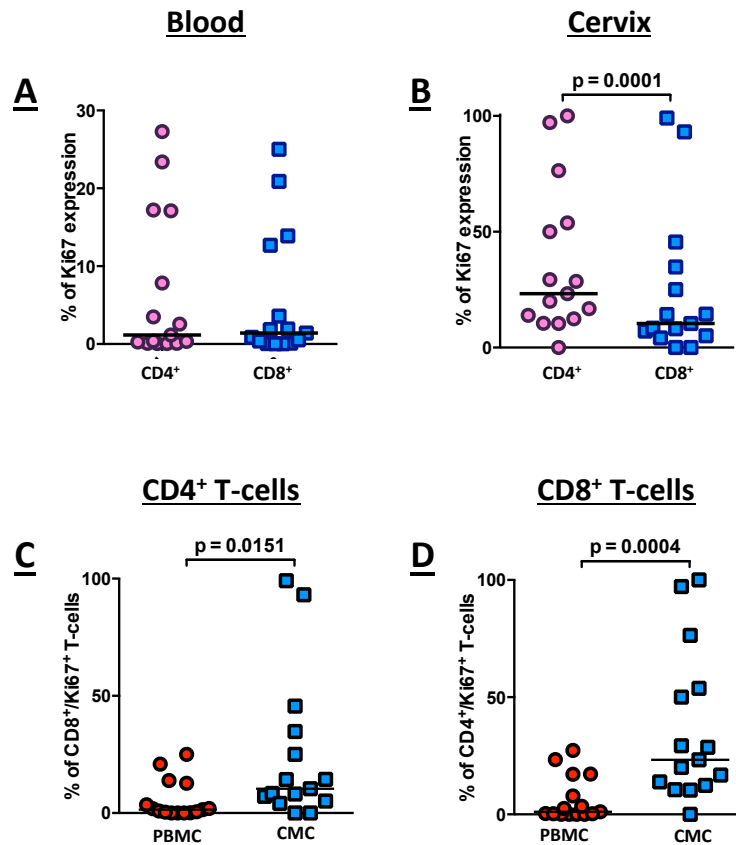


Figure 3.11. Comparison between the frequencies of proliferating (Ki67⁺) T-cell populations in blood and the female genital tract. **A.** Comparison between Ki67 expression by T-cells in blood. **B.** Comparison between Ki67 expression by T-cells in the genital tract. **C.** Comparison between blood and cervical Ki67 expression by CD4⁺ T-cells. **D.** Comparison between blood and cervical Ki67 expression by CD8⁺ T-cells. The pink and dark blue dots represent CD4⁺ and CD8⁺ T-cells in either compartment while the red dots represent T-cells from blood and the pale blue dots represent cervical T-cells. The Mann Whitney U test was used to compare each of the two groups and p values of ≤ 0.05 were considered to be significant.

Since time on HAART has previously been proposed to impact on the overall level of activation (Hunt et al., 2003b) and elevated activation of T-cells may impact on cell turnover (Catalfamo et al., 2008; Hazenberg et al., 2000), the relationship between time on HAART and the frequency of T-cells expressing Ki67 was evaluated (figure 3.12). There was no significant change in the level of expression of Ki67 over time on HAART for both CD4⁺ and CD8⁺ T-cells in blood and the genital tract.

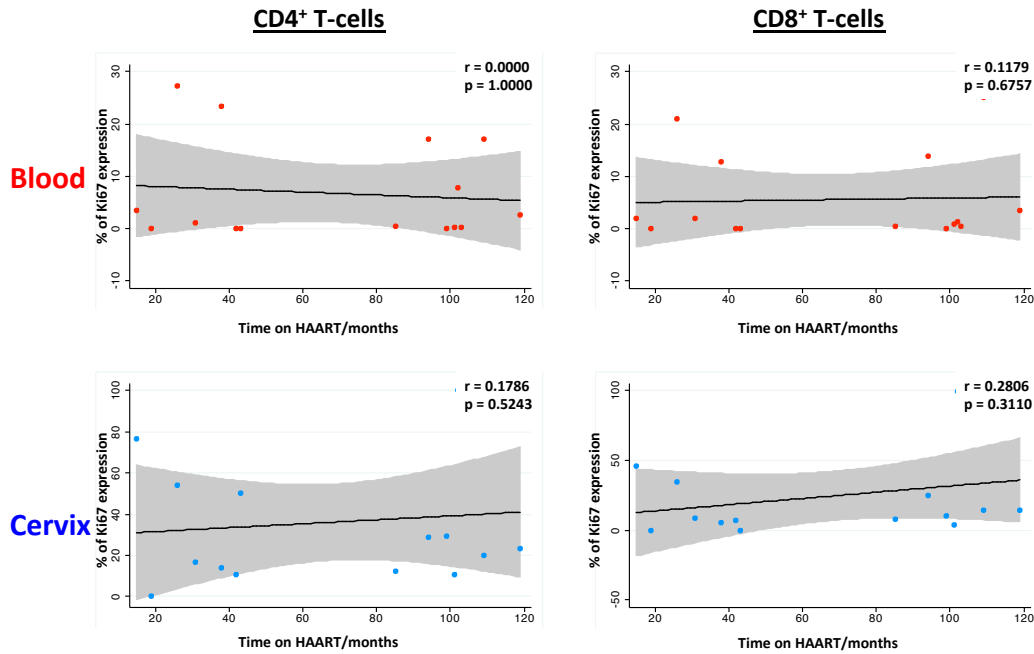


Figure 3.12. Relationship between length of time on HAART and Ki67 expression (indicating cell turnover) for T-cells in blood (A and B) and the genital tract (C and D). The black line shows the regression line and the shaded area around the line represents the 95% confidence interval for the mean. Red dots represent T-cells in blood while blue dots represent cells from the female genital tract.

Previous studies have shown that proliferating T-cells (a subset of cells which closely track with plasma viral load) are mainly memory ($CD45RO^+$), and predominantly TCM cells (Lempicki et al., 2000; Sieg et al., 2005). Thus, the memory T-cell distribution of proliferating cells for the women in this study was investigated (figure 3.13). In blood, significantly fewer naïve $CD4^+$ T-cells expressed Ki67 compared to the three more differentiated $CD4^+$ T-cell subsets (figure 3.13A), with $CD4^+$ TCM having the highest frequencies of Ki67 of all the memory subsets. Similarly, significantly fewer naïve $CD8^+$ T-cells expressed Ki67 compared to the other memory subsets (Figure 3.13B). In the genital tract, higher frequencies $CD4^+$ TCM expressed Ki67 compared to the other memory T-cell subsets (figure 3.13C). Ki67 was not expressed on the $CD4^+$ naïve and TD cells for most of the women. For $CD8^+$ T-cells, the TEM cells tended to be the most likely memory T-cell subset to express Ki67, although there was no significant difference in the percentage expression of Ki67 between any of the four naïve/memory subsets (figure 3.13D).

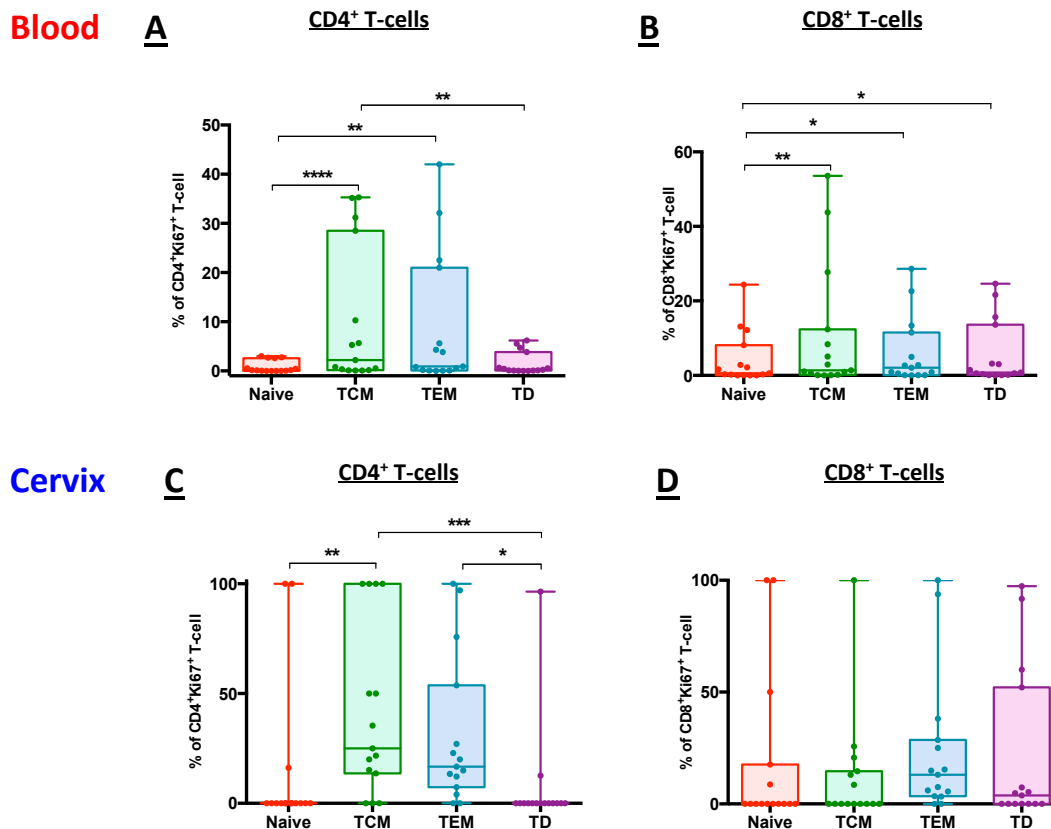


Figure 3.13. Comparison between the level of proliferation within the naïve and memory T-cell subsets in blood and the female genital tract. Ki67 expression by CD4⁺ and CD8⁺ T-cells in blood (A-B) and the genital tract (C-D). Naïve T-cells are shown as red boxes, central memory T-cells as green boxes, effector memory T-cells as blue boxes and terminally differentiated T-cells as purple boxes. One-way ANOVA was used to compare the different subsets and the comparisons that were significant after adjusting for multiple comparisons are shown. p values ≤0.05 were considered to be significant where * is p≤ 0.05; ** is p≤ 0.01; *** is p≤ 0.001 and **** is p≤ 0.0001.

Next, the extent of senescence/exhaustion in the different T-cell subsets was determined (figure 3.14). Overall, there was a significantly higher level of expression of CD57 in the CD8⁺ than in the CD4⁺ T-cell population, irrespective of which compartment it was found in (figure 3.14A and B, p<0.0001 for blood and p=0.0017 for the cervix). However, there were comparable levels of T-cell exhaustion blood and cervical cells for both CD4⁺ and CD8⁺ T-cells (figure 3.14C and D). The level of CD57 expression in both blood and the cervix was independent of the length of time for which the women were on HAART (figure 3.15).

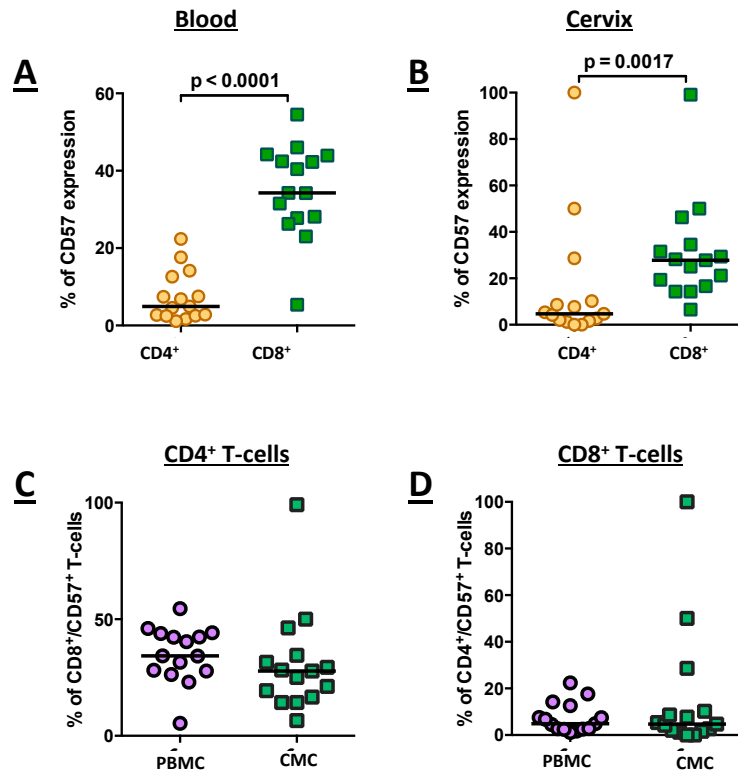


Figure 3.14. Comparison between the frequency of senescence marker expression (CD57) by T-cells in blood and the genital tract. Expression of CD57 by CD4⁺ T-cells (yellow) and CD8⁺ T-cells (green) in (A) blood or (B) genital tract. Expression of CD57 in blood (purple) and the genital compartment (green) by (C) CD4⁺ and (D) CD8⁺ T-cells.

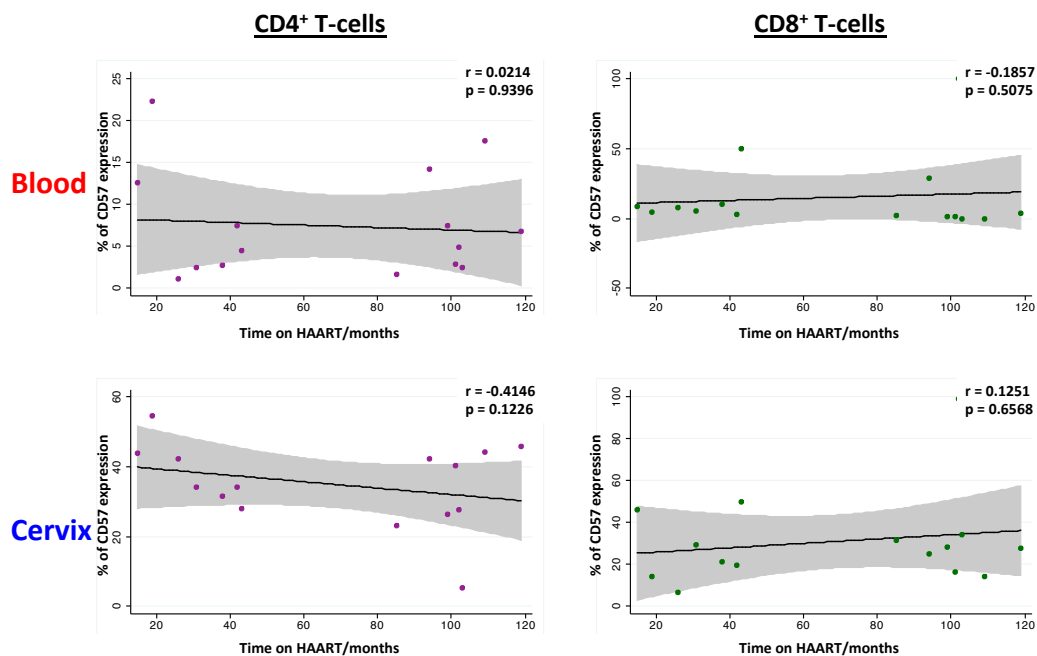


Figure 3.15. Relationship between time on HAART and T-cell exhaustion in blood and the cervix for CD4⁺ (A and B) and CD8⁺ (C and D) T-cells. The black line shows the regression line and the shaded area around the line represents the 95% confidence interval for the mean.

Previous studies have shown a positive association between CD57 expression and the differentiation status of T-cells, with higher frequencies of the most differentiated T-cells expressing CD57 than less differentiated T-cells (Hoji et al., 2007; reviewed by Appay & Sauce, 2008). In support of this, naïve CD4⁺ T-cells in blood had significantly lower levels of CD57 expression compared to all the other memory subsets (figures 3.16A and B). Higher frequencies of the two more differentiated CD4⁺ T-cell subsets (TEM and TD cells) expressed of CD57 than the two less differentiated subsets (naïve and TCM cells). Of the more differentiated subsets in blood, CD4⁺ TEM cells expressed higher frequencies of CD57 than TD cells. For blood CD8⁺ T-cells, TEM cells expressed significantly higher levels of CD57 compared to the other subsets. Similarly, in the genital tract, CD4⁺ TEM cells expressed the most CD57, although TCM had the second highest expression of CD57 (figure 3.16C). For genital tract CD8⁺ T-cells, the two more differentiated T-cell subsets expressed higher levels of CD57 (highest TEM followed by TD; figure 3.16D) than the two less differentiated T-cell subsets.

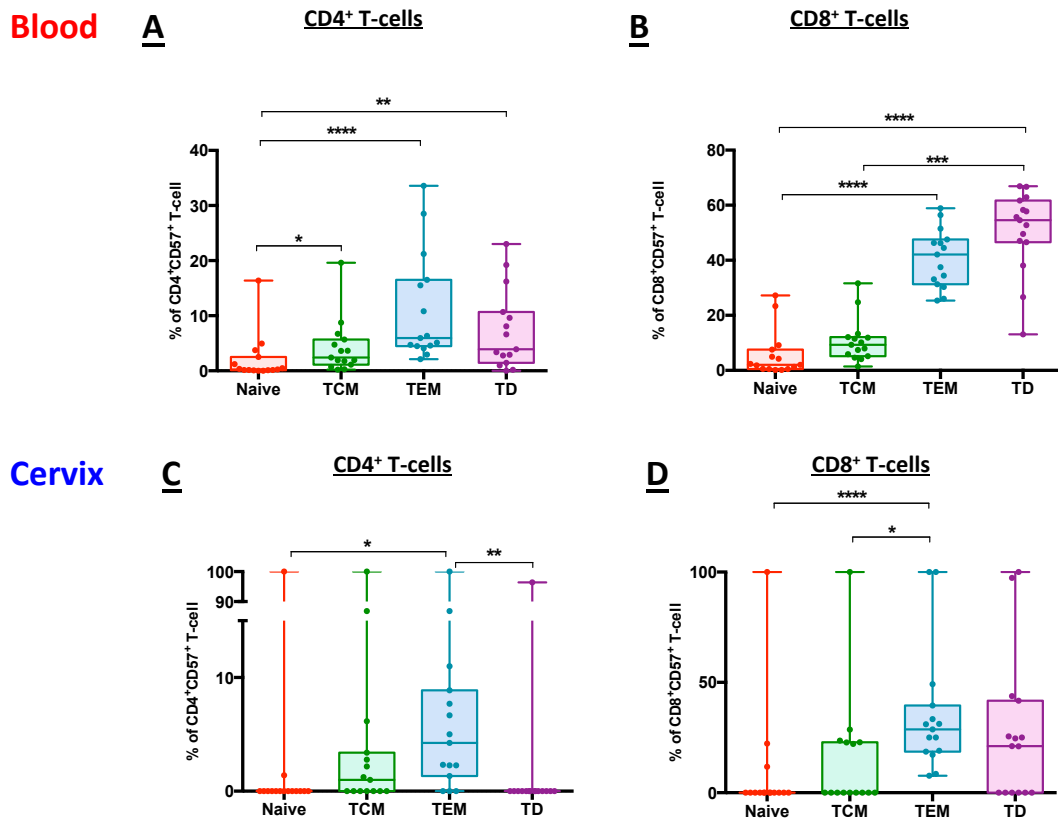


Figure 3.16. Comparison between the level of CD57 expression by memory T-cell subsets in blood and the female genital tract. **(A-B)** Differences in the percentages of CD57 in blood CD4⁺ and CD8⁺ T-cells. **(C-D)** Percentages of cervical CD57⁺CD4⁺ and CD8⁺ T-cell subsets. Naïve T-cells are shown in red, central memory T-cells in green, effector memory T-cells in blue and terminally differentiated T-cells in purple. One-way ANOVA was used to compare the different subsets and the comparisons that were significant after adjusting for multiple comparisons are shown. p values ≤0.05 were considered to be significant where * is p ≤ 0.05; ** is p ≤ 0.01; *** is p ≤ 0.001 and **** is p ≤ 0.0001.

To establish whether there was any relationship between the level of T-cell activation (HLA-DR and CD38 expression) and the level of exhaustion (CD57 expression), frequencies of the different markers of activation and the senescence marker CD57 were compared (table 3.2). For CD8⁺ T-cells, a significant positive relationship was observed between activation marker expression and CD57 expression. This suggests that the activated CD8⁺ T-cells are also those who have been through the most replicative cycles and are the most exhausted cells. The same trend was observed for CD4⁺ T-cells, although these were not significant.

Table 3.2. Relationships between T-cell activation markers and senescence.

	Marker(s)	R*	95% CI	p-value
CD8+ T-cells	CD38+	0.6167	0.1783 - 0.8508	0.0099
	HLA-DR+	0.5092	0.001886 - 0.8081	0.0460
	CD38+HLA-DR+	0.5022	0.007497 - 0.8048	0.0495
CD4+ T-cells	CD38+	0.3522	-0.1895 - 0.7296	0.1796
	HLA-DR+	0.1432	-0.3933 - 0.6069	0.5940
	CD38+HLA-DR+	0.2264	-0.3180 - 0.6585	0.3943

*Spearman correlation coefficients and 95% confidence intervals

3.3.5 Comparison between cytokine profiles in blood and the genital compartment

Concentrations of 10 cytokines were measured in matched plasma and genital tract secretions from the HIV-infected women included in this cohort who were on HAART (figure 3.17). Of these 10 cytokines, 7/10 were inflammatory (IL-1 β , IL-6, IL-8, IP-10, MIP-1 α , MIP-1 β and TNF- α), 2/10 were haematopoietic (IL-7 and G-CSF) and IL-10 was considered to be a regulatory cytokine. All 7 of the inflammatory cytokines were detected at significantly higher concentrations in the genital compartment compared to blood plasma (IL-1 β : p=0.0078; IL-6: p=0.0078; IL-8: p=0.0078; IP-10: p=0.0078; MIP-1 α : p=0.0078; MIP-1 β : p=0.0156; TNF- α : p=0.0078). In addition, 1/2 of the hematopoietic cytokines were detected at significantly higher concentrations in genital secretions versus plasma (G-CSF: p=0.0078).

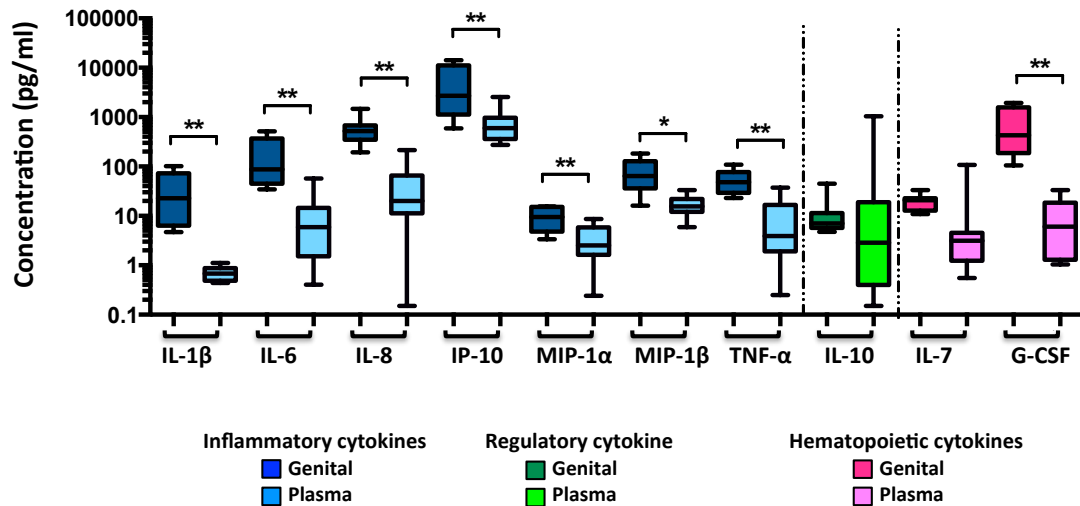


Figure 3.17. Comparison of the concentrations of the different inflammatory, regulatory and hematopoietic cytokines between blood and the genital tract. The box and whisker plots show the range of concentration for the cytokines in each compartment. The line at the centre of the box represents the median concentration, the box shows the interquartile range and the error bars below and above give the minimum and maximum values. The Wilcoxon matched-pairs signed rank test was used for statistical comparisons

3.3.6 Relationship between cytokine concentrations and T-cell activation

The relationship between the concentration of cytokines in each compartment and the respective levels of T-cell activation was evaluated (data not shown). In the genital tract, there was a significant positive association between the concentration of IL-8 and frequencies of activated CD4⁺ T-cells expressing CD38 and HLA-DR ($\rho=0.9286$ and $p=0.0009$). This association was still weakly significant after adjusting for multiple comparisons ($p = 0.054$ after applying the Bonferroni correction) In blood, no similar association was found between cytokines in plasma and CD4⁺ T-cell activation. On the other hand, plasma IP-10 correlated significantly with the total level of activation in the CD8⁺ T-cell population ($\rho=0.7143$, $p=0.0465$). The same trend was seen for the level of G-CSF and total CD8⁺ T-cell activation ($\rho=0.8503$, $p=0.0075$).

Next, linear regression modelling was used to determine whether the concentrations of cytokines could predict the level of T-cell activation in each compartment (figure 3.18). While cytokine concentrations in genital secretions had no significant prognostic value for T-cell activation (data not shown), plasma concentrations of MIP-1 β , IP-10 and G-CSF could significantly predict the level

CD38⁺ expression in CD8⁺ T-cells (MIP-1β: β coefficient = 1.0403; p = 0.0233; IP-10: β coefficient = 108.9; p = 0.0026; G-CSF: β coefficient = 1.398; p = 0.0339) as well as CD38⁺HLA-DR⁺ expression (MIP-1β: β coefficient = 7.499; p = 0.0299; IP-10: β coefficient = 836.7; p = 0.0008; G-CSF: β coefficient = 10.32; p = 0.0347). In addition, plasma IP-10 concentration could significantly predict CD8⁺HLA-DR⁺ expression (MIP-1β: β coefficient = 555.9; p = 0.0026). Figure 3.18 shows the predictive value for the expression of the two activation markers CD38 and HLA-DR and for the doubly-activated CD8⁺ T-cells.

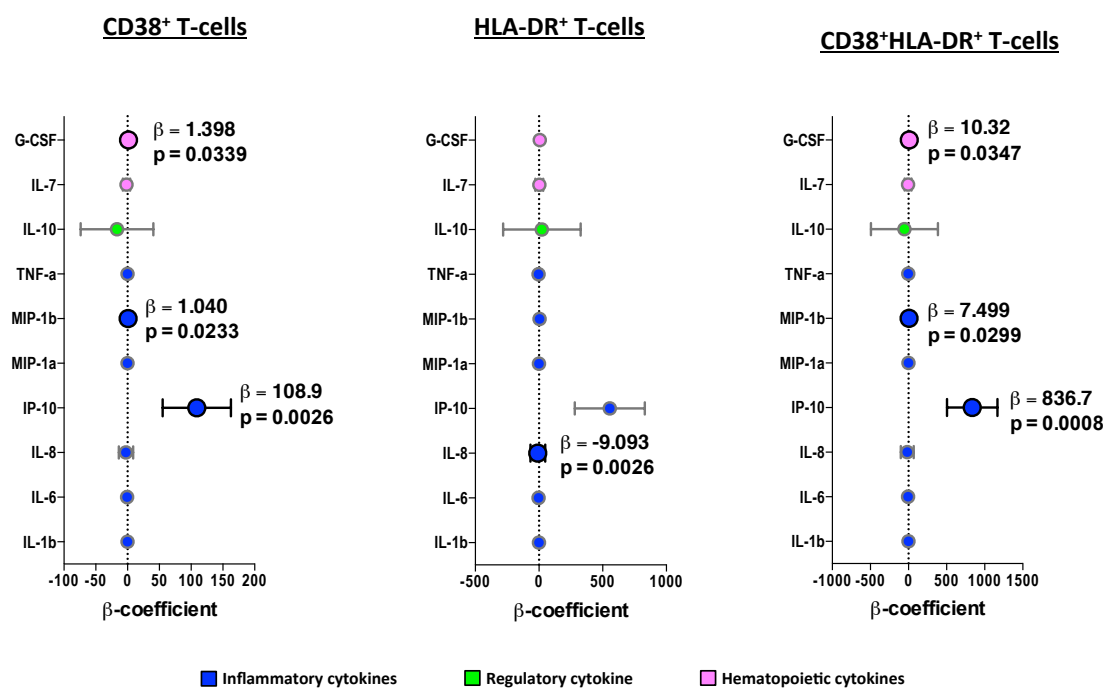


Figure 3.18. Association between the level of plasma cytokines and blood CD8⁺ T-cell activation. Cytokine concentrations were modelled against T-cells expressing the activation markers CD38 and HLA-DR, or the co-expression of both. The inflammatory cytokines are represented by blue circles (●), the regulatory one by green circles (●) and the hematopoietic cytokines by pink circles (●).

3.4 Discussion

While a number of excellent studies have investigated the effect of HAART on immune reconstitution during HIV infection (Rönsholt et al., 2012; reviewed by Guihot et al., 2011 & Hunt, 2012), comparatively few studies have described the impact of antiretroviral drugs on immune reconstitution at the genital mucosa (Jaspan et al., 2011; Mkhize et al., 2010; Nkwanyana et al., 2009). Antiretroviral drug classes have been shown to differ in their bioavailability at mucosal sites (Else et al., 2011) and some studies have noted a dysregulation between HIV suppression systemically and at the mucosa during HAART (Cummins et al., 2006; Mukura et al., 2012; reviewed by Anderson & Cu-Uvin, 2008). This chapter therefore compared the impact of HAART on the differentiation status, immune activation and exhaustion in CD4⁺ and CD8⁺ T-cells isolated from the genital mucosa from HIV-infected women, and investigated the effect of systemic versus mucosal cytokines on T-cell activation.

Although absolute CD4⁺ counts (and plasma viral load to a lesser extent) are used in clinical settings to describe the stage of an HIV infection and make treatment decisions, blood CD4:CD8 ratios have also previously been shown to be a good indicator of HIV disease progression (Margolick et al., 2006, Taylor et al., 1989). Taylor et al. (1989) showed that CD4:CD8 ratios had a slightly better prognostic value than either CD4⁺ T-cell percentages or absolute CD4⁺ T-cell counts. One of the most interesting findings from this chapter was that HIV-infected women on HAART had a CD4:CD8 ratio in blood of 0.7 and in their genital tracts of 1.2. Although all the women in this study were on HAART, it was interesting to note that their cervical CD4:CD8 ratios were more similar to those found in HIV-infected women naïve to HAART (CD4:CD8 ratio of 0.4; Nkwanyana et al., 2009) than to HIV negative African women (CD4:CD8 ratio of 2.7; Nkwanyana et al., 2009). This suggests that the HIV-infected women in this study, despite being on HAART for a median of 85 months (~7 years), had not reconstituted their CD4⁺ T-cell numbers to those typically observed pre-HIV-infection. Also, both the blood and genital CD4:CD8 ratios were independent of time on HAART.

Genital tract T-cells were found to be predominantly TEM. Genital CD4⁺ T-cells showed the highest proliferation potential (as measured by Ki67 staining) while genital CD8⁺ T-cells showed the highest levels of immunosenescence (measured by CD57 expression). Replicative senescence (CD57 expression) in the genital CD8⁺ T-cell population was positively associated with the extent of their activation. A hypothesis of this study was that genital tract inflammatory cytokine levels would positively predict higher levels of T-cell activation in the genital tract. In support of this, a positive relationship was observed between genital tract IL-8 concentrations and CD4⁺ T-cell activation (measured by the frequency of CD38⁺HLA-DR⁺ expression). IL-8 has been shown to be one of the predominant cytokines found in the genital tract ([Narimatsu et al., 2005](#)). Narimatsu et al. (2005) showed that African women on average have higher physiological levels of IL-8 in the genital cytokine milieu compared to individuals from the United States. T-cells are particularly sensitive to IL-8-chemotaxis (through the expression of IL-8-induced adhesion molecules) even in the presence of relatively low concentrations of the cytokine ([Roux et al., 2000](#)). In addition, [Jaspan et al. \(2011\)](#) showed that the level of activation at the genital mucosa may be dependent on the systemic immune activation levels. In this case, IL-8 could be one of the chemotactic factors involved in the migration of activated T-cells from the blood) to the genital mucosa ([Jaspan et al., 2011](#); reviewed by [Iwasaki, 2010](#)). Although lower levels of inflammatory cytokines were measured in matching blood plasma than in the genital tract, a similar positive association was noted between the degree of activation by blood T-cells (CD8⁺CD38⁺, CD8⁺HLA-DR⁺ and CD8⁺CD38⁺HLA-DR⁺) and plasma concentrations of MIP-1 β , IP-10 and G-CSF.

T-cells have been shown to consist of diverse populations of naïve and memory subsets, including TEM, TCM, TD and naïve T-cells (reviewed by [Mahnke et al., 2013](#)). These phenotypically different memory subsets have distinct and specific functions ([Jameson and Masopust, 2009](#); [Sallusto et al., 1999](#)). In this chapter, the distribution of the different T-cell memory subsets differed significantly between blood and the genital tract. T-cells derived from the female genital tract

were predominantly TEM, with only low frequencies of naïve and TD subtypes. Shin and Iwasaki (2013) recently reported that TCM tend to home to secondary lymphoid organs (including draining lymph nodes and spleen), while TEM circulate through non-lymphoid tissues such as the genital and gut mucosa. This may explain the higher percentage of TEM in the genital compartment compared to frequencies detected in blood. Studies have shown that CD8⁺ TEM contribute significantly to recall responses to non-lymphoid tissues (Hikono et al., 2007; Klonowski et al., 2006). The high levels inflammatory chemokines present in tissues such as the genital tract may account for recruitment of CD4⁺ and CD8⁺ TEM to the cervical tissues under a chemokine gradient (Hikono et al., 2007).

Overall, T-cells from the genital compartment of HIV-infected women on HAART in this study were more activated than those in blood, with TEM in the genital tract being the most activated of all T-cell subsets isolated from the cervix. While genital TEM and TCM cells were found to be generally more activated than those in blood, naïve and TD in blood were less activated in the genital tract. Dianzani et al. (1994) showed that, in healthy individuals, CD38 is constitutively expressed at relatively low levels by naïve T-cells and is down-regulated in the memory T-cells (Dianzani et al., 1994; reviewed by Almeida et al., 2002). However, in HIV infection, its expression increases drastically in activated memory cells, which may explain why genital TCM and TEM had the highest CD38 expression in this study (Savarino et al., 2000).

This study found that CD38 expression by blood CD4⁺ and CD8⁺ T-cells was significantly predictive of CD38 expression by T-cells derived from the genital tract. It has also previously been shown that activated CD4⁺ and CD8⁺ T-cells are recruited to the cervix in the presence of a concurrent infection or inflammation (Prakash et al., 2003; Gumbi et al., 2008). HIV in the genital tract could cause the secretion of cytokines such as IL-8, MCP-1 and MIP-1 β or antimicrobial peptides such as human β -defensin-1 and -2, which facilitate the recruitment of immune cells such as T-lymphocytes amongst others (Schaefer et al., 2005; Taub et al., 1993).

For this study, it was found that genital CD4⁺ T-cells had a higher proliferative capacity than CD8⁺ T-cells and that Ki67 expression was higher in genital T-cells than those isolated from blood. Furthermore, TEM and TCM cells in the genital tract expressed the highest frequencies of Ki67 compared to the other subsets, with CD4⁺ TCM cells expressing the highest frequencies. This confirms previous reports that Ki67 expression by T-cells during HIV infection is increased compared to HIV-negative individuals, and that this increase in cell cycling occurs mostly in the memory CD4⁺ and CD8⁺ T-cell subsets (Lempicki et al., 2000; Picker et al., 2004; Sieg et al., 2005). This is important as CD4⁺ T-cells at the genital mucosa are the main targets of HIV infection (reviewed by Bhardwaj et al., 2012). The fact that there were quite a small proportion of CD4⁺ TCM cells in the genital tract may have been influenced by this. *In-vitro* studies have shown that blood T-cells from HIV-infected individuals, particularly those that are TCM, enter the S-phase of proliferation more readily than other memory subsets or T-cells from HIV negative individuals because of bystander immune activation (Sieg et al., 2005). These cells are more susceptible to cell death by apoptosis (Patki et al., 2000), possibly leading to the depletion of this T-cell subset.

Results from this chapter show that there were significantly higher frequencies of CD57 expression in the CD8⁺ T-cells populations compared to the CD4⁺ T-cells in both blood and the genital tract of HIV-infected women on HAART, although no difference between compartments was observed. This is in agreement with data published in Liebenberg et al. (2010), where they report that CD57 expression by T-cells was similar in blood and the genital tract of chronically HIV-infected women. The higher frequency of CD57 expression in CD8⁺ than CD4⁺ T-cells could be the result of CD4⁺ T-cells being target cells for HIV, which may be depleted as a result of being infected before they become immunosenescent. An alternative explanation could be that there is a real accumulation of CD8⁺CD57⁺ T-cells. Chronic viral infections are accompanied by a dysregulation in the normal CD57⁺ apoptotic mechanisms (Wood et al., 2009), which may result in an imbalance in the rate of proliferation and apoptosis of these cells, leading to the accumulation of an exhausted CD8⁺CD57⁺ T-cell population (reviewed by Focosi et al., 2010).

An important finding from this chapter was that the extent of genital tract CD8⁺ T-cell activation was significantly associated with the degree of exhaustion in this T-cell population, with the most activated CD8⁺ T-cells (CD38⁺ HLA-DR⁺) also being the most exhausted (CD57⁺). A similar trend was not observed for CD4⁺ T-cells, although the fact that CD4⁺ T-cells are also the direct targets for HIV infection, particularly activated CD4⁺ T-cells, may have confounded this observation. Papagno et al. (2004) also showed that elevated activation and differentiation have an impact on the level of replicative senescence. Furthermore, Nixon and Landay (2010) have also previously reported that persistent T-cell activation drives proliferation and T-cell differentiation and increased expression of CD57.

In blood, plasma MIP-1 β , IP-10 and G-CSF concentrations were positively associated with the level of CD38 expression by blood CD8⁺ T-cells. Saunders et al. (2011) reported that MIP-1 β and IP-10 were involved in non-cytolytic suppression (via the secretion of still unknown soluble mediators; [Overman et al., 2007](#)) of HIV by CD8⁺ T-cells, implying that this relationship may be indirect rather than direct. The presence of viral antigens would trigger an inflammatory response involving MIP-1 β and IP-10 from CD8⁺ T-cells, usually accompanied by the upregulation of the activation marker CD38 (including the non-cytolytic mechanisms; [Appay & Sauce, 2008](#)), so as to reduce viraemia and in turn, reduce the level of systemic activation. Thus, the change in the level of expression of CD38 by CD8⁺ T-cells could match the concentration of secreted MIP-1 β and IP-10. Simmons et al. (2013) also showed that IP-10 in blood plasma correlated positively with activation of T-cells, and concentrations of this cytokine predicted the level of expression of CD38 and HLA-DR in blood CD8⁺ T-cells. IP-10 is the cognate ligand for CXCR3, a receptor which is readily induced on T-cells upon activation via TLR 7/9 in an HIV infection (reviewed by [Groom & Luster, 2011](#)). This inflammatory cytokine causes the migration of immune cells to the site of infection, including activated CD8⁺ T-cells ([Padovan et al., 2002](#)). This could account for the positive relationship between the concentration of IP-10 and the degree of plasma CD8⁺ T-cell activation. G-CSF, on the other hand, has anti-inflammatory properties and it able to modulate cytokine release by

monocytes. G-CSF secretion has been suggested to contribute to attenuating the level of activation and secretion of IFN- γ via the inhibition of the pro-inflammatory cytokines IL-12 and TNF- α (Boneberg et al., 2000), suggesting that G-CSF may have an indirect or opposite effect on the level of systemic activation.

Secretions collected from the genital tract had significantly higher concentrations of 8/10 cytokines measured in this study than blood plasma. Of these, 7/8 elevated cytokines belonged to the inflammatory class (IL-1 β , IL-6, IL-8, IP-10, MIP-1 α , MIP-1 β , TNF- α) and 1/8 belonged to the hematopoietic class (G-CSF). This suggests a comparatively higher level of inflammation at the cervix compared to blood. In chronic HIV-infected individuals on HAART, low-levels of viraemia have been shown to induce a pro-inflammatory response at the genital tract which contributes to cell recruitment (reviewed by Rajasuriar et al., 2013). Several factors could also contribute to inflammation at the genital tract (such as STIs including *Neisseria gonorrhoeae*, *Chlamydia trachomatis* or *Trichomonas vaginalis*, and bacterial vaginosis) have been associated with the recruitment of a large number of inflammatory cells (reviewed by Mayer & Venkatesh, 2011). Antigenic stimulation from these STIs or bacterial vaginosis could also trigger an inflammatory cascade and could contribute to the higher levels of cytokines in the genital compartment (Galvin and Cohen, 2004). This might also explain the higher level of activation in the T-cell populations in the genital tract compared to blood.

Previous studies have shown that systemic immune activation and inflammatory markers remain elevated in HIV-infected individuals on HAART (reviewed by Klatt et al., 2013). Sustained levels of elevated immune activation has consequences for these HIV-infected individuals because it places them at risk for non-opportunistic co-morbidities, such as cardiovascular disease, type II diabetes or liver disease (reviewed by Hunt, 2012 & Klatt et al., 2013). Persistent immune activation and inflammation in HIV-infected individuals on HAART could be driven by several factors, such as low-level viraemia despite HAART (below the level of detection), co-infections with other pathogens (such as CMV), or ongoing microbial translocation (as a result of incompletely constituted gut

mucosal barrier function). Intensification of therapy in such individuals has been used in an attempt to dampen the effect of low levels of ongoing HIV replication on T-cell activation ([Dinoso et al., 2009](#); [Hunt et al., 2013](#); [Llibre et al., 2012](#); [McMahon et al., 2010](#)). The only clinical trial that has shown some success used raltegravir for 48 weeks in participants on a HAART regimen consisting of two NRTI/NtRTI inhibitors and a PI or a NNRTI. In this study, raltegravir led to a significant decrease in residual HIV replication and CD8⁺ T-cell activation ([Llibre et al., 2012](#)). Systemic markers of microbial translocation (such as LPS and bacterial DNA) are significantly reduced in individuals who are on HAART but these do not return to pre-infection levels and are associated with increased T-cell activation and higher soluble CD14 and D-dimer levels ([Funderburg et al., 2010](#)). Other chronic viral infections also contribute to the persistent immune activation. Hunt et al. (2011) showed that treatment with Valganciclovir (an antiviral drug used to treat CMV infections) decreased CD8⁺ T-cell activation in individuals co-infected with HIV and CMV, who had elevated immune activation despite undetectable viral loads ([Hunt et al., 2011b](#)).

In this study, HIV-infected women on HAART had higher levels of activation and inflammation in the genital tract compared to blood. Previous studies have shown that there may be a link between the systemic and genital tract T-cell activation and inflammation (reviewed by [Xu et al., 2013](#)). Stimulation by HIV viral antigens in the genital tract would result in a rapid upregulation of cytokines, chemokines and chemokine receptors, leading to the recruitment of activated T-cells and DCs. These infiltrating cells have been shown to produce interferons and chemoattractants such as MIP-1 α , MIP-1 β and RANTES ([Prakash et al., 2001](#)). These, in turn, recruit more activated T-cells, especially CD4⁺CCR5⁺ T-cells. Also, Activated memory T-cells, especially memory CD4⁺CCR5⁺ T-cells, have been shown to be preferentially recruited to sites where HIV infections occur, facilitating faster and more effective immune response against the virus (reviewed by [Masopust & Schenkel, 2013](#)). Also, HIV infection of CD4⁺ T-cells and immune dysregulation at mucosal surfaces results in HIV-specific and non-specific immune activation, which continuously produces new effector T-cells (reviewed by [Xu et al., 2013](#)).

In summary, this Chapter describes both clinical and phenotypic characteristics of T-cells from blood and the female genital tract that failed to reconstitute to levels found in healthy uninfected women, even after ~7 years of being on HAART ([Nkwanyana et al., 2009](#)). This suggest that, once viral suppression occurred after initiation of HAART and the initial reconstitution of CD4⁺ T-cell counts, the immune system does not reconstitute to levels typically detected before HIV infection, even if an individual consistently uses HAART for a long period of time.

Chapter 4. Impact of initiation of HAART in chronic HIV-infected women

4.1 Introduction

Since the introduction and massive scale-up in access to HAART, HIV-infected individuals have experienced a significant improvement in both their life expectancy and quality of life. In South Africa, Johnson et al. (2013) estimated that HIV-infected individuals starting HAART have life expectancies ~80% of the normal life expectancy of HIV-negative individuals. Current HAART guidelines are based on the simultaneous administration of three drugs and have been very successful in rapidly suppressing viral replication to levels below clinical detectable levels in most patients ([Lichterfeld and Zachary, 2011](#)).

Several studies have shown that HAART plays a crucial role in reducing HIV-induced immune activation and is associated with a better clinical outcome in the long term (reviewed by [Hunt, 2012](#)). Key studies have shown that the earlier HAART is initiated during HIV infection, the better the chances of CD4⁺ T-cell reconstitution. Le et al. (2013) showed that starting HAART within the first four months of infection was associated with better CD4⁺ T-cell recovery. In a longitudinal study investigating clinical changes following initiation of HAART, Robbins et al. (2009) reported that individuals who started HAART when their CD4⁺ T-cell counts were >350 cells/ml reconstituted to levels almost similar to HIV negative individuals, but that the same immune reconstitution did not occur in individuals with lower CD4⁺ T-cell counts. Furthermore, they found that CD4⁺ T-cell numbers reconstituted in two phases: the highest rate of CD4⁺ T-cell increase occurred in the first eight weeks after initiating HAART and a more gradual increase occurring afterwards. HIV-infected individuals with baseline CD4⁺ T-cell counts >350 cells/ml had normalised CD4⁺ naïve and memory cell counts by 48 weeks, but those with CD4⁺ T-cell counts <350 cells/ml never recovered their CD4⁺ naïve/memory T-cell ratios to pre-infection levels ([Robbins](#)

[et al., 2009](#)). Irrespective of baseline CD4⁺ T-cell counts, levels of T-cell activation persisted throughout the 3 years of follow-up and the lower the CD4⁺ T-cell count was when HAART was initiated, the longer T-cell activation persisted in blood ([Robbins et al., 2009](#)).

Less is known about the impact of HAART in T-cell reconstitution and immune activation in the genital tract of HIV-infected women. Differences in the bioavailability of antiretroviral drugs in tissues such as the genital tract compared to blood may influence HAART reconstitution at mucosal sites. Studies have shown that NRTIs had a better penetrative capacity to the mucosa compared to NNRTIs (Kwara et al., 2008; Min et al., 2004). In a cross-sectional study comparing HIV-infected women on HAART with those naïve to HAART, Mkhize et al. (2010) reported that women on HAART had higher CD4⁺ T-cell percentages in the genital tract than those not taking HAART, and was also able to fully suppress the genital viral load. In about 10-27% of cases, opportunistic diseases may worsen during the period of immune reconstitution soon after the initiation of HAART ([Haddow et al., 2012](#)), leading to IRIS in both blood and the genital tract. In the genital tract, the two main causes of IRIS were the reactivation of genital herpes ([Yudin and Kaul, 2008](#)) and the re-appearance of genital warts ([Meys et al., 2010](#)), resulting in excessive immune activation and inflammation in spite of HIV-RNA suppression on HAART ([Martin-Blondel et al., 2012](#)).

The aim of this chapter was to investigate changes in immune activation, maturation and inflammation in the genital tract of HIV-infected women immediately before and 1 month after initiation of HAART, and to compare these changes at the mucosa to those observed in blood. The hypothesis being tested was that initiating HAART would improve CD4⁺ T-cell counts in the genital tract, reduce the level of T-cell activation and inflammation in the genital tract and reduce genital tract viral loads. These improvements in CD4 counts and viral load would mirror those detected in blood.

4.2 Materials and methods

For this part of the study, 11 HIV-infected women who were eligible to initiate HAART were enrolled from the Nyanga East Day Hospital (Nyanga, Cape Town) (Chapter 2 section 2.1). All women eligible for study were pre-menopausal and had not undergone a hysterectomy. 3/11 (27.3%) women were not on contraception, 5/11 (45.4%) were on Petogen and 3/11 (27.3%) on Nur Isterate. From each HIV-infected woman, blood and a cervical cytobrush sample was obtained immediately prior to initiation of HAART and after one month on therapy. Blood and a cervical cytobrush sample were collected for the extraction of PBMC and CMC, respectively (Chapter 2 sections 2.3 and 2.4). Polychromatic flow cytometry (Appendix II) was carried out to characterise the blood and cervical T-cells (Chapter 2 section 2.8). Multiplex Luminex flow cytometry was used to determine the inflammatory cytokine profile in the blood plasma and genital fluid (Chapter 2 section 2.11). In addition, the samples obtained from each woman were screened for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium* at both time points (Chapter 2 section 2.13).

4.3 Results

4.3.1 Description of participants

Eleven HIV-positive women starting HAART were enrolled into this longitudinal study immediately before and 1 month after starting HAART (table 4.1). The median age of these women was 31 years (IQR: 26-38). Importantly, these women had a median CD4 count of 200 cells/ml (IQR: 123-314) at their first visits (immediately prior to initiating HAART), despite current guidelines in South Africa recommending that HAART should be initiated with CD4 counts were 350 cells/ml. Their median plasma viral loads were 127122 copies/ml (IQR: 4002-229227). Of the 11 women, 10/11 had detectable viral loads in their genital secretions and their median genital tract viral loads were 2490 copies/ml (IQR: 1342-7981). All women were initiated on a first line HAART regimen, consisting of a NNRTI (efavirenz) and two NRTIs (tenofovir and emtricitabine/lamivudine) as per the South African antiretroviral treatment

guidelines (*The South African Antiretroviral Treatment Guidelines, 2013*). After being on HAART for 1 month, their plasma viral loads had declined to 253 copies/ml (IQR: 40-1595; with 4/11 women being completely suppressed and 7/11 women having viral loads <1500 copies per ml). Only 2/11 women were still shedding low levels of HIV in their genital tracts (183 and 872 copies/ml) and both of these women still had detectable plasma viral loads (2814 and 34848 copies/ml, respectively).

Table 4.1. Clinical description of the HIV-positive women enrolled in the study

Patient ID	Age	CD4 counts (cells/ml)	Viral load at visit 1 (copies/ml)		Viral load at visit 2 (copies/ml)		HAART regimen
			Plasma	Genital	Plasma	Genital	
HTCell1	32	153	127122	6250	323	LDL ^a	FTC ^b , EFV ^c , TDF ^d
HTCell2	24	85	139577	2490	1121	LDL	3TC ^e , EFV, TDF
HTCell3	27	123	1597123	199958	2814	183	3TC, EFV, TDF
HTCell4	20	306	4688	1572	LDL	LDL	3TC, EFV, TDF
HTCell5	33	314	2526	550	LDL	LDL	3TC, EFV, TDF
HTCell6	42	41	1122888	6716	1595	LDL	3TC, EFV, TDF
HTCell7	39	333	2600	1463	LDL	LDL	FTC, EFV, TDF
HTCell8	38	336	34350	1342	253	LDL	FTC, EFV, TDF
HTCell9	31	233	4002	LDL	LDL	LDL	FTC, EFV, TDF
HTCell10	26	200	130831	30617	34848	872	FTC, EFV, TDF
HTCell11	30	198	229227	7981	184	LDL	FTC, EFV, TDF
Median	31	200	127122	2490	253	<40 (LDL)	

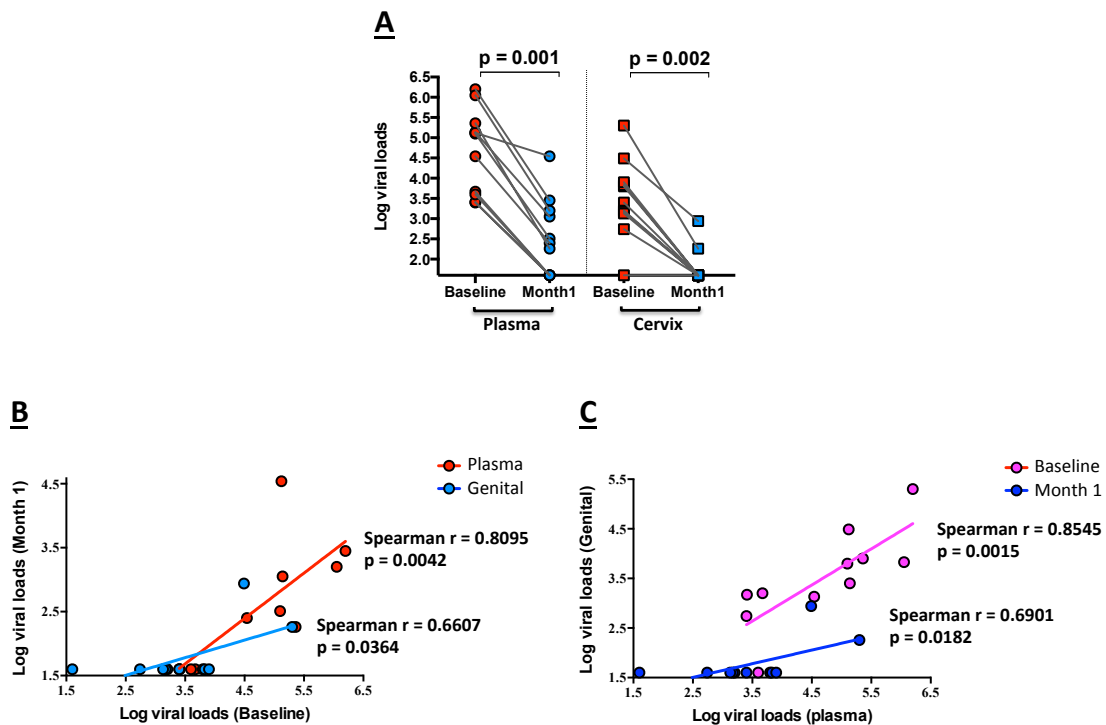
^aLDL – Lower than detectable level

^bFTC – Emtricitabine; ^cEFV- Efavirenz; ^dTDF – Tenofovir; ^e3TC – Lamivudine

4.3.2 Impact of HAART initiation on plasma and genital viral loads

Immediately before starting HAART, the 11 HIV-infected women in this study had a median plasma viral load of 127122 copies/ml (IQR: 4002-229227), with 10/11 women shedding HIV-RNA into their genital secretions (median of 4370 copies/ml, ranging from 550 to 199,958 RNA copies/ml). After one month of HAART, there was a significant decrease in both the plasma and genital viral loads ($p=0.001$ and $p=0.002$, respectively) with post-treatment plasma viral loads ranging from <40 to 34,848 RNA copies/ml and genital tract loads ranging from <40 to 872 RNA copies/ml (figure 4.1A). Within compartments, there was a

significant correlation between viral loads at the two time points (for plasma: $\rho=0.8095$ and $p=0.0025$; for cervix: $\rho=0.6607$ and $p=0.0269$; figure 4.1B), suggesting that the decrease in viral load due to HAART may depend on the viral load at initiation of therapy. There was also a positive association between the plasma and the genital viral loads both at baseline and after one month on therapy (Baseline: $\rho=0.8545$ and $p=0.0008$; for cervix: $\rho=0.6901$ and



$p=0.0188$; figure 4.1C).

Figure 4.1. Relationship between plasma and genital tract viral loads in women before and after they had initiated HAART. **A.** Difference in the log viral loads between the time of initiation of HAART (■) and after one month on treatment (■). **B.** Correlation between the baseline (immediately prior to initiating HAART) and month 1 viral loads for the blood (●) and cervical compartments (●). **C.** Correlation between the plasma (●) and cervical supernatant (●) log viral loads at baseline and month 1. The Wilcoxon Signed Rank test for paired samples for used for **A** while the Spearman rank correlation test was used for **B** and **C**. A p value of ≤ 0.05 was considered to be statistically significant.

4.3.3 Changes in T-cell distribution in the genital tract and blood following initiation of HAART

CD4:CD8 T-cell ratios from blood and cervical cytobrushes, as an indicator of CD4⁺ T-cell reconstitution, were measured immediately prior to the start of therapy and 1 month after initiation of HAART (figure 4.2). An inverted CD4:CD8

ratio of less than 1.0 is a characteristic of an HIV infection (shown as a grey dotted line in figure 4.2), and has previously been shown to be a useful correlate of absolute CD4⁺ T-cell counts (Sainz et al., 2013). An inverted ratio is an indication that T-cells were predominantly CD8⁺ T-cells as opposed to the predominant CD4⁺ T-cell profile found in healthy uninfected individuals (Margolick et al., 2006). In South African women, Nkwanyana et al. (2009) showed that healthy HIV-negative women had a CD4:CD8 ratio of 2.7 in their genital tracts but didn't report on blood CD4:CD8 ratios. Margolick et al. (2006) reported that HIV negative African American women had a blood CD4:CD8 ratio of 1.6, although they didn't report on genital tract CD4:CD8 ratios.

Overall, there were no differences in the frequencies of CD4⁺ and CD8⁺ T-cells obtained before and after HAART initiation in either the genital tract or blood. Immediately before starting HAART, the median blood CD4:CD8 ratio for these HIV-infected women was 0.466 (IQR: 0.148-0.614). After 1 month on HAART, their median blood CD4:CD8 ratio was largely unchanged at 0.391 (IQR: 0.222-0.689), indicating that no observable CD4⁺ T-cell reconstitution in these women within the first month of HAART (despite significant improvements in plasma viral loads, table 4.1). In contrast to blood, CD4:CD8 ratios in the genital tract were a median of 0.096 (IQR: 0.034-0.391) immediately prior to initiating HAART indicating that the women enrolled in this study had almost undetectable CD4⁺ T-cell counts in their cervical cytobrushes (CD8⁺ T-cells outnumbered CD4⁺ T-cells 10:1). After 1 month on HAART, genital tract CD4:CD8 ratios were still very low with a median of 0.194 (IQR: 0.113-0.396).

The median CD4:CD8 ratios in this cohort of HIV-infected women who had just started HAART were compared with the 15 HIV-infected women who had been on HAART for a long period of time (median of 85 months; figure 4.3). The HIV-infected women in this chapter who had only recently initiated HAART had significantly lower CD4:CD8 ratios than those from women who have been on therapy for 85 months (blood: $p = 0.0359$; cervix: $p = 0.0025$). This suggests that a longer period of time may be needed for HAART to restore CD4⁺ T-cell counts

despite early reductions in plasma viral loads and HIV shedding in genital secretions.

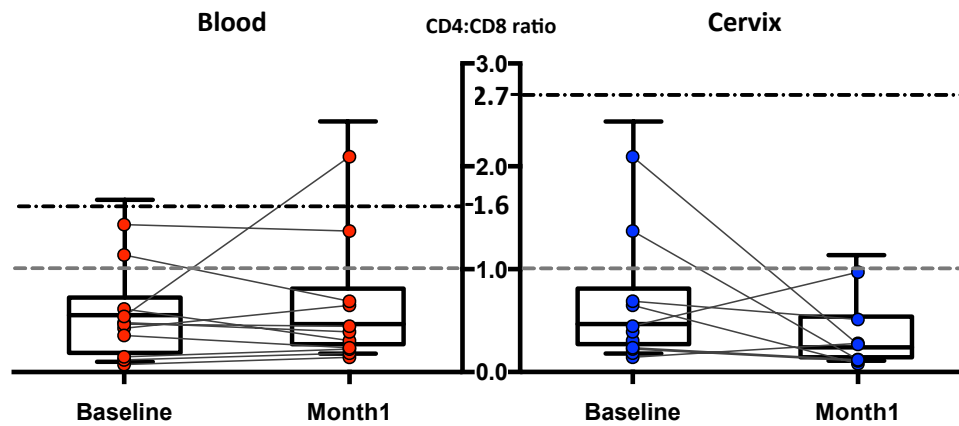


Figure 4.2. Impact of HAART initiation on CD4:CD8 ratios in both the blood (●) and genital (●) compartments. Baseline indicates the time point immediately prior to initiation of HAART; and 1 month after initiation of HAART. The grey dotted line shows the threshold (plasma CD4:CD8 ratio=1) below which the ratio becomes inverted while the black dotted line shows the average ratio for healthy uninfected women.

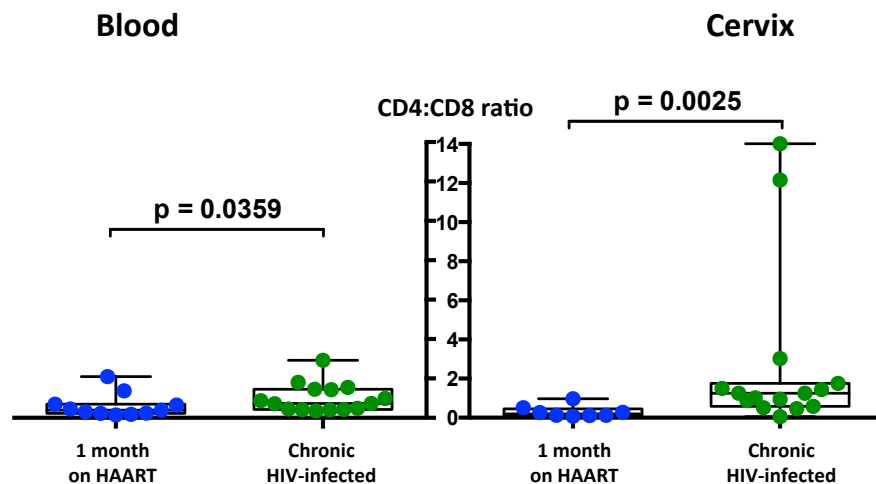


Figure 4.3. Comparison between the CD4:CD8 ratios of HIV-infected women who have been on HAART for 1 month with HIV-infected women who have been on HAART for longer in the blood (●) and cervical (●) compartments.

4.3.4 Impact of HAART on T-cell maturation marker expression in the genital compartment and blood

Untreated HIV infection has been characterised by an imbalance in the distribution of the different naïve/memory T-cell subsets, because some T-cell subsets are preferentially depleted during infection and may be more sensitive

to activation induced cell death than other subsets ([Hellerstein et al., 2003](#)). The memory composition of T-cells in blood and in the genital tract (from cervical cytobrushes) was compared in women immediately before and 1 month after they started HAART. Generally, HAART did not seem to influence the predominant T-cell memory subsets in either the genital tract or blood. Genital CD4⁺ and CD8⁺ T-cells were mostly TEM irrespective of HAART status, with the other memory subsets being present at much lower proportions (figures 4.4 B, D, F and H). Blood CD4⁺ T-cells consisted mainly of naïve and TEM cells with TD being present at the lowest frequencies (figures 4.4 A and C). Blood CD8⁺ T-cells, in contrast, TD and TEM cells were found in the highest proportions, with TCM being found at the lowest frequencies (figures 4.4 E and G).

It was interesting to note that genital frequencies of TEM decreased, which was accompanied by an increase in the proportion of TCM for both CD4⁺ and CD8⁺ T-cells, significantly so for the CD4⁺ TCM subset ($p = 0.0313$). Reconstitution in the proportion of the long-lived memory subsets has previously been reported to be an early sign of immune reconstitution ([Hellerstein et al., 2003](#)). For this study, however, one month on HAART may not be enough time to correct the immune dysfunction seen in chronic HIV infection.

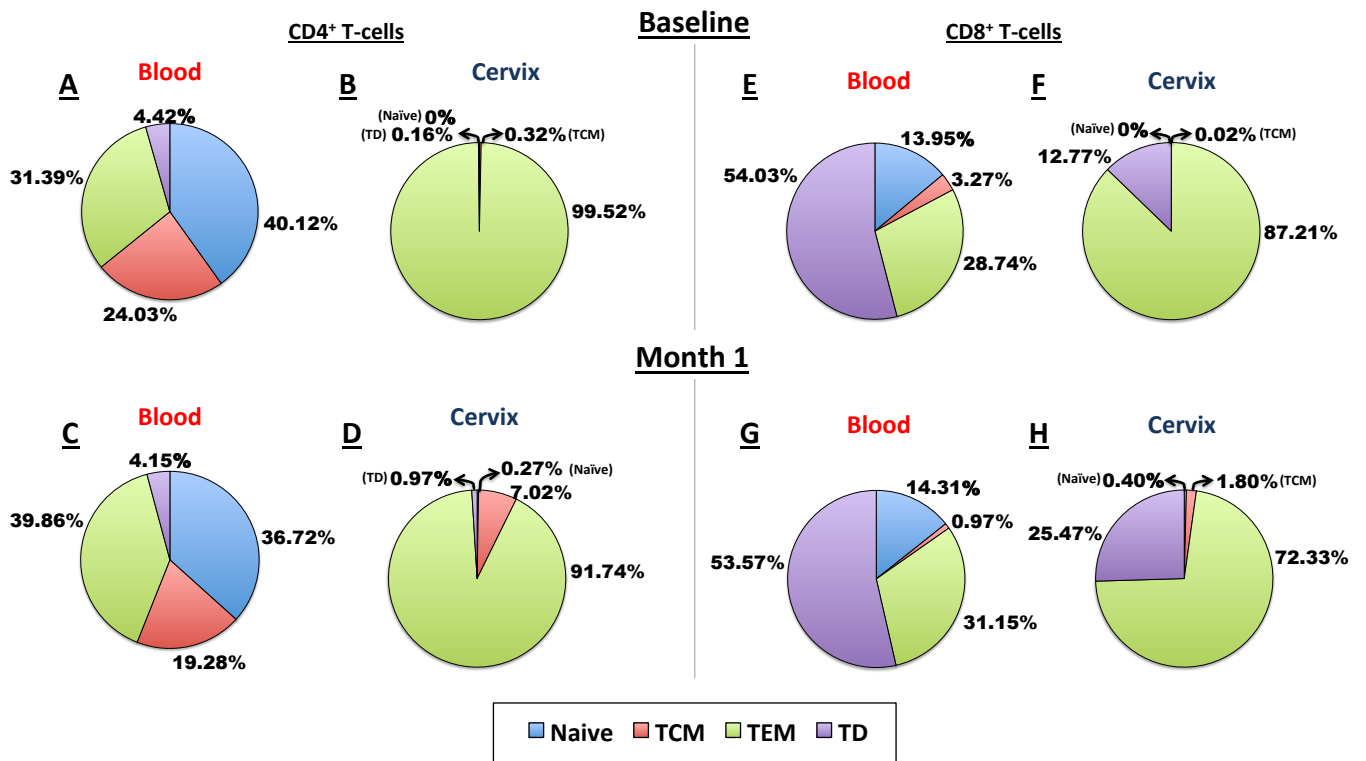


Figure 4.4. Distribution of the different naïve/memory subsets for the CD4⁺ and CD8⁺ T-cell populations in blood and the genital tract of HIV-infected women immediately before initiation of HAART (A and B) and after 1 month on HAART (C and D). The blue, red, green and purple slices represent the percentage of naïve, central memory, effector memory and terminally differentiated T-cells respectively.

4.3.5 Impact of HAART on T-cell activation in blood and the genital compartment

Previous studies have shown that T-cell activation in blood decreases significantly after HAART is initiated, despite the poor recovery of gut-associated lymphoid CD4⁺ T-cells (Giorgi et al, 1998; reviewed by Brenchley & Douek, 2008). It was suggested that, in an HIV infection, it is the level of HIV replication that mainly drives immune activation and the rapid viral suppression after starting therapy leads to an equally rapid and marked decrease in immune activation (reviewed by Hunt, 2007). Studies comparing changes in immune activation and inflammation after initiation of HAART and the effect on CD4⁺ T-cell repopulation have not been carried out in the genital tract.

To determine whether 1 month of HAART had an effect on activation of T-cells, frequencies of CD4⁺ and CD8⁺ T-cells expressing CD38 and HLA-DR were compared between the two time points (figure 4.5). In blood, activation of T-cells was generally lower following 1 month of HAART compared to before initiation of HAART (CD4⁺CD38⁺: p=0.001; CD4⁺CD38⁺HLA-DR⁺: p=0.0322; CD4⁺total activation: p=0.001; CD8⁺CD38⁺: p=0.0029; CD8⁺HLA-DR⁺: p=0.0068; CD8⁺CD38⁺HLA-DR⁺: p=0.0137; CD8⁺total activation: p=0.002). In contrast, HAART initiation did not seem to reduce genital T-cell activation. The frequency of activated CD8⁺ T-cells from the genital tract was similar before and after initiation of HAART (figure 4.5D). In more than half of the women studied, CD4⁺ T-cells from the genital tract actually seemed slightly more activated (HLA-DR, CD38 and total activation) after 1 month on HAART compared to before initiating HAART, although this was not significant (figure 4.5C)

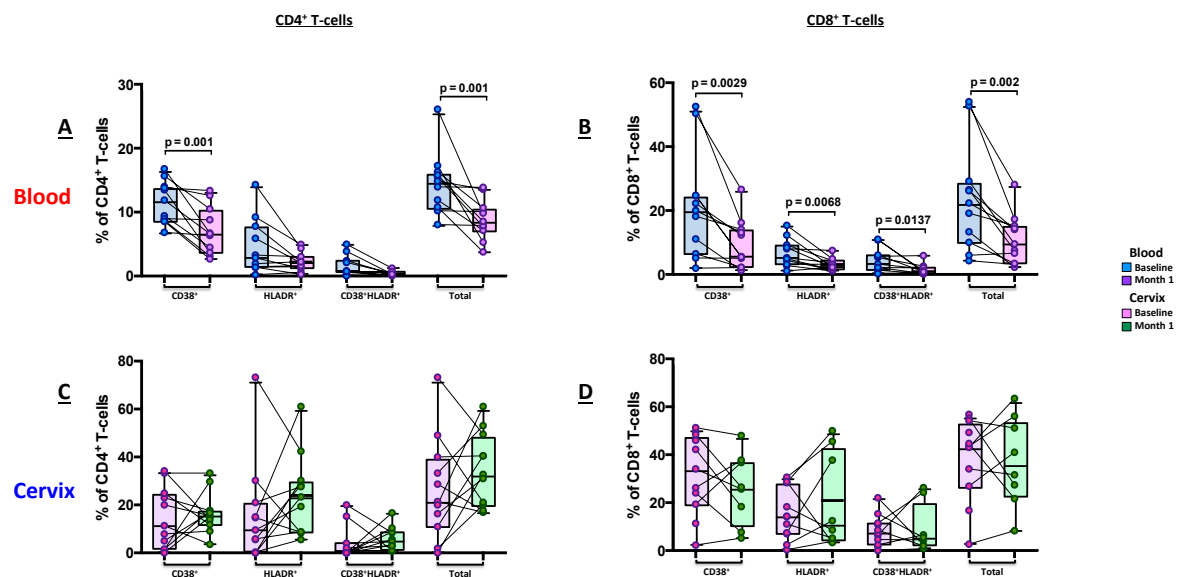


Figure 4.5. Comparison of pre- versus post-HAART activation in genital tract and blood T-cell populations. **(A-B)** Differences in the percentages of CD38⁺, HLA-DR⁺, CD38⁺HLA-DR⁺ and total activation in blood CD4⁺ and CD8⁺ T-cells (shown in blue and purple). **(C-D)** Differences in the percentage of the different activation markers in genital CD4⁺ and CD8⁺ T-cells (shown in pink and green).

4.3.6 Effect of HAART initiation on T-cell proliferation and exhaustion in the genital tract and blood

To determine the effect of initiating HAART on the level of T-cell proliferation and exhaustion, frequencies of Ki67⁺ and CD57⁺ T-cells were compared immediately before and 1 month after starting HAART, respectively. No change in the level of proliferation (Ki67 expression) by T-cells from blood and the genital tract was observed (data not shown). Similarly, HAART initiation did not seem to influence frequencies of T-cells in blood expressing CD57.

In contrast, in the genital compartment, frequencies of T-cells expressing CD57 were significantly elevated after 1 month of HAART ($p=0.0322$ for CD4⁺ and $p=0.0078$ for CD8⁺ T-cells; figure 4.6). When the genital cells were subdivided into naïve/memory subsets, it was found that this increase in CD57 expression was largely restricted to increased expression by TEM and TCM subsets (figure 4.7).

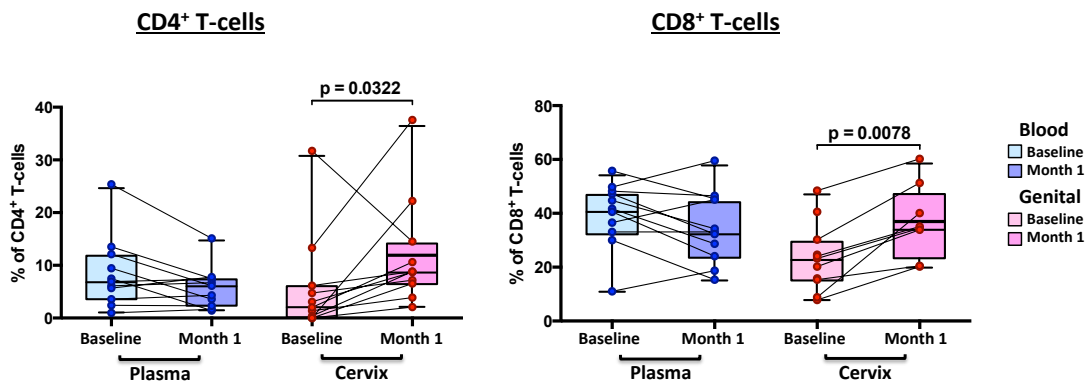


Figure 4.6. Comparison between the frequency of T-cells expressing the senescence marker CD57 before and 1 month after starting HAART. **A.** Comparing the level of expression of CD57 in CD4⁺ T-cells. **B.** Percentage of CD57⁺ cells in the blood and genital CD8⁺ T-cells. The peripheral blood mononuclear cells are represented by the light and dark blue box-and-whisker plots and the cervical mononuclear cells by light and dark box-and-whisker plots.

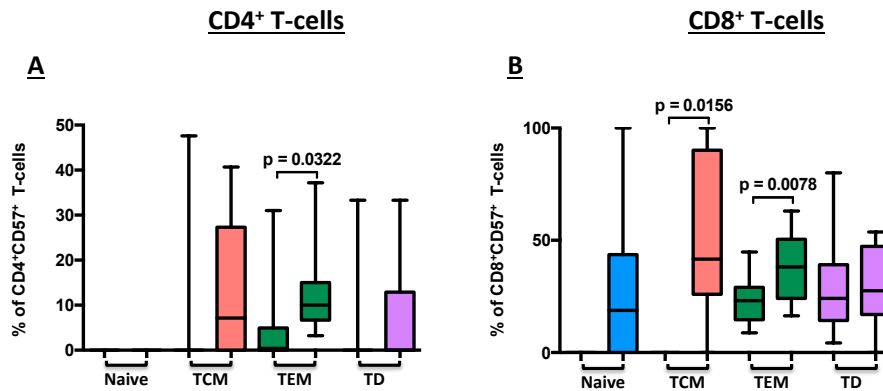
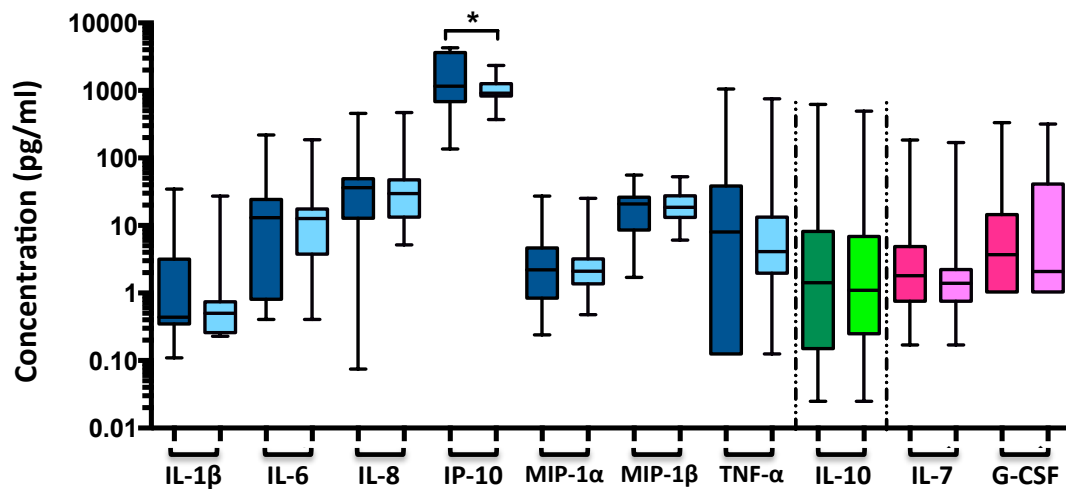


Figure 4.7. Comparing the level of exhaustion within the naïve/memory subsets in the cervix **A.** Differences in the percentages of CD57 in CD4⁺ T-cells. **B.** Percentages of CD57 in CD8⁺ T-cell subsets. Naïve T-cells are shown in blue, central memory T-cells in orange, effector memory T-cells in green and terminally differentiated T-cells in lavender.

4.3.7 Effect of HAART on cytokine concentrations in blood and genital secretions

The concentrations of seven inflammatory cytokines (IL-1 β , IL-6, IL-8, IP-10, MIP-1 α , MIP-1 β and TNF- α), two hematopoietic cytokines (IL-7 and G-CSF) and a regulatory cytokine (IL-10) were measured in blood plasma and genital secretions collected immediately before and after 1 month on HAART (figure 4.8). In blood plasma, most cytokines remained unchanged after 1 month on HAART with only IP-10 being significantly lower after 1 month of HAART ($p=0.05$). Similarly, in genital secretions, most cytokines remained unchanged after 1 month on HAART with only IL-10 being significantly lower after treatment ($p=0.0068$). This suggests that one month on therapy may not be long enough to impact significantly on blood and genital cytokine levels. The fact that the isolated changes in the cytokine levels was different between blood and the genital tract could be due compartmentalization between the two sites (Coombs et al., 2001). The concentrations of cytokines in the genital tract could also be influenced by other confounders such as the levels of immune activation (Sodora and Silvestri, 2008), hormone regulation or the presence of other sexually transmitted diseases (Ghanem et al., 2005; Kaushic et al., 2011). Each of these factors would have a specific impact on the type of inflammatory response and which cytokines would be preferentially secreted locally.

Blood



Cervix

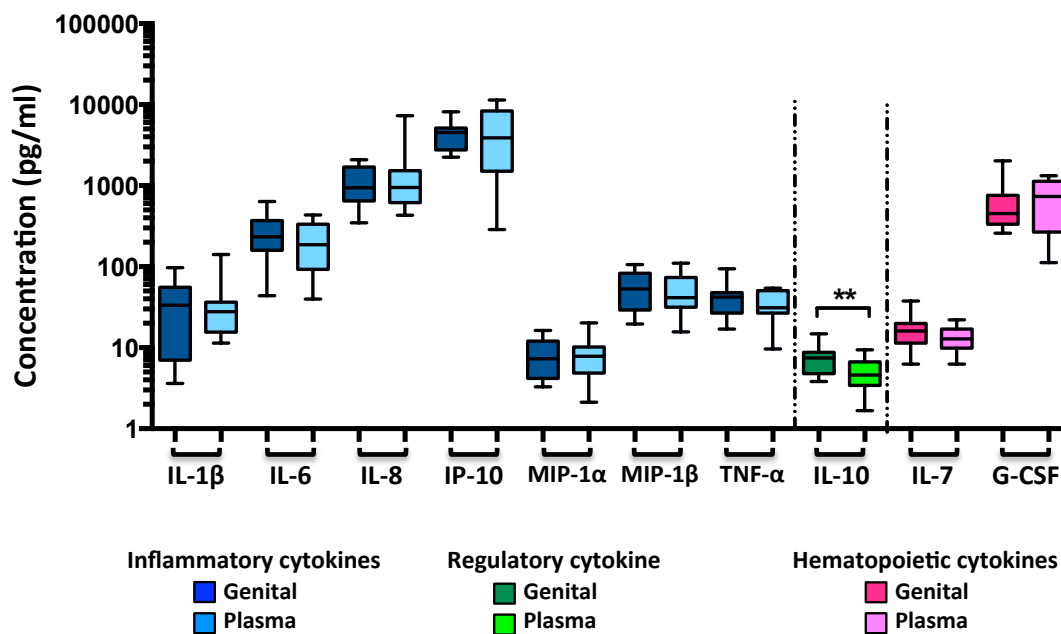


Figure 4.8. Comparison of the cytokine concentrations from the time of initiation of HAART to one month on antiretroviral therapy in blood and the cervical compartment. The dark and pale blue box-and-whisker plots represent the concentrations of inflammatory cytokines at baseline and after one month respectively. The dark and light green plots show the concentrations of the regulatory cytokine at the two time points and the dark and light pink plots represent the hematopoietic cytokine concentrations at the two time points.

The change in plasma concentrations of IP-10 correlated significantly with a similar decrease in the level of CD4⁺ T-cell activation in blood (table 4.2). In addition, there was also a significant relationship between the difference in the

chemokine level and the decrease in plasma viral load seen after one month of therapy where larger decreases in IP-10 concentrations predicted a higher degree of plasma viral load suppression (table 4.2).

Table 4.2. Correlation between plasma IP-10 and CD4⁺ T-cell activation on HAART

		Spearman r	p value
Activation markers	CD38 ⁺	0.7000	0.0204
	HLA-DR ⁺	0.8636	0.0012
	CD38 ⁺ HLA-DR ⁺	0.7909	0.0055
	Total activation	0.8091	0.0039
Plasma viral load		-0.6818	0.0208

4.3.8 Relationship between cytokine levels at baseline and 1 month on HAART

To determine whether the level of each cytokine before initiation of HAART has an impact on the its respective concentration after 1 month on HAART, linear regression models were used, comparing the baseline concentrations to those after one month on HAART (figure 4.9). Initial cytokine concentrations in plasma were found to significantly predict concentrations measurable 1 month after starting HAART, with IP-10 having the highest β coefficient (β coefficient = 1.9364; $p = 0.0021$). In the genital tract, a similar trend was seen although only the baseline IL-8 concentrations were significantly predictive of IL-8 concentrations at 1 month on HAART (β coefficient = 0.1980; $p = 0.0343$). This suggests that, while baseline plasma concentrations of cytokines influence the extent of decrease observed on HAART in blood, the relationship in the genital tract between starting cytokine concentrations and their decrease during HAART is possibly also influenced by other factors driving local inflammation, such as the acquisition of concurrent STIs.

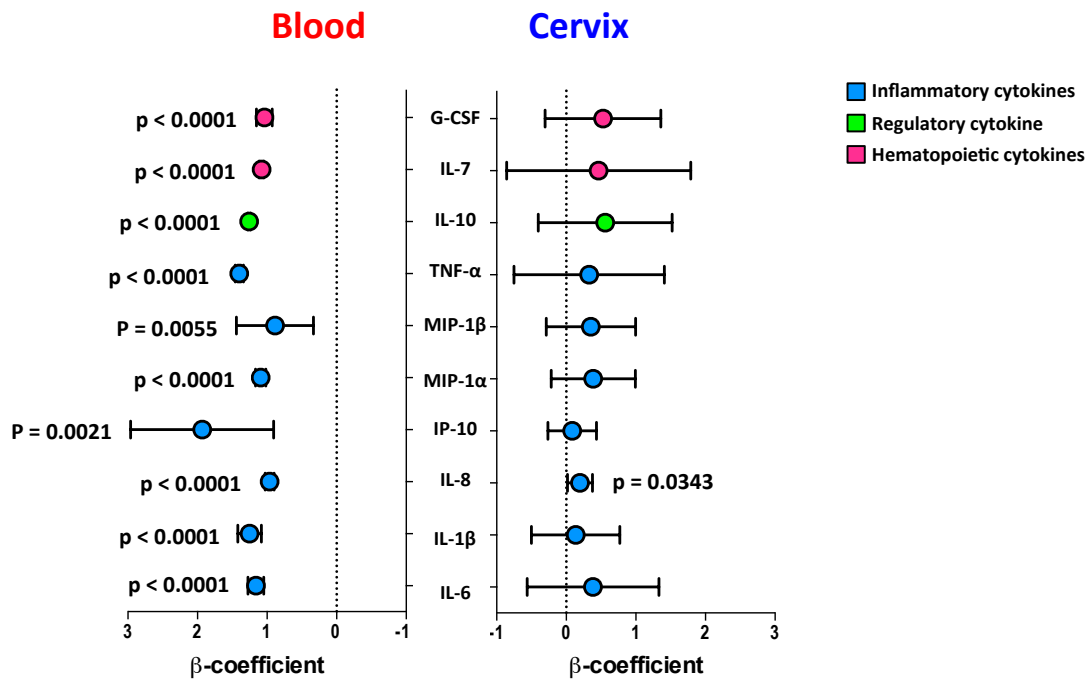


Figure 4.9. Relationship between cytokine concentrations at baseline and after 1 month on HAART in blood and the genital tract. The inflammatory cytokines are shown in blue, the regulatory cytokine in green and the hematopoietic cytokines in pink.

4.3.9 Impact of HAART on STI prevalence

Previous studies have shown that initiation of HAART is associated with reactivation of sub-clinical STIs including HSV-2 and HPV (Yudin & Kaul, 2008; Meys et al., 2010), possibly as the result of IRIS. To investigate the impact of HAART on prevalence of common STIs in this study, the presence of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium* was determined in women before and 1 month after they had initiated HAART (table 4.3). While no women in this study had gonorrhoeae or infection with *T. vaginalis*, 4/11 women were infected with *M. genitalium* and 2/11 women were infected with *C. trachomatis*. After initiating HAART, 3/4 of the women infected with *M. genitalium* were still infected, the 2 women with *C. trachomatis* infections cleared their infections while another 1 women became infected, and 2/11 women became infected with *T. vaginalis*. Since HPV and HSV-2 were not measured in this study, no inference can be made about reactivation of these viral STIs.

Table 4.3. STI prevalence immediately before starting HAART and after 1 month

Patient ID	Visit 1 (Baseline)				Visit 2 (Month 1)			
	NG	CT	TV	MG	NG	CT	TV	MG
HTCell1				■		■		■
HTCell2				■				■
HTCell3				■				■
HTCell4				■				
HTCell5								
HTCell6							■	
HTCell7								
HTCell8								
HTCell9								
HTCell10		■						
HTCell11		■					■	

NG - *Neisseria gonorrhoeae*; CT - *Chlamydia*; TV - *Trichomonas vaginalis*; MG - *Mycoplasma genitalium*

To investigate whether these fluctuations in STI prevalence before and after initiating HAART influenced cytokine concentrations in genital secretions, standardised genital cytokine concentrations were compared in women with and without an STI at each study visit (figure 4.10). Median cytokine levels did not differ in women with or without an STI for either of the two visits. Before starting HAART, the women with no STIs tended to have a higher level of IL-1 β , IL-8, IL-7, MIP-1 β , TNF- α and IL-10 and lower levels of IL-6, MIP-1 α , and G-CSF compared to those with an STI. After starting HAART, women with no STIs had higher levels of MIP-1 β , IL-7 and IL-10 and lower levels of IL-1 β , IL-6, IP-10, TNF- α and G-CSF. Unlike other studies showing that that STIs cause an increase in inflammation in the genital tract, no similar findings were observed in this small cohort of women.

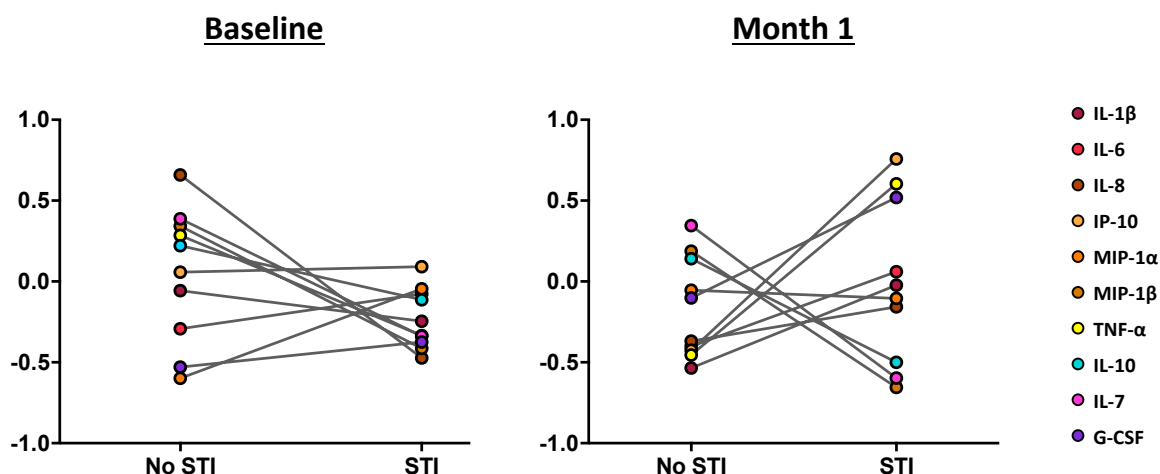


Figure 4.10. Influence of STIs on the difference in cytokine concentrations between women before and 1 month after starting HAART. The coloured dots represent the standardised concentrations of each cytokine and the grey lines show the difference in levels of each cytokine between the two groups of women at each time point. Cytokine concentrations were standardised to ensure that all variables contribute evenly to the analysis. This was done by dividing the distance of each data point from the mean by its standard deviation, meaning that each point on the graphs have a mean of 0 and a standard deviation of 1.

In a more detailed analysis, the relative cytokine profile of each woman was compared between visits 1 and 2, and ranked according to whether the women had an STI or not (figure 4.11). Participant HTCell10 had a relatively lower cytokine profile after one month on HAART compared to pre-HAART, which could be attributed to the fact that she had chlamydia at visit 1 which cleared by the 1 month visit. Participant HTCell2, who had a *M. genitalium* infection at both visits but no other changes in STI profile, had a marked increase in the level of cervical inflammatory cytokines during the first month of HAART. Chronic *M. genitalium* infection has been associated with a potent inflammatory response due to the recruitment of monocytes and macrophages to the cervical mucosa. Except for these two women, the overall concentrations of cytokines in the genital secretions for these women was relatively conserved over time, irrespective of whether they had an STIs or not. Although common bacterial STIs were evaluated in this study, a limitation was that HSV-2 reactivation and HPV infection were not measured. Fluctuations in these viral infections may have accounted for fluctuations in genital cytokine levels.

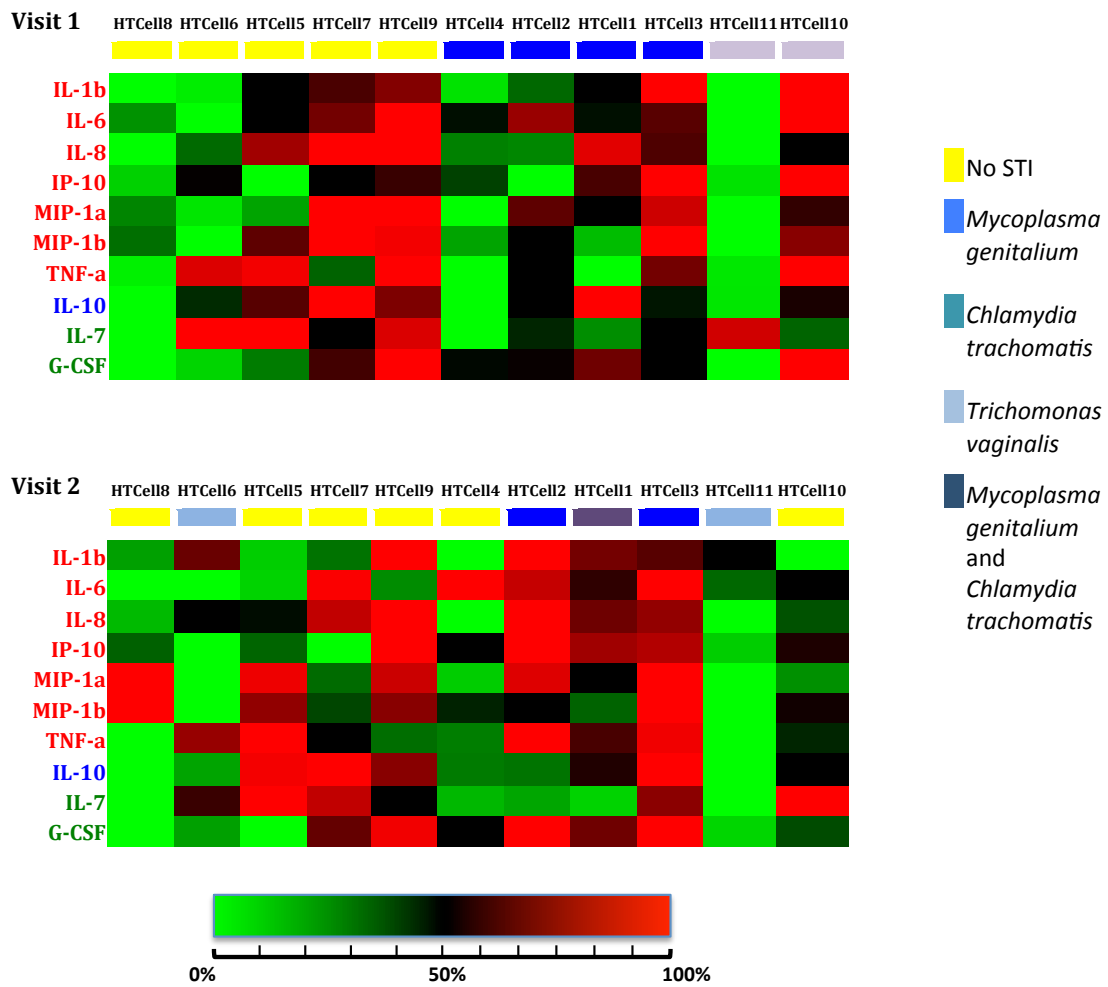


Figure 4.11. Heat map showing the relative cytokine concentrations for each women immediately before and one month after starting HAART. The lightest green colour represents the 10% lowest concentrations for each individual cytokine, black shows the intermediate concentrations and the brightest red represents the 10% highest concentrations for each cytokine. The STI status is shown above each heat map.

4.4 Discussion

The impact of starting HAART on the immune reconstitution and change in immune activation and inflammation in peripheral blood has been well studied (Keating et al., 2011; reviewed by Hunt, 2012 & Klatt et al., 2013) but there are fewer studies on similar changes in the female genital tract. These studies showed that initiating HAART brought significant improvements to the systemic immune activation and is associated with viral suppression, reconstitution of CD4⁺ T-cell counts and a decrease in HIV-specific immune response, even though it was not completely restored to levels found in HIV-negative individuals (reviewed by Hunt, 2012; Le et al., 2013). On the other hand, previous studies have argued that due to factors such as poorer tissue bioavailability of certain antiretroviral drugs, ongoing low-level viral replication and a highly inflammatory environment, the same immune reconstitution was not seen in the genital tract (Kwara et al., 2008; Mkhize et al., 2010).

The aim of this chapter was to measure the change in the levels of immune activation, maturation and inflammation in the genital tract immediately before and 1 month after initiation of HAART in both the female genital tract and blood. The women in this study only started HAART much later in infection when the disease had already progressed and the CD4⁺ T-cell counts were well below 350 cells/ml. In this longitudinal study, one month on HAART had some clear gains in terms of both clinical and phenotypic characteristics of the 11 chronically HIV-infected women in this study. Treatment was successful in suppressing the plasma and genital viral loads in most women and the degree by which the genital viral load decreased was directly proportional to the change in each woman's plasma viral load. Also, the decrease in plasma viral load after 1 month on HAART was directly proportional to her viral load in plasma at immediately prior to initiating HAART. Despite marked improvements in viral load and HIV genital shedding, significant differences in the reconstitution of T-cell numbers were not observed during the short follow-up in this study. While there was no significant difference in the distribution of the different naïve/memory T-cell subsets in blood after one month on HAART, therapy was accompanied by an increase in the proportion of the TCM subset and a decrease in the TEM subset

for both genital CD4⁺ and CD8⁺ T-cells. Initiating HAART caused a significant decrease in blood T-cell activation. However, a different trend was seen in the genital compartment where no significant change in T-cell activation was observed. HAART had no significant impact on the level of CD57 in blood T-cells. On the other hand, there was a significant increase in the level of genital T-cell senescence, which occurred mainly in memory T-cell subsets. A significant decrease in plasma IP-10 concentration was observed after one month on HAART and this change correlated significantly with a similar decrease in plasma viral loads as well as to the decrease seen in CD4⁺ T-cell activation. It was found that the resulting plasma cytokine concentrations after one month on HAART could significantly be predicted based on the concentrations at initiation of treatment. In the genital tract, most cytokines remained largely unchanged pre-versus post-treatment with the exception of IL-10 which was significantly lower after HAART treatment than before. Finally, the STIs tested for in this study (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium*) appeared to have no overall qualitative effect on the cytokine levels in the genital tract.

Several studies have shown that the strongest predictor of being able to detect HIV in the genital tract is plasma viral load (Kaul et al., 2008; Mukura et al., 2012), and this was shown to be true for women on HAART as well (Kovacs et al., 2001). HIV-infected women on HAART who were not fully suppressed systemically were the ones who more frequently shed the virus (Kovacs et al., 2001). In this study, the two women who were still shedding after one month on HAART (HTCell3 and HTCell10) were also those who had the highest plasma viral loads on treatment (2814 and 34848 copies/ml respectively). There could also be other factors influencing the genital viral load, such as the presence of sexually transmitted infections (*M. genitalium* in the case of HTCell3 and/or other STIs not tested for in this study), compartmentalization of the infection or the differential bioavailability of the antiretroviral drugs for each participant. However, in this study, all the participants were on first line regimens consisting of antiretroviral drugs with similar bioavailabilities. The only difference noted was that some women were on emtricitabine while others were on stavudine,

but these two drugs have the same efficiency in mucosal tissues ([Dropulic and Cohen, 2010](#)). There was a significant relationship between the two visits for both the plasma and genital viral loads, suggesting that the extent to which HAART induces viral load suppression may be dependent on the viraemia when HAART was initiated.

Margolick et al. (2006) found that the median blood CD4:CD8 ratio in African American HIV-negative individuals was 1.6. Nkwanyana et al., (2009) reported that the median genital CD4:CD8 ratio in HIV negative black South African women was 2.7. In this study, blood and genital CD4:CD8 ratios were below these previously reported thresholds, even after one month on HAART (0.391 for blood and 0.194 for the genital tract). There were also no significant differences in the blood and genital tract CD4:CD8 ratios between the time of initiation of HAART and one month after, suggesting that there was no significant CD4 immune reconstitution. Compared to women who have been on HAART for a longer period of time (~85 months; Chapter 3), CD4:CD8 ratios measured in women in this Chapter were significantly lower. This indicates that one month on treatment is unlikely to be long enough to result in a significant reconstitution in CD4⁺ T-cell numbers and to correct the immune imbalance.

HAART appeared to have no effect on the memory distribution of blood T-cells. On the other hand, in the cervix, being on HAART was accompanied by an increase in long-lived TCM cells and an associated decrease in TEM for both CD4⁺ and CD8⁺ T-cells. Central memory T-cells are long-lived cells and have been reported to have a stronger proliferative capacity than TEM ([Jameson and Masopust, 2009](#)). The formation of long-lived memory T-cells has previously been shown to be predictive of a better outcome in HIV-infected individuals on HAART and a lower mortality rate ([Rönsholt et al., 2012](#)). Less differentiated cells such as TCM typically produce the early cytokine IL-2, especially in the CD4⁺ T-cell population ([Kalia et al., 2010](#)). This cytokine plays an important role in the differentiation and survival of antigen-specific T-cells ([Seder et al., 2008](#)). Exposure to IL-2 was also found to enhance the survival of short-lived CD8⁺ T-cells ([Kalia et al., 2010](#)). A bigger proportion of antigen-specific T-cells would

allow better control of HIV infection and this could be one of the reasons why the presence of TCM is associated with a better outcome during HIV infection.

Importantly, there was an overall decrease in the frequency of activated T-cells in blood following initiation of HAART, suggesting that HAART impacts on the level of activation in blood relatively quickly although reconstitution in absolute CD4⁺ T-cell counts may take a bit longer to achieve. In the genital tract, however, there was no significant change in T-cell activation. This sustained T-cell activation could be due to the presence of other opportunistic diseases and common infections such as STIs or helminthes infections. Certain STIs such as HSV-2 and HPV have also been found to trigger excessive immune responses and inflammation (as part of IRIS) soon after immune reconstitution occurs on treatment with HAART. Cohen et al. (2011) have shown that, even in the absence of an HIV infection, individuals in sub-Saharan Africa have high frequencies of activated genital T-cells. They speculate that these differences could be attributed to environmental factors, co-infections, or due to host genetics (Cohen et al., 2011; reviewed by Mouser et al., 2012). Similar factors might be masking the effect of HAART on the level of activation in the genital tract in this study.

HAART treatment did not influence the level of exhaustion of T-cells (measured by expression of CD57) in blood. In contrast, there was a significant increase in genital tract T-cell senescence, particularly for the CD8⁺ T-cell population. This increase in CD57 expression by genital tract T-cells was predominantly in the memory subsets (TCM and TEM). Migueles et al. (2009) similarly reported that genital T-cell senescence persists even after starting HAART, in addition to broader T-cell dysfunction such as defective proliferative capacity and loss of their cytotoxic capacity. The more severe degree of exhaustion in CD8⁺ T-cells could be attributed to the fact that the HIV infection resulted in smaller numbers of helper CD4⁺ T-cells to maintain the CD8⁺ T-cell population (Virgin et al., 2009).

Fleury et al. (1998) showed that there is a late increase in the fraction of proliferating cells in individuals starting HAART (measured at 24 weeks after initiation of HAART) in both blood and lymph nodes. The lack of change in Ki67

expression seen in this study could simply be because one month is too short a period for quantifiable differences to occur. Another explanation could be that there was there was a large percentage of CD57⁺ exhausted T-cells, which have been shown in previous studies to be proliferation-incompetent ([Brenchley et al., 2003](#); [Migueles et al., 2009](#); [Palmer et al., 2005](#)).

Of the cytokines measured in blood, concentrations of IP-10 were significantly lower following 1 month on HAART compared to pre-HAART concentrations. In addition, decrease in plasma viraemia (which was significantly lower post-HAART initiation) was positively associated to the change in blood IP-10 concentrations. Roberts et al. (2010) similarly reported that elevated IP-10 levels were associated with higher plasma viral loads. Simmons et al. (2013) also showed, in a cross-sectional study comparing pre-ART individuals to those on HAART, that IP-10 plasma concentration was most significantly associated with HIV-1 viral load. Furthermore, this chapter shows that the decrease in plasma IP-10 concentrations during the first month on treatment was accompanied by a proportional decrease in CD4⁺ T-cell activation. IP-10 is produced in response to type I or II interferons (reviewed by [Hunt, 2012](#)). An interferon response is crucial in enhancing T_h1 responses in an adaptive immune response ([Nuvor et al., 2012](#)). A decrease IP-10 could be an indirect measure of lower interferon responses and thus, could be linked to the decrease in activation.

In the genital tract, a significant decrease in IL-10 concentration was observed in women who had initiated HAART compared to their matching pre-HAART concentrations. This finding could reflect the slight increase observed in genital CD4⁺ T-cell activation. IL-10, which is considered to be an anti-inflammatory cytokine, has also been shown to cause down-regulation of T_h1 cytokine production by monocytes and macrophages (reviewed by [Couper et al., 2008](#)) and directly act on CD4⁺ T-cells by inhibiting production of certain pro-inflammatory cytokines ([Joss et al., 2000](#)). Thus, a marked decrease in the production of this cytokine in the genital tract could lead to an increased inflammatory response and a rise in the level of expression of activation markers seen in the genital CD4⁺ T-cell population. Aside from IL-10, all of the other

cytokines measured were not different in the genital tract pre- versus post-HAART initiation.

In blood, concentrations of all 10 cytokines before initiation of HAART were found to significantly predict their respective concentrations after one month on HAART (particularly for IP-10). However, in the genital tract, this relationship was not as strong with only IL-8 concentrations between time points correlating. The weaker associations pre- versus post-HAART in the female genital compartment could potentially be due to the potentially larger number of confounders present at the genital mucosa. For example, cytokine production and changes in the genital inflammatory environment could largely be driven by infections with secondary STIs rather than directly from HAART-related effects. Behavioural disinhibition in individuals who have initiated HAART may also influence the results in this study. Several studies have reported that initiation of HAART was linked to increases in riskier sexual behaviour for a variety of reasons ([Boily et al., 2004](#); [McClelland et al., 2006](#); [Wilson et al., 2004](#)). These individuals may perceive their risk of transmission to their sexual partners to be lower because their viral loads are reduced. Wilson and Minkoff (2001) reported increasing inconsistencies in condom use as the women on HAART believed that they would not be transmitting the virus to their partner if they were on HAART. Another explanation could be that women start to feel better as their clinical symptoms subside. Treatment with HAART is typically accompanied with an improvement in the physiological and psychological effects of infection, leading to more risky behaviour (such as lack of condom use or having multiple partners) once the feeling of illness decreases ([Wilson et al., 2004](#)). These practices would put the individuals at risk for other STIs, which may in turn influence the rate of reconstitution in the genital tract cytokine milieu. Alternatively, immune reconstitution in the genital tract could be influenced by the development of IRIS after starting HAART. For example, the reactivation of HSV-2 or the formation of genital warts soon after genital HIV-RNA suppression could cause a marked increase in local inflammatory responses ([Worsley et al., 2010](#)).

Although previous studies have shown that STIs result in increased mucosal levels of inflammatory cytokines ([Galvin and Cohen, 2004](#); [Modjarrad and Vermund, 2010](#)), STIs measured in this small cohort of women did not seem to influence the inflammatory environment in their genital tracts. Apart from sample size, the lack of association between the level of inflammation and the presence of STIs may be due to a number of additional factors, including STIs which were not tested for, the stage in menstrual cycle at which the participants were sampled ([Wira and Fahey, 2008](#)), their choice of hormone contraceptive ([Baeten et al., 2001](#); [Ghanem et al., 2005](#)), or vaginal hygiene practices ([Hilber et al., 2007](#)). Some studies have reported a higher degree in higher HIV shedding during the luteal phase of the menstrual cycle, which would influence the cytokine concentrations detected in the genital fluid at the time of sampling ([Benki et al., 2004](#); reviewed by [Anderson & Cu-Uvin, 2011](#)). Progesterone-based contraceptives such as depot medroxy-progesterone acetate also have an effect on HIV shedding and the regulation of immune responses in the female genital tract (reviewed by [Stringer & Antonsen, 2008](#)).

In summary, while changes in immune reconstitution and dampening in immune activation was observed in blood in this study, similar reconstitution and dampening of activation were not observed in the genital mucosal environment. Several studies have reported that HAART improves systemic immunity as CD4⁺ T-cell number reconstitute. However, improvements in immunity after initiation of HAART can lead to other clinical manifestations due to the dysregulation of the immune system or due to an inflammatory response to previously sub-clinical pathogens and residual antigens that were not being recognised during HIV-induced immune suppression (IRIS). These infections then become clinically relevant as the immune system starts to reconstitute on HAART (reviewed by [Chahroudi & Silvestri, 2012](#) & [Dhasmana et al., 2008](#)). The women in this study met many of the criteria that would put them at risk for HIV-related IRIS, as they started HAART when their CD4⁺ T-cell counts were low (median of 200 cells/ml) and treatment with HAART was accompanied by a rapid decrease in HIV-RNA (reviewed by [Dhasmana et al., 2008](#)). Antonelli et al. (2010) showed that HIV-infected individuals who develop IRIS had elevated frequencies of highly

activated CD4⁺ T-cells in blood compared to those who did not develop IRIS ([Antonelli et al., 2010](#)). Genital immune reconstitution, occurring in the women in this study who were initiating HAART, may have had a stronger immune response against previously subclinical genital tract infections, leading to higher levels of immune activation and inflammation following HAART initiation.

Chapter 5. Discussion and Conclusion

Despite extensive research describing changes in immune activation and inflammation in blood of HIV-infected individuals initiating HAART, relatively little is known about local changes occurring at the genital mucosa. The aim of this study was to compare the impact of HAART on inflammation and local immune reconstitution in the female genital tract of chronically HIV-infected women by comparing genital mucosal and blood cytokine profiles, T-cell differentiation status, level of activation, proliferation and exhaustion. This study found that HIV-infected women initiating HAART experienced a rapid suppression of plasma and genital tract viral loads (within 1 month of starting therapy; Chapter 4). While this suppression in viraemia was associated with significantly lower levels of T-cell activation in blood, the immune activation and inflammation levels in the genital tract were not reduced during early HAART. In the female genital tract, short term HAART therapy was accompanied by the accumulation of an exhausted memory T-cell population that was not observed in blood. Furthermore, this study showed that long term HAART (~7 years; Chapter 3) in HIV-infected women did not completely reconstitute T-cells to levels found in HIV-negative women, with a marked T-cell imbalance and high levels of inflammation still being present, especially in the genital tract.

5.1 Development of tools to investigate immune reconstitution during HAART

To address the objectives of this study, a robust 10-colour flow cytometry panel was designed and optimised to measure the phenotypical characteristics of blood and genital CD4⁺ and CD8⁺ T-cells. This involved the selection of specific markers that would be most relevant to be study (described in Appendix II). The panel selected was aimed at measuring the frequency of CD3⁺ T-cells and the CD4⁺ and CD8⁺ T-cell subpopulations. The cellular markers CCR7 and CD45RO were used to identify the naïve, memory and differentiated T-cells. The degree of

immune activation (CD38 and HLA-DR expression), proliferation (Ki67) and exhaustion (CD57) was also measured.

5.2 T-cell defects and incomplete immune reconstitution detected in the female genital tract and blood of HIV-infected women on long-term HAART

Chapter 3 compared the impact of long-term HAART (~7 years) on markers of immune activation, inflammation, T-cell differentiation, and exhaustion in the female genital tract and blood of women chronically infected with HIV. Women in this study had CD4:CD8 ratios similar to HIV-infected but HAART-naïve individuals, despite having been on HAART for about 7 years. Although this part of the study was not longitudinal, comparing long-term HAART CD4:CD8 ratios to previously published ratios ([Margolick et al., 2006](#); [Nkwanyana et al., 2009](#)) indicates that CD4⁺ T-cells had not reconstituted to pre-infection levels. CD4:CD8 ratios were used as a clinical indicator of HIV disease progression in this study ([Margolick et al., 2006](#); [Taylor et al., 1989](#)). The fact that genital CD4:CD8 ratios were more similar to that of HIV-infected women who were naïve to HAART than HIV-negative women [as reported by [Nkwanyana et al. \(2009\)](#)] showed incomplete CD4 reconstitution at the genital mucosa.

In this study, genital T-cells were shown to be predominantly TEM cells. Although TEM cells are known to circulate through non-lymphoid tissue (including the genital mucosa; reviewed by [Shin & Iwasaki, 2013](#)), accumulation of this memory T-cell subset in the genital mucosa could also be the result of chemokine/cytokine-dependent recruitment of activated and differentiated T-cells ([Gumbi et al., 2008](#); [Hikono et al., 2007](#); [Prakash, 2003](#)). Interestingly, the level the T-cell activation in blood (measured by CD38 expression) was shown to significantly predict levels of T-cell activation in genital T-cells. This suggests that elevated levels of activation systemically may provide a large pool of activated cells to traffic to mucosal tissues such as the genital mucosa. Furthermore, genital CD4⁺ T-cell activation was significantly associated with genital IL-8 concentrations, a chemotactic cytokine which may be implicated in

the recruitment of activated T-cells to the genital tract ([Narimatsu et al., 2005](#)). This suggests that chemotactic recruitment of activated T-cells may also play a role in homing of these cells to the genital mucosa. Previous studies have described a relationship between chronic T-cell activation and continuous T-cell proliferation and differentiation, leading to the accumulation of an activated, senescent T-cell population ([Nixon and Landay, 2010](#); [Papagno et al., 2004](#)). In support of this, this study showed that genital CD8⁺ T-cell activation was positively associated with the extent of exhaustion in this T-cell population.

An important observation from this study was that immune activation in blood and at the genital mucosa does not completely subside with long periods of time on HAART. This has long-term consequences for these HIV-infected women as persistent immune activation puts them at a higher risk of non-opportunistic diseases such as cardiovascular disease, type II diabetes or cancer (reviewed by [Hunt, 2012](#); [Klatt et al., 2013](#)). In addition to having higher levels of immune activation (especially in the CD4⁺ T-cell population) compared to blood, the genital tract had significantly higher levels of inflammation (measured by expression of 10 inflammatory, anti-inflammatory and haematopoietic cytokines). The comparatively elevated levels of immune activation and inflammation in the genital tract of these HIV-infected women who have been on long-term HAART could be due to several factors such as the presence of opportunistic infections or low-level viraemia (reviewed by [Rajasuriar et al., 2013](#)). It has been proposed that ongoing HIV replication, occurring below the detection limits of current assays, could be triggering a pro-inflammatory response (hence the elevated levels of cytokines) which leads to the recruitment of activated T-cells to the genital tract ([Mavigner et al., 2009](#); [Ostrowski et al., 2008](#)). Alternatively, antigenic stimulation from opportunistic pathogens, including STIs and BV, could also induce an inflammatory response, contributing to the highly inflammatory milieu in the genital tract (reviewed by [Mayer & Venkatesh, 2011](#)). Findings from this study also showed that the heightened inflammatory levels and immune activation in the genital tract was also accompanied by the accumulation of exhausted T-cells and the continued

imbalance in the proportions of the different memory T-cells subsets, leading to an overall immune dysfunction.

Several groups explored the inclusion of anti-inflammatory or immunomodulatory drugs, in conjunction with HAART, to dampen the ongoing immune activation and lower inflammation (reviewed by [Hunt, 2012](#)). One strategy which has shown some success was intensification of treatment with raltegravir, which led to a significant decrease in T-cell activation ([Llibre et al., 2012](#)). According to the authors, this selective reduction of immune activation in CD8⁺ T-cells was a result of raltegravir blocking residual productive viral replication occurring despite suppressive HAART. Another strategy that has been tested was to treat common co-infections to reduce immune activation. For example, treatment of CMV co-infection with Valganciclovir resulted in a marked decrease in CD8⁺ T-cell activation ([Hunt et al., 2011b](#)). Statins and glucocorticoids such as atorvastatin and prednisolone have potent anti-inflammatory and anti-viral properties which were found to cause a significant decrease in CD8⁺ T-cell activation and the production of molecular drivers of immune activation such as soluble CD14 or LPS-binding protein ([Ganesan et al., 2011](#); [Kasang et al., 2012](#)). These approaches could be another way to correct the immune imbalance caused by the chronic HIV infection and allow a more efficient CD4 reconstitution and a better long-term outcome in the chronic HIV-infected individuals on HAART.

5.3 Early gains from HAART initiation in reducing immune activation and inflammation in blood but not at the genital mucosa

Chapter 4 assessed the early temporal changes occurring in clinical and phenotypic characteristics of HIV-infected women from the time of initiation of HAART to after one month on treatment. Treatment for a month was successful in suppressing the plasma and genital viral loads in most HIV-infected women. In this study, the decrease in genital viral loads was proportional to that seen in blood, confirming that the plasma viral load was a strong predictor of genital

shedding ([Kaul et al., 2008](#); [Kovacs et al., 2001](#)). Despite early marked viral suppression, however, there was no similar evidence of early CD4 reconstitution (indicated by CD4:CD8 ratios). CD4:CD8 ratios were significantly lower in HIV-infected women who had just initiated HAART (Chapter 4) compared to women who had been on long-term HAART (~7 years; Chapter 3), suggesting that one month of treatment was too short for any discernable CD4 reconstitution.

While one month on HAART appeared too short for the correction of the T-cell imbalance in blood, there was an increase in the proportion of long-lived central memory T-cells, accompanied by a decrease in the short-lived effector memory T-cells in the genital tract. This could be indicative of an improvement in the genital tract immunity as the formation of long-lived memory T-cells was found to predict an overall better outcome and a lower mortality rate in HIV-infected individuals ([Rönsholt et al., 2012](#)).

There was a significant decrease in systemic immune activation after starting HAART (measured by CD38 and HLA-DR expression by T-cells), which coincided with significant reductions in plasma viral loads. In contrast, a different scenario was seen in the genital tract where there was no significant change in T-cell activation, where a slight increase, if anything, in CD4⁺ T-cell activation was seen after one month on HAART. This lack of change in genital immune activation could be the result of a number of occurrences that have contrasting effects on the overall immune response. The positive gains due to HAART on HIV-RNA suppression and immune activation could have been masked by the negative effects of other opportunistic infections in the genital tract, as has been documented to happen during IRIS. Within the first few weeks of starting HAART, a significant proportion of HIV-infected individuals experience IRIS ([Meintjes et al., 2012a](#)). IRIS refers to a syndrome where, where soon after immune reconstitution occurs, the immune system starts responding to subclinical infections in an uncontrolled manner, resulting in excessive immune activation and inflammation, despite being on HAART (reviewed by [Chahroudi & Silvestri, 2012](#)). In this study, treatment with HAART was accompanied by an early accumulation of exhausted CD4⁺ and CD8⁺ T-cells in the genital tract, which could

be due to the general T-cell dysfunction caused by chronic HIV infection, where T-cells become proliferation-incompetent and also lose their cytotoxic potential (Migueles et al., 2009).

Treatment with HAART resulted in significantly lower concentrations of IP-10 in blood of HIV-infected women. The decrease in plasma IP-10 after one month on HAART correlated with a decrease in CD4⁺ T-cell activation and plasma viral load. Since IP-10 is known to be induced by type I and II interferon responses as a result of TLR7/8 signalling by HIV (reviewed by Chang & Altfeld, 2010 & Hunt, 2012), this decreased production of IP-10 could be an indirect measure of type I and II interferon responses, a consequence of lower viral loads (reviewed by Hunt, 2012; Nuvor et al., 2012).

In the genital tract, a significant decrease in the concentration of the anti-inflammatory cytokine IL-10 was observed in HIV-infected women who had just initiated HAART. IL-10 has been shown to directly suppress T-cell activation and the production of pro-inflammatory cytokines such as IFN- γ (Donnelly et al., 1999). Although it is difficult to infer causation, the reduction in IL-10 found in this study could influence CD4⁺ T-cell activation in the genital tract, which was slightly, although not significantly, elevated post- versus pre-HAART initiation. Since it has immunomodulatory properties, a decrease in IL-10 could result in up-regulation of T_h1 cytokines in response to pathogen-specific recognition by the genital immune cells, leading to uncontrolled increases in immune activation and inflammation (reviewed by Price et al., 2009; Tadokera et al., 2013).

In this study, common bacterial STIs (including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium*) did not seem to impact on the genital inflammatory milieu. This suggests that other factors, such as common viral STIs not tested for in this study (including HSV-2 and HPV), the type of hormonal contraceptive the women may have been using or the phase of the menstrual cycle at the time of sampling, may have been at play (Baeten et al., 2001; Wira and Fahey, 2008). Ratnam et al. (2006) showed that the reactivation

of HSV-2 and HPV infections in the female genital tract of women initiating HAART were the two most common features of IRIS in the genital mucosa.

Uncontrolled immune activation and inflammation are detrimental to the overall outcome of treatment with HAART as they may slow down the rate of CD4 reconstitution in HIV-infected individuals initiating treatment. A strategy to counteract the possible genital HIV-associated IRIS-type signatures emerging from the current study might be to treat women with anti-inflammatory drugs, in conjunction with the prescribed HAART regimen, during the first few weeks of therapy. Corticosteroids (such as prednisolone) and NSAIDs have been suggested for the dampening of systemic immune activation ([Meintjes et al., 2012a](#)). A similar approach, aimed at reducing genital immune activation and inflammation, could be useful in controlling the extent of local immune responses. Mucosal long-lived T-cells are drastically depleted during chronic HIV infection and are slow to be reconstituted ([Grossman et al., 2006](#); [Mehandru et al., 2004](#)). Reducing the excessive inflammatory environment in the genital tract might help in forming and maintaining a larger pool of resting long-lived memory T-cells and contribute to improving the overall genital immune reconstitution on HAART.

5.4 Some of the limitations of this study

A limitation of this study was that absolute CD4⁺ T-cell counts were not available for the women in this study although CD4:CD8 ratios, a reliable measure of disease progression, were used in both chapters as an indirect measure of CD4⁺ T-cell reconstitution. Information about hormonal contraception was only available for the 11 women in the longitudinal study and data about any other medication was not recorded. In chapter 3, correlations were carried out between the different variables and time on HAART but the wide range of times on HAART (15-119 months) may have limited the statistical significance of the tests. Furthermore, both the cross-sectional and the longitudinal studies had a relatively small sample size (including only 15 and 11 women respectively) and this is likely to have influenced the statistical power for the study. Testing for

additional genital tract infections (particularly HSV-2 and HPV infections) and collecting information about hormone contraceptive choice would have given more insight on the what the confounders of the effect of HAART on genital T-cell activation and inflammation could have been.

5.5 Conclusion

This study showed that the chronically HIV-infected women on long term HAART were not fully immune reconstituted despite being on HAART for a ~7 years, in both their genital compartment and in blood. Ongoing T-cell activation and inflammation could impair the ability of an HIV-infected individual's immune system to reconstitute and to correct immune imbalances caused by the chronic HIV infection but could also have long term consequences. The inability to dampen the elevated levels of activation and inflammation can also lead to non-AIDS-related non-immunological diseases (reviewed by [Klatt et al., 2013](#)). Uncontrolled immune activation in individuals developing HIV-associated IRIS could have a significant negative impact on morbidity and mortality ([Barber et al., 2012](#)). In the genital tract, persistent inflammation and immune activation could increase the risk of HIV genital shedding even though viral suppression may have been achieved systemically. Excessive immune responses in the genital tract would prevent the reconstitution of mucosal long-lived memory T-cells and could also induce the reactivation of certain STIs such as HSV-2 or HPV, putting the women at a greater risk of shedding and transmitting the infection to potential partners.

This study suggests that treatment with HAART would be more effective in correcting immune dysfunction in the genital tract in a situation where the excessive immune activation and elevated pro-inflammatory cytokine production could be prevented. Just as immunomodulatory or anti-inflammatory drugs have been used to control immune activation in blood ([Ganesan et al., 2011](#); [Hunt et al., 2011b](#); [Meintjes et al., 2012a](#)), dampening of the genital tract immune activation or inflammation could be achieved through the simultaneous use of different formulations of similar anti-inflammatory drugs such as statins

or glucocorticoids, many of which are already licensed for topical use in humans. This would allow for better local immune reconstitution and lower the risk of both, shedding of HIV and infection with potentially subclinical STIs present in immunosuppressed individuals.

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Appendix I

DESCRIPTION OF THE CYTOKINES/CHEMOKINES MEASURED IN THE STUDY

Table A1.2. Function of the different cytokines relevant to this study

Class	Cytokine	Function
Pro-inflammatory	IL-1 β	HIV-infected individuals in the acute phase show markedly higher levels of IL-1 β , which is then suppressed on HAART. Involved in HIV expression and replication via binding of NF κ β to the HIV-1 long terminal repeat ^{1,2} .
	IL-6	Is important in the transition between acute and chronic inflammation ³ . Is one of the major stimulator of acute phase protein production. Induces the development of cellular and humoral immune responses including B-cell differentiation, antibody secretion and T-cell activation ⁴ .
	IL-8/ CXCL8	Has inflammatory and anti-microbial properties. Secretion is triggered by presence of Nef ⁵ . Is present at much higher levels in HIV-infected individuals ⁶ and is one of the predominant cytokines in the female genital tract during infection ⁷ . Stimulates chemotaxis of basophils and T-cells and induces neutrophils to release lysosomal enzymes ⁸ .
	MIP-1 α / CCL3	One of the major components of HIV-suppressive activity as they can bind to CCR5 T-cells. Important in T-cell chemotaxis to inflamed areas. Causes the upregulation of activation markers and pro-inflammatory mediators secretion. Causes T _h 1 skewing ^{5,9} .

Pro-inflammatory	MIP-1 β / CCL4	Has anti-HIV activity as it can bind to CCR5. Also involved in T-cell chemotaxis to inflamed tissues and the upregulation of inflammatory and activation markers. Causes IL-12 secretion from DCs, enhancing T _h 1 immunity ^{5,9} .
	TNF- α	Activates NF κ β , which initiates the inflammatory cascade in HIV infection ⁵ . Is part of a feedback loop where TNF- α stimulates production of IL-10, which, in turn, modulates inflammation by decreasing TNF- α production ¹⁰ .
	IP-10/ CXCL10	Induces chemotaxis in macrophages, DCs, NK cells and T-cells towards inflamed areas. Facilitates infection by stimulating HIV replication in macrophages and lymphocytes ¹¹ . Has been associated with immunological treatment failure in individuals on HAART ¹² .
Adaptive	IFN- γ	Is the most well characterized antiviral cytokine. Critical for both innate and adaptive immunity. Is mostly produced by NK and NKT-cells in innate immunity and by T _h 1 effector cells as part of the antigen-specific immunity ¹³ . Is important for both self-activation and activation of nearby immune cells ¹⁴ . Is bifunctional, has both inhibitory and stimulatory effects on HIV. IFN- γ can suppress HIV replication but chronic exposure to IFN- γ has negative consequences on T-cell homeostasis and survival ¹⁵ .
Regulatory	IL-10	Is a T _h 2 cytokine and can modulate T-cell activation ¹⁶ . Depending on its concentration. Can have both inhibitory and inductive effects on HIV <i>in-vitro</i> . Inhibits replication in macrophages via the release of TNF- α and IL-6. Lower concentrations have been associated with enhanced replication in monocyte-derived macrophages ⁵ .

Hematopoietic	IL-7	Is elevated in seropositive individuals ¹⁷ . Is crucial for T-cell development and maintenance of T-cell homeostasis ¹⁸ . Participates in CTL development ¹⁹ . However, may also induce HIV replication in chronic-infected individuals ²⁰ .
	G-CSF	Is both a hematopoietic growth factor and a mediator of anti-HIV and inflammatory responses. Is considered to be useful in immune reconstitution in patients on HAART ^{21,22} .

¹Kedzierska & Crowe, 2001; ²Boulware et al., 2011; ³Kaplanski et al., 2003; ⁴Gabay, 2006; ⁵reviewed by Alfano & Poli, 2005; ⁶Matsumoto et al., 1993; ⁷Narimatsu, Wolday & Patterson, 2005; ⁸Roux et al., 2000; ⁹reviewed by Maurer & von Stebut, 2004 and Menten, Wuyts & Van Damme, 2002; ¹⁰Leghmari et al., 2008; ¹¹Lane et al., 2003; ¹²Stylianou et al., 2000; ¹³Schoenborn & Wilson, 2007; ¹⁴Schroder et al., 2004; ¹⁵Catalfamo et al., 2011; ¹⁶Boulware et al., 2011; ¹⁷Boulassel et al., 2003; ¹⁸Fry & Mackall, 2001; ¹⁹Kim et al., 1997; ²⁰Scripture-Adams et al., 2002; ²¹Hübel, Dale & Liles, 2002; ²²Von Aulock & Hartung, 2002

Appendix II

DESIGN AND OPTIMISATION OF THE POLYCHROMATIC FLOW CYTOMETRY

PANEL

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DESIGN AND OPTIMISATION OF THE POLYCHROMATIC FLOW CYTOMETRY PANEL FOR EVALUATING THE QUALITY AND LEVEL OF ACTIVATION OF BLOOD AND CERVICAL T-CELLS

To address the objectives of this study, a ten-colour antibody panel was optimized to measure the frequency of T-cells (based on expression of CD3, CD4 and CD8), their memory phenotype (based on expression of CD45RO and CCR7) and their level of activation (based on expression of CD38 and HLA-DR), proliferation (based on expression of Ki67) and exhaustion (based on expression of CD57). A viability marker, violet viability reactive dye (VIVID), was used to identify live cells. A dump channel was used to exclude B-cells and monocytes (CD14 and CD19) in order to improve the accuracy of the panel to identify T-cell-specific markers. This chapter describes the steps involved in designing the 10-colour antibody panel, and optimizing the staining protocol.

A2.1 Configuration of the LSR Fortessa

A BD Fortessa™ flow cytometer was used for this study. The BD Fortessa™ cytometer used in this study had four lasers (a violet laser at 405 nm, a blue laser at 488nm, a green laser at 544nm and a red laser at 640nm), capable of detecting ≤18 colours (figure A2.1).

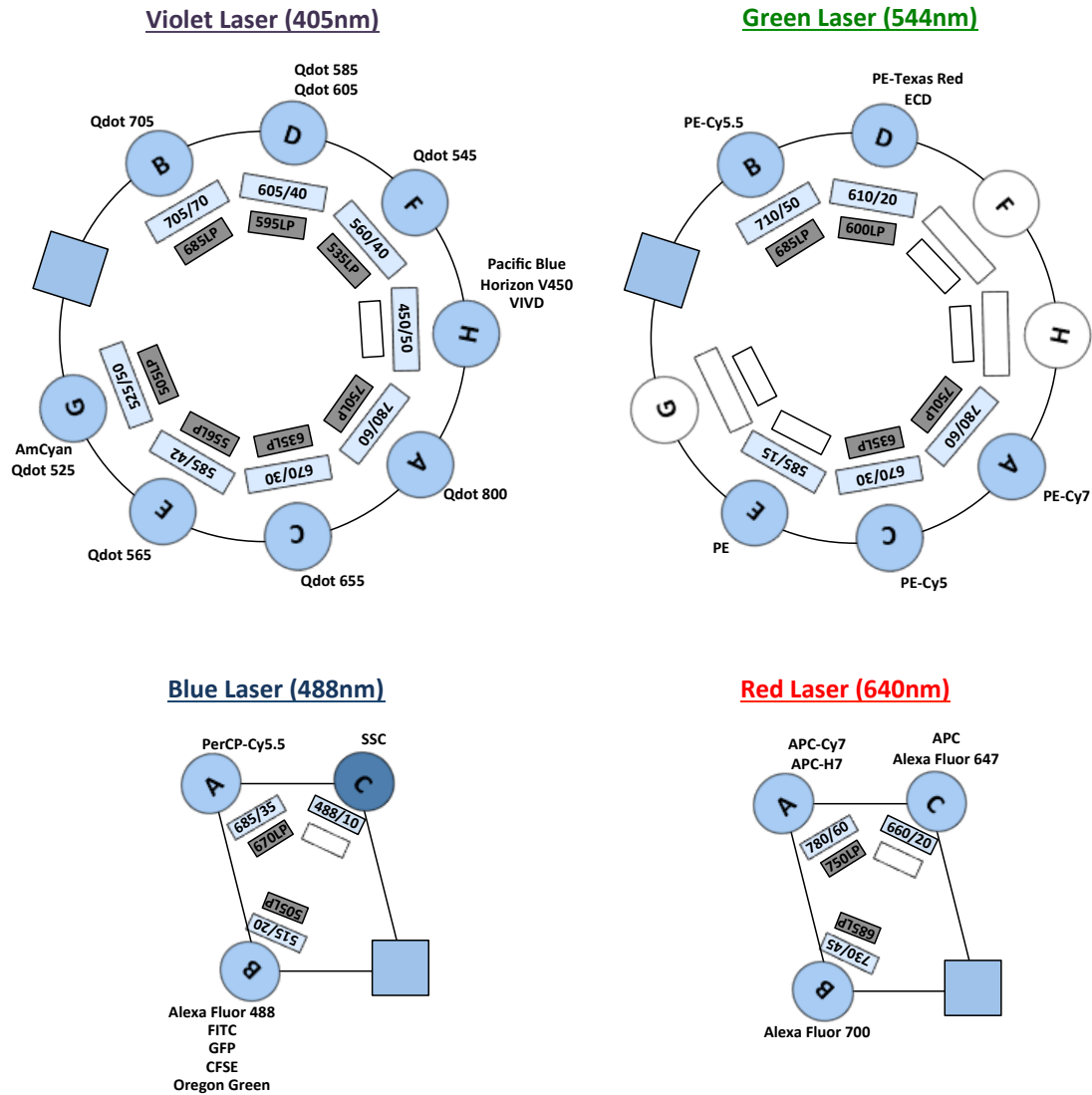


Figure A2.1. Configuration of the BD LSRFortessa used in this study, showing the different lasers and the number of channels for each of them.

A2.2 Selection of antibodies

Antibody selection for this study was dependent on several factors including the configuration of the Fortessa flow cytometer, the level of expression of each phenotypic, memory or activation marker, the stain index of each antibody (a measure of the effective brightness of the antibody-fluorochrome conjugate, which would impact on the overall level of background responses) and the amount of spectral overlap from each fluorochrome (Maecker and Trotter, 2012). In general, the antibodies with the brighter fluorochromes are matched with markers that are expressed at lower levels while the dimmer fluorochromes can be used for the markers found at a higher frequency. For this panel, fluorochromes such as FITC and PE (which have a higher staining index on the

Fortessa ([BD Biosciences, 2012](#)) were used for antigens like Ki67 and CD57 as these are the markers that are expressed at a lower frequency on or inside cells, compared to the other markers being measured. Furthermore, dimmer fluorochromes such as APC-Cy7, APC and Pacific Blue were then kept for the more densely expressed markers such as HLA-DR, CD3 and Vivid (the dead cell marker).

Another factor to be considered for selection of fluorochromes was the amount of spillover of one fluorochrome into the other channels. One of the ways to deal with it is to use compensation beads (as described in section 2.9). Spectral compensation is important because it ensures that cells that fluoresce in one channel will have no effect in other channels ([Maecker and Trotter, 2012](#)). However, in the case of some of the brightest fluorochromes, compensation does not get rid of all the noise, leading to a decrease in resolution or sensitivity. In this case, it is recommended that a dimmer antibody be used to minimize spectral overlap. For example, in the panel designed for this study, PerCp-Cy5.5 (used for the CD4 marker) was included together with APC (for the CD3 marker) instead of other brighter markers, for example PE-Cy5, as this would result in a lower level of background fluorescence and higher resolution sensitivity for APC.

A2.3 Titration of antibodies

Titration was carried out for all the fluorochromes in the panel to ensure that the volumes used in each experiment allowed for optimal separation between the positive and negative populations, and excluded the maximum background fluorescence possible. Titration allowed the determination of each antibody's saturation titre, defined as the volume of antibody (or the highest dilution factor) that allows complete staining of the particular cellular marker in a sample. For these experiments, 6-8 fold serial dilutions were carried out for each of the antibodies titrated. Surface and/or intracellular staining with the antibody was carried out as described in section 2.8. Pseudo-colour plots and histograms were generated to determine the degree of separation and the amount of background fluorescence with each antibody volume. To confirm that the correct saturation titre was chosen, signal-to-noise ratio plots were also generated from the data.

This was obtained by plotting the ratio of the MFI of the positive population to that of the negative population against the volume of antibody used in each case. From the graphs, saturation occurs at the point where the curve plateaus. The titration results for CD57 (detected by surface staining in this study) and CD3 (detected by intracellular staining) are shown as examples (figure A2.2). Staining for CD3 was done intracellularly due to the internalization of the receptor that may occur on activated cells (Schrum et al., 2003). For titration of CD57, the best separation between the positive and negative populations was determined to correspond with a volume of 5 μ l of antibody (indicated by the red arrow; figure A2.2A and B). The signal-to-noise ratio curve in figure A2.2C shows that this was the optimal volume because difference in fluorescence between the positive and the negative cell populations plateaus at a volume of 5 μ l. For the CD3 antibody titration, the saturation point could not be easily determined from the signal-to-noise ratio curve (figure A2.3C). Since the most distinct separation between the positive and negative populations occurred with a volume of 2.5 μ l, this was chosen to be the optimal volume (as shown by the red arrow in figure A2.3A and B).

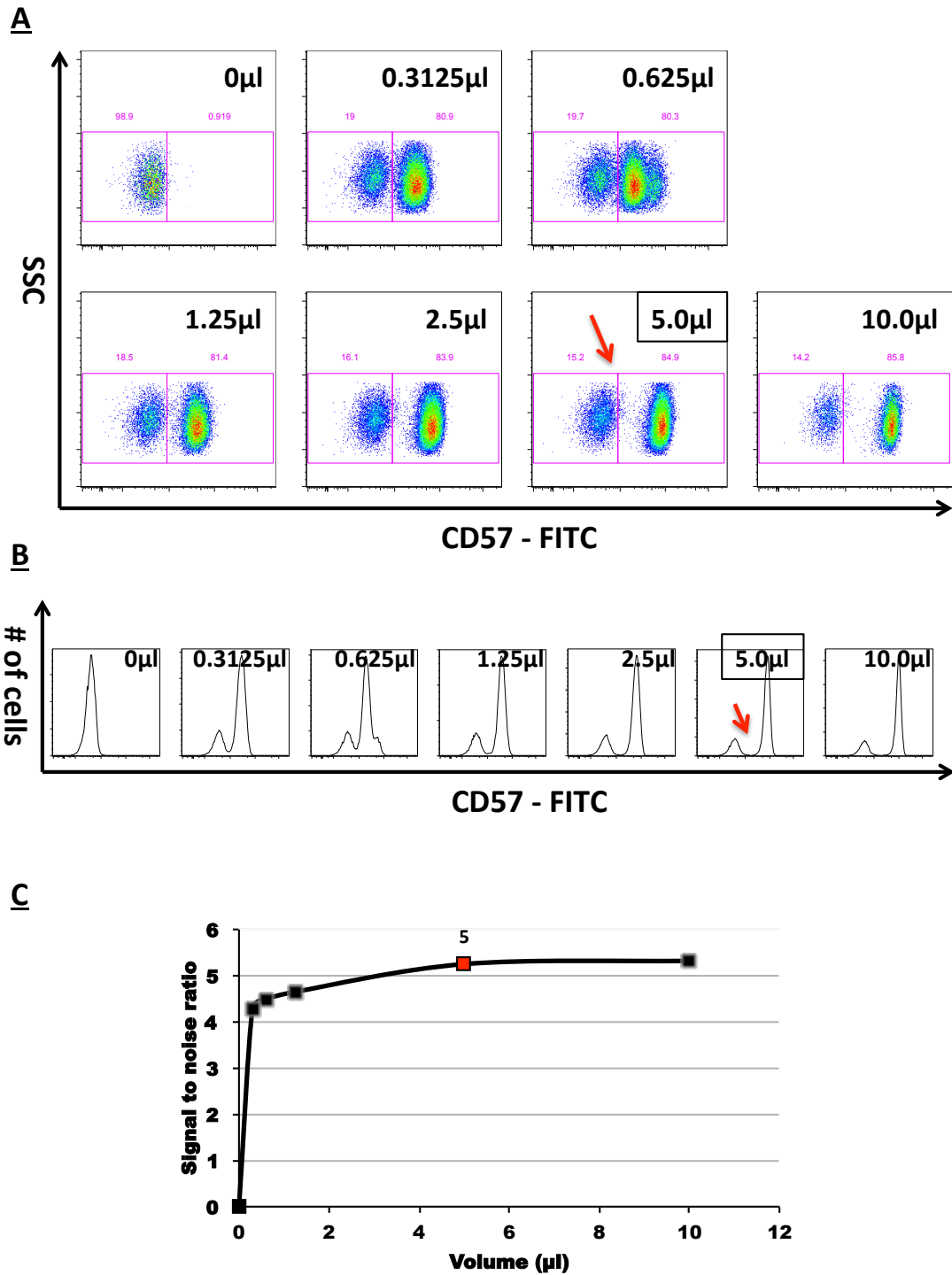


Figure A2.2. CD57-FITC antibody titration. **A.** Pseudo-colour plots with gates showing the position of the positive and negative populations and the degree of separation between the two. The CD57⁺ cells were obtained after first gating on live cells, singlets, lymphocytes and then CD3⁺ T-cells. The volume of antibody used in each case is shown on the upper right corner of each plot. **B.** Histograms showing the relative fluorescence of the positive and negative populations. **C.** Graph of the signal-to-ratio comparing the frequency of the positive events against the negative ones.

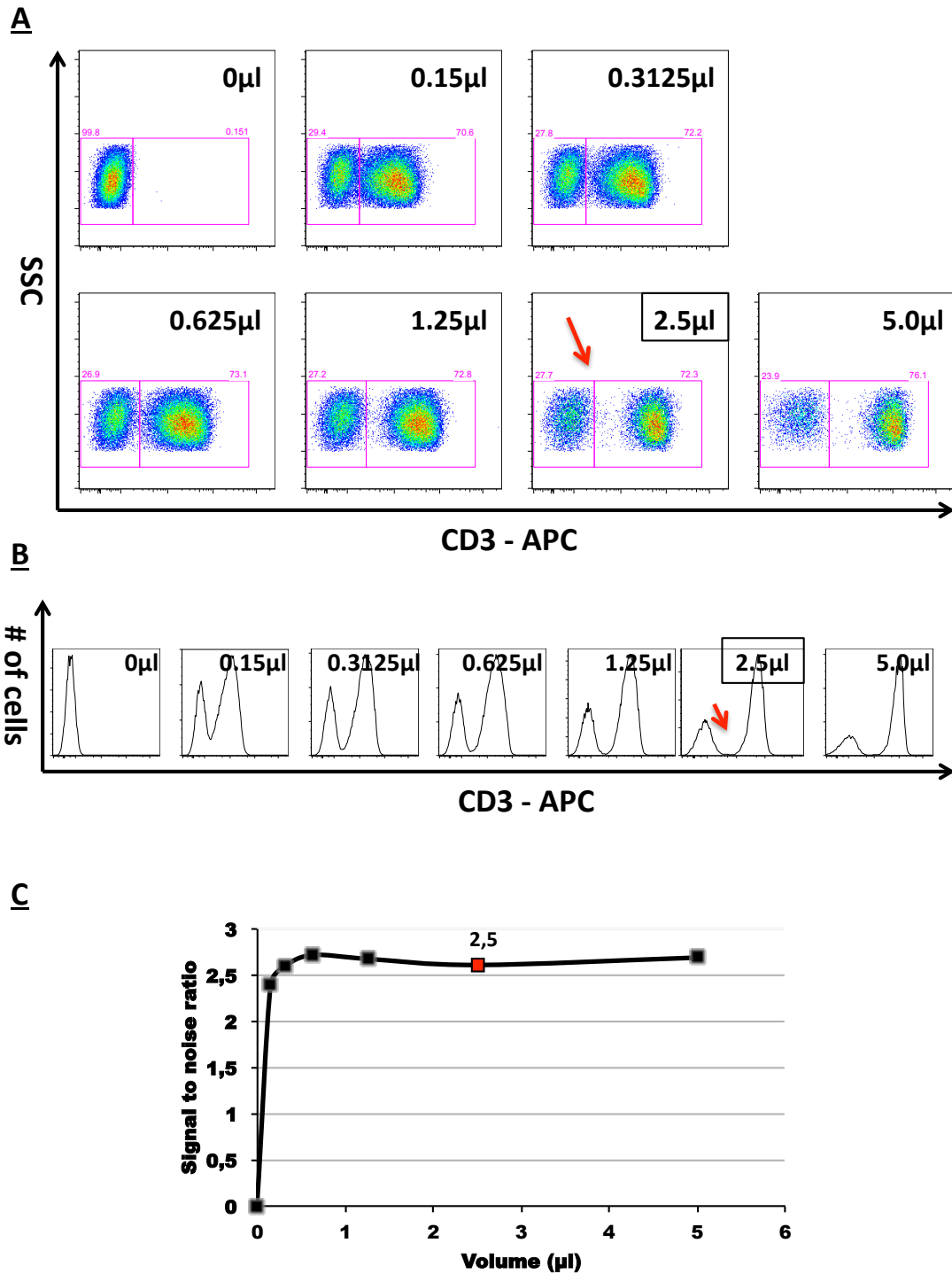


Figure A2.3. CD3-APC antibody titration. **A.** Pseudo-colour plots with gates showing the position of the positive and negative populations and the separation between the two. The CD3⁺ cells were obtained after first gating on live cells, singlets and then lymphocytes. The volume of antibody used in each case is shown on the upper right corner of each plot. **B.** Histograms showing the relative fluorescence of the positive and negative populations. **C.** Graph of the signal-to-ratio comparing the frequency of the positive events against the negative ones.

A2.4 Inclusion of a dump channel

To improve the quality the data obtained through flow cytometric analysis and to avoid non-specific binding of antibodies to non-T-cells, antibodies for markers specific for monocytes (CD14) and B-cells (CD19) were also included as part of a dump channel (figure A2.4). Antibodies included in the dump channel were selected to use the same channel (Pacific Blue) as Vivid. In this way, a single channel could be used to exclude all B-cells, monocytes and dead cells, thereby decreasing background responses and giving better data.

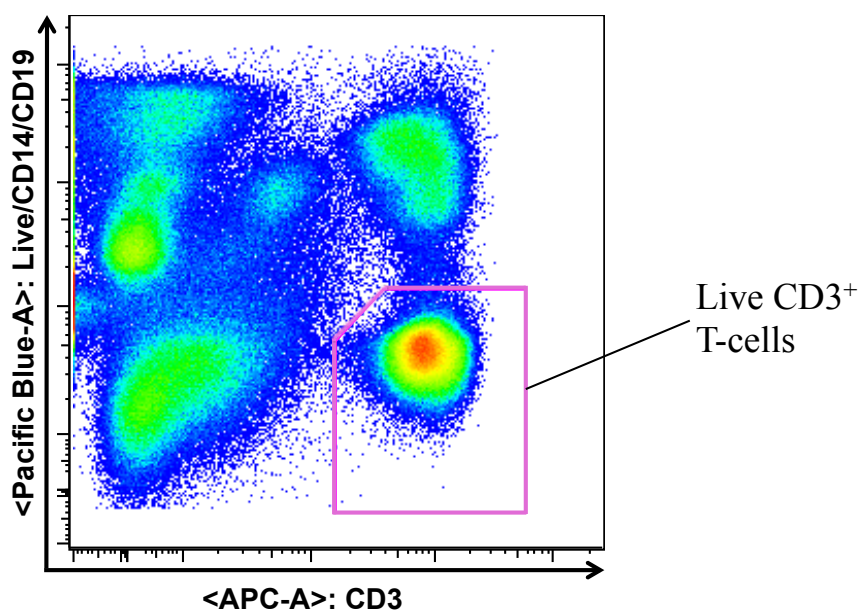


Figure A2.4. Gates showing only the CD3⁺ T-cells after the Pacific Blue channel was used to exclude all dead cells, B-cells and monocytes.

A2.5 Overview of the antibody panel

Table 3.1 provides a list of the antibodies that were included in the optimized 10-colour polychromatic flow cytometry panel used in this study.

Table A2.1. Antibody specificity and fluorochromes included in the flow cytometry panel

Marker	Fluorochrome	Clone	Manufacturer	Optimal volume/ μ l	Staining
CD3	APC	UCHT1	eBioscience	1.25	Intracellular
CD8	Qdot 605	3B5	Invitrogen	5 (1:10 dilution)	Extracellular
CD4	PerCP-Cy5.5	OKT4	eBioscience	1.0	Extracellular
CCR7	PE-Cy7	3D12	BD Biosciences	2.5	Extracellular
CD45RO	ECD	UCHL1	IO Test	5.0	Extracellular
CD38	Alexa Fluor 700	HIT2	eBioscience	5.0	Extracellular
HLA-DR	APC-Cy7	L243	BD Biosciences	3.0	Extracellular
KI67	PE	20Raj1	eBioscience	1.0	Intracellular
CD57	FITC	NK1	BD Biosciences	5.0	Extracellular
CD14	Pacific Blue	M5E2	BD Biosciences	1.25	Extracellular
CD19	Pacific Blue	MHCD1928	Invitrogen	2.5	Extracellular

The panel makes use of all the four lasers used by the Fortessa: the fluorochromes Pacific Blue and Qdot 605 are excited by the violet laser, ECD, FITC and PerCP-Cy5.5 by the blue laser, PE and PE-Cy7 by the green laser and Alexa Fluor 700, APC and APC-Cy7 by the red laser.

A2.6 Fluorescence Minus One (FMO) controls

FMO controls were included in each experiment in order to allow more accurate gating and determine the presence of false positives or background responses (potentially due to degradation of conjugated antibody-fluorochrome molecules, to spillover or antibody reactions especially when large panels are used). In each staining experiment, aliquots of 1×10^6 PBMCs were stained with the full panel excluding one individual antibody in each case. This way, fluorescence from secondary channels is visible, as the main antibody has been left out as shown for each antibody in figure A2.5. Using FMOs is crucial especially for markers that are usually expressed at low levels.

For the panel optimized for this study, very low levels of background noise in all the different channels was noted. The highest level of background was seen in the Pacific Blue (0.107; figure A2.5A), PE-Cy7 (0.101; figure A2.5B) and the PE Texas Red (0.118; figure A2.5C) channels. Since these fluorochromes are conjugated to highly expressed markers, the level of background responses could be considered negligible compared to the level of fluorescence emitted by the primary fluorochrome. The frequency of the background responses for each parameter were recorded and subtracted from analyses carried out on all the samples collected.

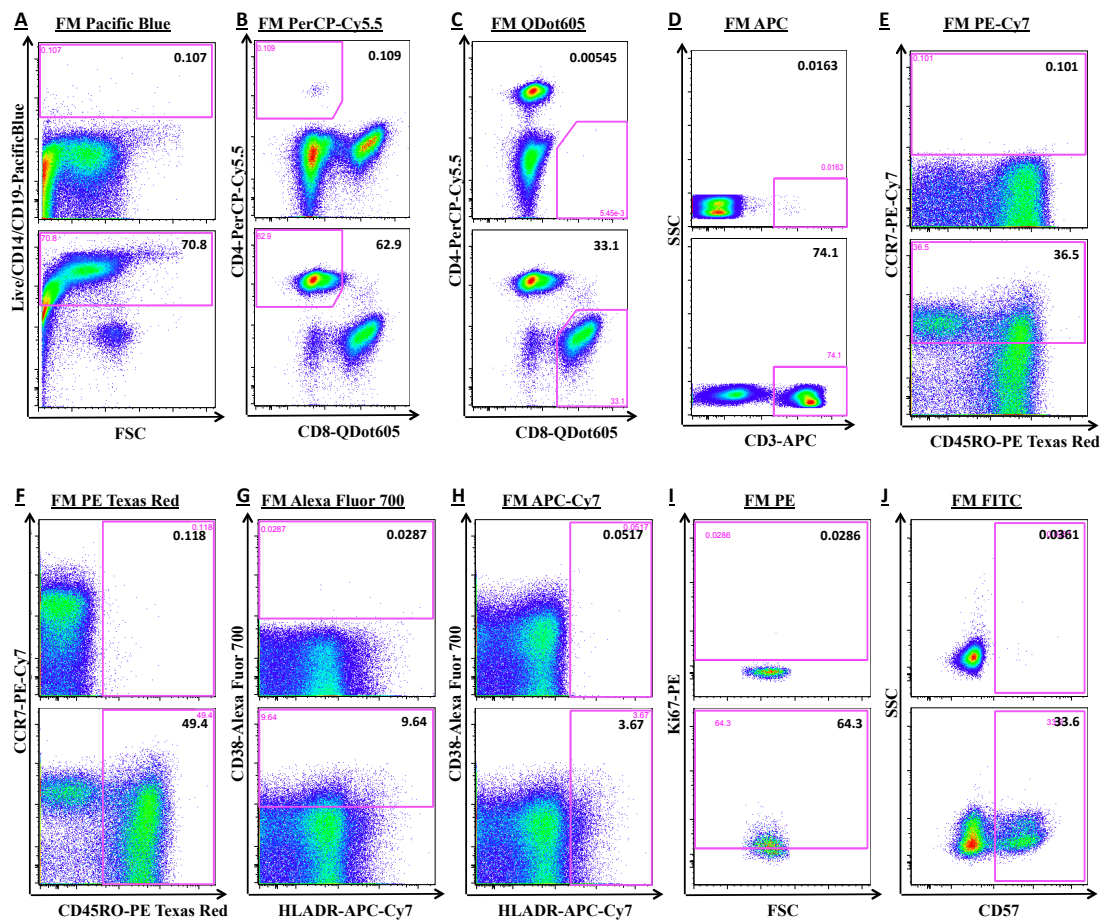


Figure A2.5. FMO controls for the full 10-colour antibody panel. The title of each individual set of plots indicates which antibody has been excluded in each case. All plots were first gated on live cells, singlets, lymphocytes and CD3⁺ T-cells except for the Pacific Blue channel that shows the events gated from singlets. Below each FMO plot is the corresponding pseudo-colour plot that had been gated on a fully stained PBMC sample. FM: Fluorescence minus.

A2.7 Time gates

Before gating for the different phenotypic and functional markers included in the panel, the cells were first gated based on time (figure A2.6). This gating strategy allows exclusion of artefacts from acquisition like bubbles or small blockages in the fluidic system of the apparatus or other issues that went unnoticed during the acquisition process. Including time gates in the gating strategy provided a good method for identifying and excluding irregularities in the flow cytometer's pressure and laser output. The time gates were drawn in such a way as to only include the part where the event rate and fluorescence signals were constant over time.

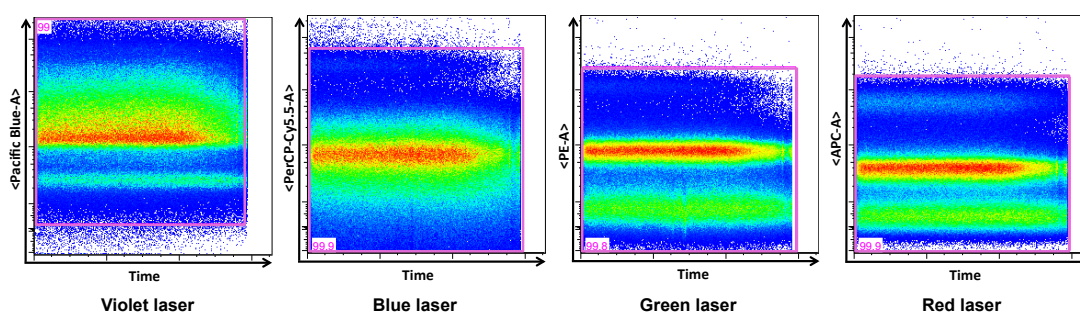


Figure A2.6. Time gates drawn for each of the four lasers used by the LSR Fortessa.

A2.8 Gating strategy

After all the optimization steps, a gating strategy was then designed to determine the level of expression of the different markers for each sample. Figure A2.7 shows the final gating strategy that was used for the analysis of all the flow cytometry data for the study. The total number of events recorded on the flow cytometer were first analysed according to event size (forward scatter (FSC)-height against FSC-area) to select for singlets only and exclude doublets or larger cellular aggregates. The Pacific Blue channel was used to gate on the live T-cells, while eliminating all dead cells, B-cells and monocytes. Lymphocytes were gated on according to their size and level of granularity (side scatter-area against FSC-area). CD3⁺ T-cells were gated on, followed by the CD4⁺ and CD8⁺ T-cells while making sure the double positive cells were not included. Gates specific for the memory markers (CCR7 and CD45RO), the activation markers (CD38 and HLA-

DR), the proliferation marker (Ki67) and the senescence marker (CD57) were then drawn for both the CD4⁺ and the CD8⁺ T-cells.

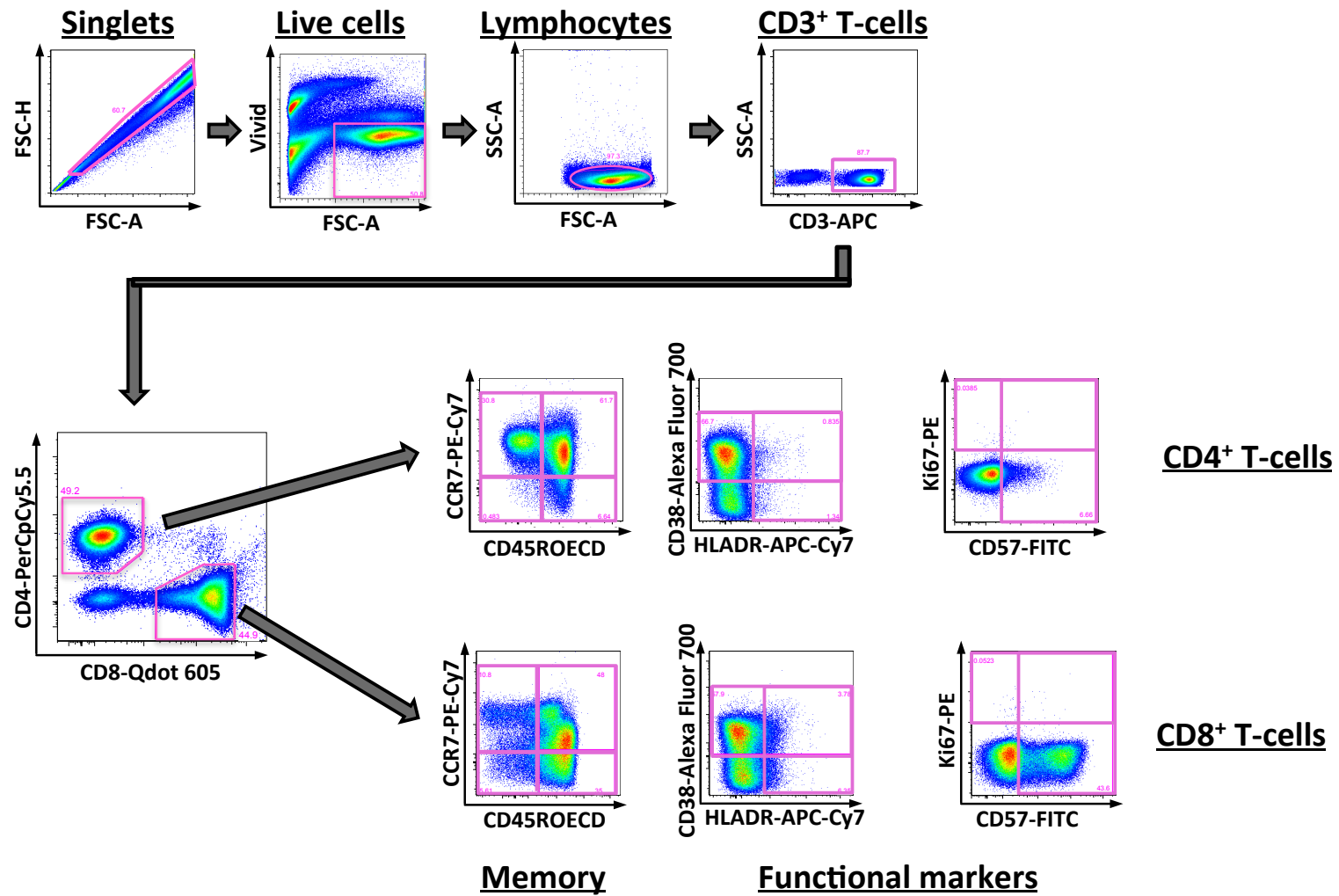


Figure A2.7. Final gating strategy using the optimized panel. The memory and functional markers were gated on CD4⁺ and CD8⁺ T-cells. The position of the gates for all the markers were based on the FMOs carried out as described earlier (section 6).

A2.9 Discussion

The aim of this Chapter was to develop and optimize a polychromatic flow cytometry panel to accurately measure specific markers on CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cells in both peripheral blood and cervical cytobrush samples of HIV-positive women. Cellular markers were selected to differentiate between the different memory subsets (using CCR7 and CD45RO) and to quantify the degree of immune activation (based on the level of expression of CD38 and HLA-DR), proliferation (Ki67) and senescence (CD57). All the optimization experiments were carried out on PBMC samples from HIV-negative individuals, before testing the final panel on PBMCs from HIV-positive individuals. The antibody panel used was designed and optimized by (1) identifying the relevant markers that would give the most comprehensive data relevant to the study and choosing the appropriate fluorochrome conjugates; (2) titrating the reagents for optimal results; and (3) determining the level of background noise/false positive events and correcting for it (using FMOs).

Polychromatic flow cytometry is especially useful when the samples are difficult to obtain or when the cells being analysed are rare. This is relevant in this study using cervical cytobrush samples that are known to contain a relatively few T-lymphocytes ([Bere et al., 2010](#)). This technique allows for analysis of specific cell populations with a higher degree of accuracy and a large volume of information can be obtained from a single sample. Overall, an optimized 10-colour flow cytometry panel was developed that reliably generated information about the T-cell phenotype and its functions in HIV-positive women included in this study.