

**Impact of immune activation and inflammation on
the susceptibility to HIV infection and disease
progression in HIV serodiscordant and
seroconcordant couples**

by

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LIST OF ABBREVIATIONS

ACTG	AIDS Clinical Trials Group
AGM	African green monkey
AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
APC	Antigen presenting cells
ART	Antiretroviral therapy
BALT	Bronchus-associated lymphoid tissue
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CO₂	Carbon dioxide
cm	Centimeter
CMC	Cervical mononuclear cell
CMV	Cytomegalovirus
CNS	Central nervous system
CRP	C-reactive protein
CTL	Cytotoxic T-lymphocyte
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DMPA	Depo-Provera
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
EC	Elite controller
ELISPOT	Enzyme linked immunosorbent spot
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence Minus One

FSW	Female sex worker
g	Gram
GALT	Gut-associated lymphoid tissue
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp120	Glycoprotein 120
HCV	Hepatitis C virus
HEPS	Highly exposed persistently seronegative
HESN	Highly exposed seronegative
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSV	Herpes simplex virus
IFN-γ	Interferon-gamma
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
LNTP	Long term non-progressor
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MALT	Mucosa-associated lymphoid tissue
MCP-1	Monocyte chemotactic protein-1
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
ml	Milliliter

mM	Millimolar
MSM	Men who have sex with men
MTCT	Mother-to-child transmission
NaN₃	Sodium azide
NFκβ	Nuclear factor-kappa beta
NHLS	National Health Laboratory Service
NIH	National Institutes of Health
NK	Natural killer
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE-Cy	Phycoerythrin-Cyanine
PerCP-Cy	Peridin Chlorophyll Protein-Cyanine
pg/ml	Picogram per milliliter
PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulated on activation normal T-cell expressed and secreted
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SHIV	Simian/Human immunodeficiency virus
SIV	Simian immunodeficiency virus
SM	Sooty mangabey
STD	Sexually transmitted disease
STI	Sexually transmitted infection
T-Reg	Regulatory T-cell
TB	Tuberculosis
Th	T-helper
TLR	Toll-like receptor

TNF-α	Tumour necrosis factor-alpha
UNAIDS	Joint United Nations Programme on HIV and AIDS
UCT	University of Cape Town
WHO	World Health Organisation
°C	Degree celcius
μg	Microgram
μl	Microliter

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Abstract

The biological correlates of protection against HIV infection remain poorly characterized, hindering the development of an effective prevention strategy. Studies of individuals who resist HIV infection or progress more slowly after being infected are important for the conception of appropriate approaches for mimicking the effective responses against HIV infection or progression. The role of immune activation and chronic inflammation in the modulation of HIV acquisition risk and/or rate of HIV disease progression has been proposed as one of the most important mechanisms determining risk and pathogenesis but is not fully understood. A state of immune quiescence has been associated with protection against HIV infection and slower disease progression. To explore potential risk factors associated with HIV transmission and HIV disease progression, this dissertation investigates the relationship between clinical and biological biomarkers and resistance to HIV infection or disease progression (including viral load, CD4 counts, cellular activation, soluble inflammatory and regulatory cytokines, and HIV co-receptor expression) in stable long-term HIV seroconcordant and serodiscordant couples.

Chapter 2 shows that HIV+ seroconcordant individuals (with HIV+ partners) had 0.5 log₁₀ higher plasma viral loads than HIV+ serodiscordant ones ($p < 0.0001$), although this did not predict worse disease outcome (measured by absolute blood CD4 counts at later time points). Plasma viral loads were determined to account for 35% of the variability in CD4 counts in HIV+ individuals in this study, confirming previous findings that plasma viral load is not a reliable predictor of rate of CD4 loss during HIV infection. However, CD4 T-cell counts at enrolment into this study were found to be a stronger predictor of subsequent variability in CD4 counts, accounting for 77% of the fluctuation in CD4 counts.

Chapter 3 shows that HIV+ seroconcordant individuals had greater frequencies of susceptible, activated T-cells (CD38⁺CCR5⁺) in blood compared to their HIV+ serodiscordant counterparts. Manifestation of a higher plasma viral load resulted in significantly higher frequency of CD4⁺CCR5⁺ ($p = 0.007$), CD8⁺CD38⁺ ($p = 0.01$) and CD8⁺HLA-DR⁺CD38⁺ ($p = 0.01$) T-cells compared to individuals with lower HIV plasma load (< 1500 cps/ml). HIV+ seroconcordant individuals also had significantly higher plasma concentrations of the pro-inflammatory cytokines IL-1 β and TNF- α than HIV+ serodiscordant individuals. Multivariate regression analysis identified CD4⁺HLA-DR⁺CD38⁺

T-cell frequencies and concentrations of TNF- α in plasma as the most influential factors determining plasma viral loads, independently of CD4 counts. Modeling to determine the variation in CD4% revealed that inclusion of systemic immune activation markers substantially improved predictive capacity by 16% from the univariate model with plasma viremia as predictor.

In Chapter 4, HIV+ seroconcordant women also had significantly higher viral loads in their genital compartments compared to HIV+ serodiscordant women ($p=0.001$), with plasma viral loads being significantly associated with genital tract viral loads ($Rho=0.65$; $p<0.0001$). Despite HIV+ seroconcordant women having higher genital tract viral loads compared to HIV+ serodiscordant women, genital tract cytokine concentrations were similar between the two groups although women shedding HIV in their genital tracts had significantly higher mucosal inflammatory, regulatory and chemotactic cytokine concentrations (IP-10, $p=0.002$; IL-1 α , $P=0.007$; IL-1 β , $p=0.004$; IL-6, $p=0.005$; IL-8, $p=0.008$; MCP-1, $p=0.03$; MIP-1 β , $p=0.01$; IL-10, $p=0.01$; G-CSF, $p=0.002$) compared to non-shedders. Mathematical modeling identified genital IL-1 β and plasma HIV levels as being predictors of genital HIV shedding.

Chapter 5 evaluates cellular activation markers in blood and plasma cytokine concentrations in HIV-exposed but uninfected individuals (HIV- serodiscordant with HIV+ partners) compared to HIV- unexposed controls (HIV- seroconcordant with HIV- partners). HIV- serodiscordant individuals had lower frequencies of activated CD8⁺ T-cell in blood ($p<0.01$) but also significantly lower expression levels of CCR5 than HIV- seroconcordant individuals ($p<0.05$ for CD4⁺ and $p<0.05$ for CD8⁺).

Chapter 6 compares soluble cytokine biomarkers of HIV risk in matched plasma and genital tract secretions from HIV- serodiscordant and HIV- seroconcordant women. HIV- serodiscordant women had significantly elevated genital tract concentrations of IL-1 α compared to HIV- seroconcordant women ($p=0.001$), which could suggest HIV exposure. However, no other differences were noted between HIV- serodiscordant and seroconcordant individuals in any of the other 13 cytokines measured in genital fluid, suggesting that no broad inflammatory signatures of risk were evident between groups. Concentration of 3/14 cytokines (IL-1 β , IL-6, IL-10) were evaluated in matching plasma and genital secretions, and data from this analysis suggest that the female genital tract and blood are immunologically separate compartments.

In conclusion, this dissertation suggests that HIV+ individuals who have not transmitted to their partners (in HIV+ serodiscordant relationships) and HIV- individuals who have not become infected despite being exposed (HIV- serodiscordant relationships) share an immune quiescent phenotype in that they both exhibit reduced frequencies of activated CCR5-expressing T-cells than their seroconcordant counterparts. Studies of immune activation, inflammation, HIV-specific immune responses and immunoregulatory mechanisms both in blood and at the genital mucosal level in serodiscordant couples are important to characterise some of the determinants of transmissibility and protective mechanisms against HIV transmission. CCR5 expression on the surface of T-cells, especially the activated ones, is central to the perpetuation of HIV replicative cycle and confirms CCR5 agonists as appropriate targets in the design of an anti-HIV strategy.

CHAPTER 1

Literature Review

1.1 The status of the HIV/AIDS pandemic

1.1.1 The global perspective

It is estimated that 2.5 million people were newly infected with the human immunodeficiency virus (HIV) worldwide in 2011 (Figure 1.1; UNAIDS, 2012), bringing to 34 million the total number of people living with HIV infection (Figure 1.2; UNAIDS, 2012). For the first time since the start of the HIV pandemic, UNAIDS (2012) reported a 24% reduction in global acquired immunodeficiency syndrome (AIDS)-related deaths (comparing statistics for 2011 to 2005). Despite these gains, HIV and AIDS remain a major global priority, with 1.7 million AIDS-related deaths in 2011 (Figure 1.1; UNAIDS, 2012).

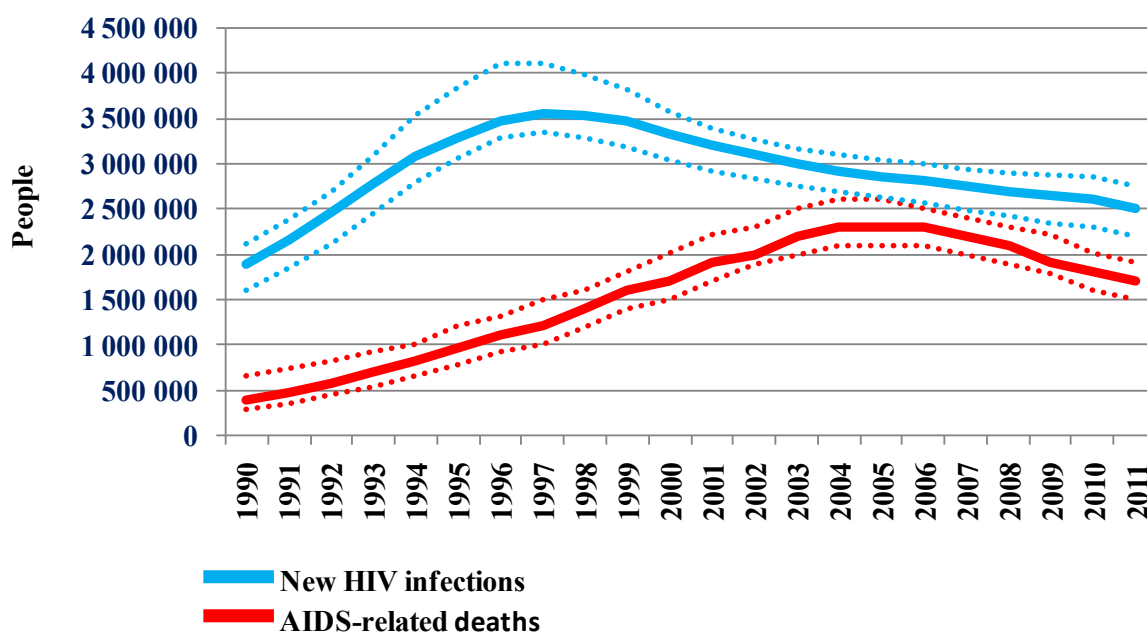


Figure 1. 1 New HIV infections and AIDS-related deaths worldwide, 1990-2011. Twenty five countries have seen a 50% or greater drop in new HIV infections since 2001. Sub-Saharan Africa remains the epicentre of the pandemic contributing 72% to the global pool of newly HIV infected individuals and accounting for 71% of global AIDS-related deaths in 2011. Source: UNAIDS Report on the Global AIDS Epidemic, 2012.

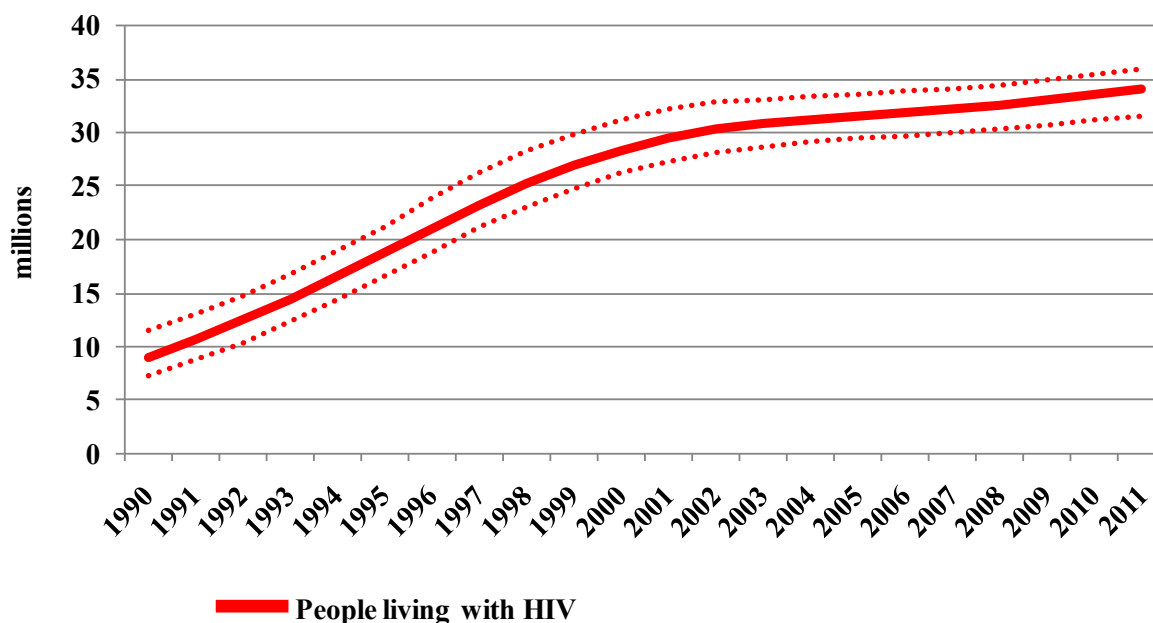


Figure 1. 2 People living with HIV worldwide, 1990-2011. Sub-Saharan Africa accounted for 69% of HIV prevalence globally in 2011. It remains the most affected region with nearly 1 in every 20 adults living with HIV. Source: UNAIDS Report on the Global AIDS Epidemic, 2012.

The scaling up of antiretroviral therapy (ART) in low- and middle-income countries has led to a substantial downturn in recent years in AIDS-related morbidity and mortality (a decline of 24% from 2005 to 2011; Figure 1.1 and Figure 1.3). As a result, HIV prevalence worldwide has reached a plateau, with the absolute number of people living with HIV remaining fairly stable over the past 5 years, having increased steeply for more than 10 years (from less than 10 million infections in 1990 to over 30 million in 2002; Figure 1.2). Despite rollout of ART globally, uneven progress has been made in different regions with significantly lower access to ART in regions like Eastern Europe and Central Asia and Middle-East and North Africa compared to the Caribbean and Sub-Saharan Africa (Figure 1.3). These regional differences in ART rollout translated into increases in AIDS-related death in areas of low access to ART (21% increase in Eastern Europe and Central Asia and 17% increase in Middle-East and North Africa), compared to decreases in the Caribbean (48%) and Sub-Saharan Africa (33%) over the period 2005 to 2011 (UNAIDS, 2012).

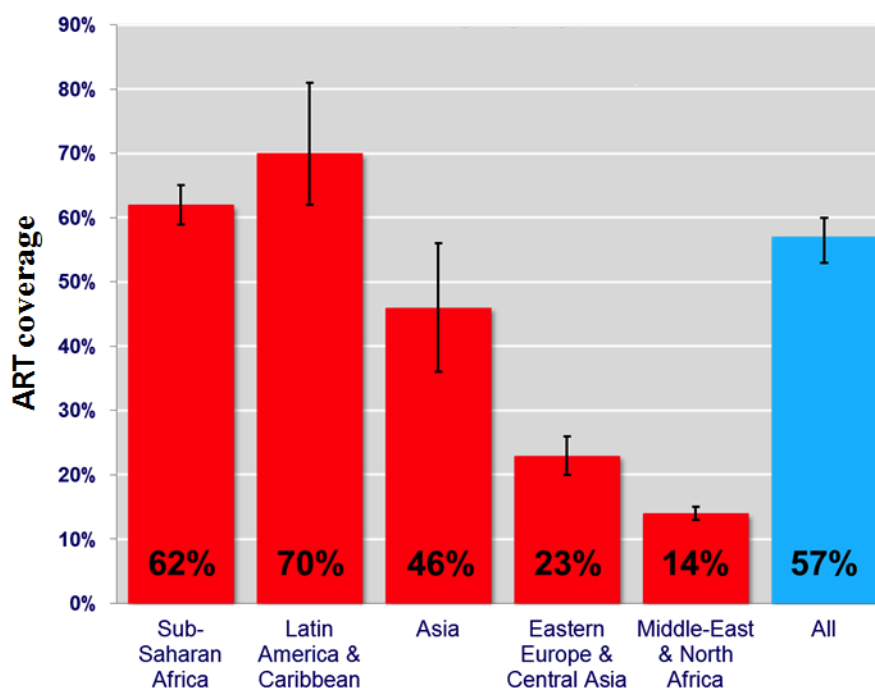


Figure 1. 3 ART coverage between regions in adult populations, 2011. In 2011, more than 8 million people living with HIV had access to antiretroviral therapy, with the number increased by 63% from 2009 to 2011. However, there remain 7 million people eligible for HIV treatment who still do not have access, most notably children (72% globally). Source: UNAIDS Report on the Global AIDS Epidemic.

1.1.2 The South African / Sub-Saharan African perspective

Unlike other regions of the world, the majority of people living with HIV in Sub-Saharan Africa are women (58%; UNAIDS, 2012), leading some to say that the Sub-Saharan epidemic wears a ‘woman’s face’ (Annan, 2002). Of the countries making up Sub-Saharan Africa, Swaziland has the highest HIV prevalence rate at 26% (UNAIDS, 2012). Although South Africa only has the fourth highest HIV prevalence rate, it has the highest number of HIV-infected individuals of all countries in the region with 5.6 million infected people (UNAIDS, 2012). The HIV epidemic in South Africa is reflective of the Sub-Saharan region, whereby women represent 57% (UNAIDS, 2012) of infected individuals, with women aged 20-24 being 4 times more at risk of having an HIV infection than men of the same age group (21.1% versus 5.2%; South African Global AIDS Response Report, 2012). In South Africa, 30-34 year old women carry the highest burden of the HIV epidemic (HIV prevalence rate of 42.6%) and 30.2% of pregnant women in South Africa aged 15-49 were living with HIV (South African Global AIDS Response Report, 2012).

The HIV epidemic in South Africa is mainly heterosexual, with mother-to-child transmission being the other main infection route. It has been suggested that the reproductive biology in the female genital tract makes women 2-8 times more vulnerable to HIV infection than men (Nicolosi *et al.*, 1994, Padian *et al.*, 1994). Male-to-female transmission could occur more efficiently relative to female-to-male, on the account of extended exposure to seminal fluid (Cummins and Dezutti, 2000; Hirbod and Broliden, 2007).

1.1.3 HIV exposed seronegative individuals, HIV seroconcordance and serodiscordance

Since the discovery of HIV in the early 1980s (Barré-Sinoussi *et al.*, 1983), a number of studies from different parts of the world have reported on individuals who were repeatedly exposed to HIV but remain uninfected, sometimes called ‘HIV resistant’, HIV-exposed seronegative (HESN) or highly exposed persistently seronegative (HEPS; Kaul *et al.*, 2000; Broliden *et al.*, 2001; Iqbal *et al.*, 2005; Liu *et al.*, 1996). Because these individuals appear to be less susceptible to HIV infection, they have been the focus of intensive investigation to understand the genetic, immunologic, and environmental factors that may alter susceptibility to HIV (Horton *et al.*, 2010). One of the best and clearest mechanisms of HIV resistance that has been documented is in individuals that are homozygous for the CCR5 Δ 32 deletion mutation, who lack a CCR5 co-receptor that HIV is able to use to gain entry into host cells (Liu *et al.*, 1996). This homozygous genotype is rare (detected in ~1% of individuals globally) and has primarily been described in people of European descent (Gupta and Padh, 2012).

The best characterized cohorts of HESN have included individuals with high risk sexual behaviours such as commercial sex workers (Kaul *et al.*, 2000; Broliden *et al.*, 2001; Iqbal *et al.*, 2005), men who have sex with men (MSM), injection drug users, infants exposed to vertical transmission and HIV serodiscordant couples (Ruzagira *et al.*, 2011; Vernazza *et al.*, 2011; Abaasa *et al.*, 2013). The HIV seronegative partner in a HIV discordant couple represents the ideal cohort for the identification of factors affecting susceptibility to HIV. It is however important to bear in mind that these different cohorts from which HESNs are recruited also have varying degrees of relative HIV risk; determined according to their respective frequency and type of sex acts, whether their partners have initiated ART, or their frequency of condom usage. It is generally very difficult to accurately and objectively quantify the risk profile of HESN individuals. Even though HIV infection risk may be lower

in these individuals, the identification of genuinely resistant subjects is challenging. However, HIV serodiscordant couples remain the most accessible and most representative population (with regard to global epidemic) for the identification of biological correlates of protection from a heterosexual transmission perspective.

For the purposes of this dissertation, a couple is described as seroconcordant negative when both partners are HIV negative (HIV-) and seroconcordant positive when both are HIV positive (HIV+). A serodiscordant couple is one in which one partner is HIV+ and the other is HIV-. Guthrie and colleagues (2007) argued that the manner in which HIV serodiscordancy arises may differ in less mature compared to mature HIV epidemics. They suggest that most serodiscordant couples arise in early epidemics when HIV is introduced into a pre-existing relationship through sex outside of the relationship (such that both partners may be HIV- at the start of a relationship and one becomes infected from outside the relationship). A mature epidemic is characterised by a plateau in the incidence of HIV, accounted for by a balance in the number of new HIV infections and AIDS-related deaths. In more mature HIV epidemics, a greater proportion of HIV serodiscordant couples are formed with one partner already being HIV infected prior to a couple formation. Countries with hyperendemic, generalised and mature epidemics, like South Africa (Abdool Karim *et al.*, 2011), are characterised by heterosexual transmission, HIV serodiscordant relationships are common and it has been estimated that transmission in stable serodiscordant couples accounts for >50% of new HIV infections in Africa (Curran *et al.*, 2012; Eyawo *et al.*, 2010).

Because they represent a key HIV risk group, HIV serodiscordant individuals have been the focus of many important biomedical trials to prevent or reduce HIV transmission, including medical male circumcision (Gray *et al.*, 2000; Wawer *et al.*, 2009), ART for the HIV+ partner (CDC, 2009; Cohen *et al.*, 2011), pre-exposure prophylaxis (Vernazza *et al.*, 2011; Mastro *et al.*, 2011), post-exposure prophylaxis (van der Straten *et al.*, 2000), herpes simplex virus type 2 (HSV-2) suppression (Lingappa *et al.*, 2009; Mujugira *et al.*, 2011), microbicides (Abaasa *et al.*, 2013), and HIV vaccines (Horton *et al.*, 2010; Ruzagira *et al.*, 2011).

1.2 The natural history of HIV infection

1.2.1 Systemic HIV-specific immune responses, CD4⁺ T-cell depletion and HIV viral load

HIV infects CD4⁺ T helper lymphocytes using the CD4 receptor (Rubbert *et al.*, 2006) and CCR5 or CXCR4 co-receptor (Moore *et al.*, 1997). HIV hijacks the cellular machinery of CD4⁺ host cells to replicate within them and eventually lyses the cells as newly formed virions are released extracellularly to infect other CD4⁺ host cells (Fauci *et al.*, 2003). As a consequence, CD4⁺ T-cells are progressively depleted during the course of HIV infection (Meyaard *et al.*, 1993). In parallel, the host immune system becomes progressively crippled because of CD4⁺ T-cell depletion, which are pivotal in directing and coordinating the functions of other adaptive immune cells (CD8⁺ T-lymphocytes, B-lymphocytes, monocytes and macrophages) (Zhu and Paul, 2008; Wan and Flavell, 2009).

Fiebig and collaborators (2003) have demonstrated that HIV RNA is detectable in blood by PCR as early as 10-14 days post-infection. Within 4 weeks, there is an exponential increase in HIV plasma viremia coupled with the massive depletion of CD4⁺ T-lymphocytes (Figure 1.4). The emergence of cytotoxic CD8⁺ T-cell responses and the production of antibodies by B-cells that bind to HIV coincide with lowering of HIV load in blood to a steady state, termed viral set-point. Viral set point is generally reached by 6 months post-infection. Along with this initial decline in plasma viremia comes a transient replenishment of CD4⁺ T-cell numbers, but to levels lower than before infection (Simon *et al.*, 2006). In the absence of ART, infection with HIV leads to a prolonged and gradually progressive disease which eventually results in opportunistic infections and ultimately death. Depending on a number of host and viral characteristics (such as host immunity, host genetics, viral virulence), or when ARTs were initiated, the asymptomatic phase of HIV infection may last ten years or more. In the absence of ART, the functional capacity of the immune system is impaired with time and the clinical manifestations of AIDS become progressively apparent (Pantaleo and Fauci, 1996). In HIV+ individuals, absolute CD4⁺ T-cell counts in blood predict the development of opportunistic infections and death due to AIDS (Gulick *et al.*, 1997; Hammer *et al.*, 1997; Cameron *et al.*, 1998; Hulgán *et al.*, 2006).

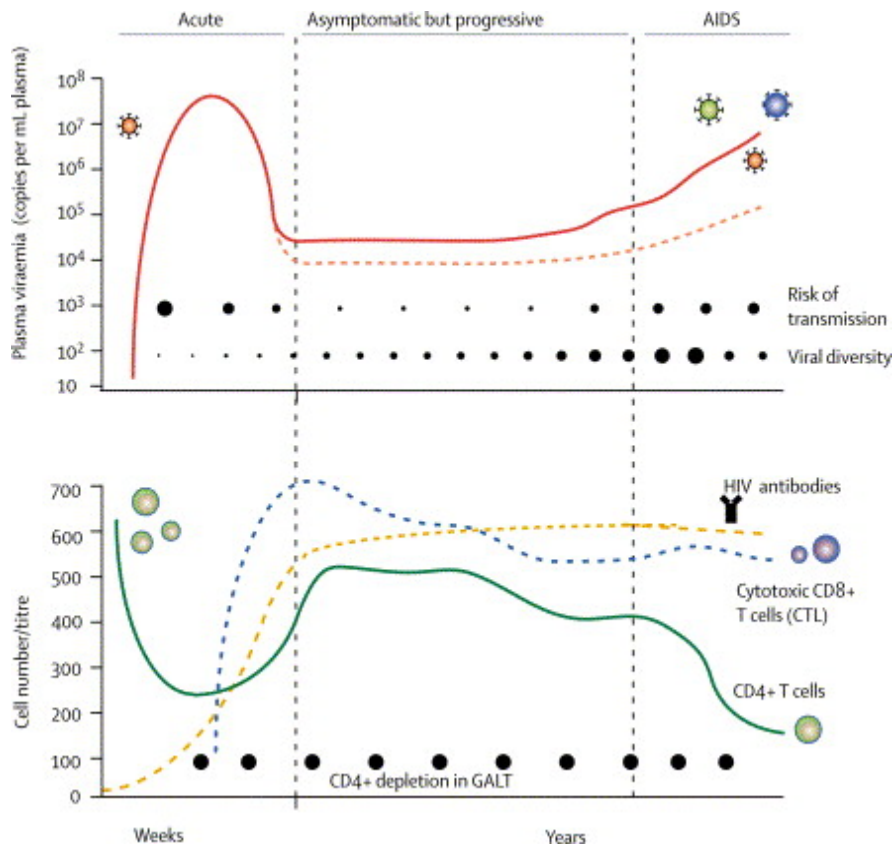


Figure 1. 4 Course of HIV-1 infection defined by the level of viral replication. Plasma viraemia (top), and dynamic changes of the CD4⁺ T-lymphocyte compartments (bottom). Primary infection characterised by high plasma viraemia (redline, top), low CD4 cells (green line, bottom), and absence of HIV-specific antibodies (orange line, bottom). Viraemia drops as cytotoxic CD8⁺ T-lymphocytes (CTL) develop (blue line, bottom) and an individual viral-load set point is reached during chronic infection. Viral set points differ greatly among individuals (e.g., red dotted line, top) and predict disease progression. Viral diversity increases throughout the disease (closed circles, top). The risk of transmission is highest in the first weeks when viraemia peaks (closed circles, top). GALT = gut-associated lymphoid tissues (Adapted from Simon *et al.*, 2006).

HIV-specific T-cells emerge at about 2 weeks following infection, generally persist throughout the course of disease, and systemic HIV-specific CD8⁺ T-cells are considered to be one of the best correlates of immune control (McMicheal and Rowland-Jones, 2001; Betts *et al.*, 2005; Jiao *et al.*, 2006). Gag-specific CD8⁺ T-cells are the best characterized and the magnitude and breadth of Gag-specific CD8⁺ T-cell IFN- γ responses are associated with lower viral loads, independently of human leukocyte antigen (HLA) (Edwards *et al.*, 2002; Kaufmann *et al.*, 2004; Kiepiela *et al.*, 2004). Furthermore, flow cytometry based studies including multiple immune parameters simultaneously have suggested that polyfunctional

CD8⁺ T-cells, which have the capacity to secrete multiple cytokines, are associated with better control of HIV (Betts *et al.*, 2006; Valor *et al.*, 2008; Pantaleo and Koup, 2004).

Several lines of evidence in humans and primates have suggested that HIV- and SIV-specific CTL responses are associated with protection during infection, including: (1) peak viremia declined rapidly during acute HIV infection in humans following the appearance of HIV-specific CD8⁺ T-cells (Borrow *et al.*, 1994; Koup *et al.*, 1994); (2) plasma viral loads increased following depletion of CD8⁺ T-cells in SIV-infected macaques (Jin *et al.*, 1999; Schmitz *et al.*, 1999); (3) HIV⁺ individuals with low viral loads or slower disease progression generally have protective HLA alleles (O'Brien and Nelson, 2004; Fellay *et al.*, 2007); and (4) HIV and SIV clearly demonstrate the ability to mutate in CTL epitopes under CTL pressure (called CTL escape mutations; Leslie *et al.*, 2004; Oxenius *et al.*, 2004).

In addition to CD8⁺ T-cells, the ability of HIV-specific CD4⁺ T-cells to secrete either IL-2 alone, or both IL-2 and IFN- γ has also been shown to correlate with viral control during chronic HIV infection (Harari *et al.*, 2004). Similarly, studies in vaccinated macaques have demonstrated that the presence of T-cells secreting both IFN- γ and IL-2 was associated with protection from infection following SHIV challenge (Sadagopal *et al.*, 2005). In HIV⁺ individuals who are able to control their HIV plasma load and sustain a normal CD4 count in absence of ART (called long term non-progressors), Gag-specific IL-2 and IFN- γ secreting CD4⁺ T-cell responses best predicted control of viremia (Boaz *et al.*, 2002). Both CD8⁺ and CD4⁺ T-cells produce a variety of cytokines during HIV infection and among the best described are IFN- γ , IL-2, TNF- α and MIP-1 β (Brenchley *et al.*, 2008; Ferrando-Martinez *et al.*, 2012; Riou *et al.*, 2013).

Despite a generally robust HIV-specific T-cell response during infection, which has the ability to significantly reduce viremia during the chronic stages of infection, the human immune response to HIV is generally unable to completely eradicate or suppress HIV or halt CD4 decline over time. This indicates that other host and viral factors may facilitate incomplete suppression and ultimately loss of control. Immune activation of T-cells has been demonstrated to play an important role in ongoing CD4⁺ T-cell loss, even in individuals controlling their viral loads.

1.2.2 Antiretroviral therapy (ART)

Morbidity and mortality associated with HIV / AIDS has been curbed with the advent of ARTs. The health benefits of ARTs is well documented, with different ART drug classes targeting various stages of the HIV life cycle, from binding and infection of target cells, to the budding of newly formed virions (Hogg *et al.*, 2001; Kitahata *et al.*, 2009; Zolopa *et al.*, 2009). The public health benefits of ART are not limited to HIV+ individuals. Studies have shown that the drastic decline in HIV genital shedding in HIV+ individuals on ART translated to a significant decreased risk of heterosexual HIV acquisition in their uninfected partners (Attia *et al.*, 2009; Donnell *et al.*, 2010; Cohen *et al.*, 2012). The use of ART by HIV+ women during pregnancy and breast feeding has considerably diminished the incidence of paediatric HIV infection resulting through mother-to-child transmission (MTCT) (Tonwe-Gold *et al.*, 2007; Kumwenda *et al.*, 2008).

The World Health Organisation (WHO) has been supplying guidelines on various aspects of HIV diagnosis, treatment and care since 2002 and strongly recommends the administration of ART in HIV+ persons (Table 1.1). A classification system for staging of HIV disease and AIDS for HIV+ adults and adolescent that has been defined by the Centre for Disease Control and prevention (CDC) and adopted by the WHO. Both the ARV guidelines and AIDS staging rely on specific biomarkers or predictors of HIV disease (CD4 count and plasma viral load) outcome that are necessary for administration of the right treatment at the right time.

Table 1. 1 Eligibility criteria for starting ART regimen for adults and adolescents. Guidelines recommended by WHO. Source: The South African Antiretroviral Treatment Guidelines, 2013.

Eligible to start Lifelong ART
<ul style="list-style-type: none"> ▪ CD4 count ≤ 350 cells/mm³ irrespective of WHO clinical stage <p style="text-align: center;">OR</p> <ul style="list-style-type: none"> ▪ Irrespective of CD4 count <ul style="list-style-type: none"> ○ All types of TB (In patients with TB drug resistant or sensitive, including extra pulmonary TB) ▪ WHO stage 3 or 4 irrespective of CD4 count
Require fast track (i.e. ART initiation within 7 days of being eligible)
<ul style="list-style-type: none"> ▪ HIV positive women who are pregnant or breast feeding <p style="text-align: center;">OR</p> <ul style="list-style-type: none"> ▪ Patients with low CD4 <200 <p style="text-align: center;">OR</p> <ul style="list-style-type: none"> ▪ Patients with Stage 4, irrespective of CD4 count <p style="text-align: center;">OR</p> <ul style="list-style-type: none"> ▪ Patients with <u>TB/HIV co morbidity with CD4 count < 50</u> (Patients with Cryptococcus meningitis or TB meningitis (defer ART for 4-6 weeks)
Patients with CD4 above 350, Not yet eligible for ART
<ul style="list-style-type: none"> ▪ Transfer to a wellness programme for regular follow-up and repeat CD4 testing 6-monthly. ▪ Advise on how to avoid HIV transmission to sexual partners and children ▪ Initiate INH prophylaxis if asymptomatic for TB ▪ Provide counselling on nutrition and contraception and do annual pap smear

1.3 Immune activation and HIV pathogenesis

Recruitment of activated immune cells to control or eliminate an infection is a key feature of an efficient host immune response. During a T-cell response, priming through cytokine signalling or antigen recognition in association with HLA molecules leads to cellular functions such as cytotoxic, proliferative, secretory, differentiation, or regulatory being ‘switched on’. During HIV infection, the molecular mechanisms causing immune activation are still incompletely understood but are likely to be multifactorial. During an immune response to HIV infection, almost all components of innate and adaptive immune system become activated (including T cells, B cells, NK cells, macrophages and monocytes, Lane *et al.*, 1983; Giorgi *et al.*, 1999; Deeks *et al.*, 2004; Fauci *et al.*, 2005; Brenchley *et al.*, 2006; Catalfamo *et al.*, 2011; van Grevenynghe *et al.*, 2012).

Infection with HIV leads to significant changes in the level of activation of lymphocytes as measured by activation markers such as CD25, CD38, CD69, CD71, CD95 and HLA-DR (Imlach *et al.*, 2001; Mueller *et al.*, 2001; Li *et al.*, 2012). The expression of CD38 and HLA-DR on CD4⁺ and CD8⁺ T-cells has been shown to increase dramatically during chronic infection. Kestens *et al.* (1994) showed that changes in expression of HLA-DR and CD38 on CD4⁺ T-cells correlated with similar changes on CD8⁺ T-cells and as well as with the presence of HIV antigen in systemic circulation and disease stage.

1.3.1 Immune activation in natural hosts challenges the ‘classical’ model of HIV pathogenesis

There is compelling evidence from both human and macaque studies that markers of immune activation and T-cell proliferation are elevated in blood and gastrointestinal tract during HIV and SIV infections, and that these strongly predict with the rate of disease progression (d’Ettorre *et al.*, 2011). Although the course of SIV infection in rhesus macaques is generally similar to the natural history of HIV infection in humans, macaques are not the natural primate hosts for SIV with Sooty mangabeys and African green monkeys recently being used as representative natural host primate models for SIV infection (Chahroudi *et al.*, 2012). Sooty mangabeys and African green monkeys can be chronically infected with SIV without developing severe disease, have high viral loads throughout the course of infection but do not

experience persistent immune activation or CD4 decline (Silvestri *et al.*, 2007). Primary SIV infection in these natural hosts was associated with a modest and transient decline of systemic blood CD4 count, which was accompanied by drastic depletion of CD4 cells in the gut associated lymphatic tissues (GALT) and lungs (Gordon *et al.*, 2007; Pandrea *et al.*, 2007). During chronic infection however, the level of immune activation was persistently low in spite of high SIV loads in blood (Figure 1.5).

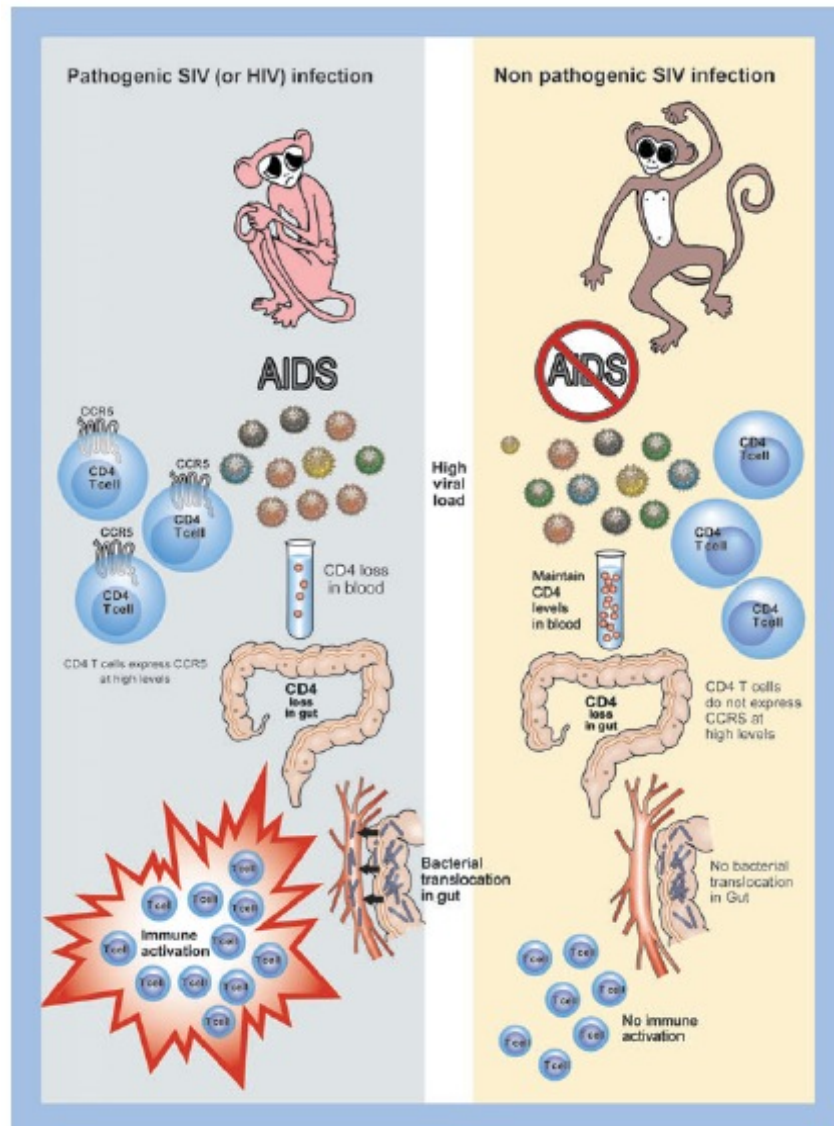


Figure 1. 5 Comparison of SIV infection in non-natural (left panel) and natural host (right panel). SIV infection in the non-natural host is characterised by high viral load, a progressive depletion of CD4⁺ T-cells in blood and mucosal compartments, microbial translocation from the GALT and chronic immune activation. In contrast, SIV infection of natural host is non-pathogenic associated with a stable CD4⁺ T-cell count and absence of immunodeficiency, chronic immune activation and bacterial translocation in spite of high viremia and depletion of mucosal CD4⁺ T-cells (taken from Silvestri, 2009).

The course of SIV infection of the natural primate hosts differs significantly to the clinical course of HIV infection in most humans, who almost invariably progress to AIDS if left untreated. A similar phenomenon of decreased or lack of pathogenicity in humans could be drawn from a rare group of HIV-infected individuals, termed long-term non-progressors (LTNPs), who typically maintain stable levels of systemic CD4⁺ T-cells. These LTNPs similarly also exhibit lower levels of immune activation and microbial translocation than individuals who progress normally during HIV infection (Silvestri *et al.*, 2007). In contrast to high levels of viremia observed in SIV-infected natural hosts, LTNPs are typically able to control virus replication. Some studies have ascribed better control of viral replication in LTNPs to strong and broad immune responses to the HIV, while the natural hosts generally show only low levels of SIV-specific immune responses (Dunham *et al.*, 2006). Whilst the absence of hyperimmune activation in LTNPs can be explained by lower viral loads (resulting in lower concentrations of antigen), the reason for only minimal immune activation in the highly viremic SIV-infected natural hosts is unclear. Table 1.2 summarizes the events unfolding during chronic HIV infection in “normal” human progressors, LTNPs, and SIV infection in natural hosts like SM and AGM.

Table 1. 2 Similarities and differences between HIV-infected Progressors, LTNPs and natural SIV hosts

	Progressors	LTNPs	Natural Hosts
AIDS	Yes	No	No
CD4 Depletion in blood	Yes	No	No
CD4 Depletion in MALT	Yes	Yes	Yes
Viral load	High	Low	High
Microbial translocation	High	Low	Low
Immune activation	High	Low	Low
CCR5 on CD4⁺ T-cell	High	High	Low

1.3.2 Causes of systemic immune activation during HIV infection

The establishment of chronic immune activation during HIV and SIV infection involves both direct and indirect mechanisms. Direct antigenic stimulation by the virus represents the most common cause of T-cell activation (Catalfamo *et al.*, 2008). Approximately 20% of circulating CD8⁺ T-cell and ~3% of CD4⁺ T-cells recognize HIV antigens (Betts *et al.*, 2001; Papagno *et al.*, 2002). Although these T-cells are generally protective during disease course

(Section 1.2.2), they can conversely also be harmful to the host because these cells become chronically activated in the presence of chronic antigen stimulation during the course of HIV infection (Sauce and Appay, 2008). Another mechanism of direct HIV-mediated immune activation comes from interaction of HIV proteins (such as gp120 and Nef) with T-cells. Several studies have shown that the envelope glycoprotein gp120 can either directly activate cells or enhance their responsiveness to activation through binding to CD4 and/or CCR5 (Merrill *et al.*, 1989; Lee *et al.*, 2003; Sailaja *et al.*, 2007). HIV accessory protein Nef induces direct lymphocyte activation (Wang *et al.*, 2000; Simmons *et al.*, 2001) or through the infection of macrophages (Swingler *et al.*, 1999). Expression of Nef in primary macrophages, in an adenovirus recombinant system, was enough to induce expression of macrophage inflammatory-1 alpha and beta (MIP-1 α and MIP-1 β). Schindler and collaborators (2006) reported that differences in Nef function within humans during HIV infection and in pathogenic SIV infections in macaques partly accounted for much higher level of T-cell activation during HIV and pathogenic SIV infection when compared to non-pathogenic SIV infections.

Indirect antigenic stimulation from sources other than HIV, such as opportunistic infections with Epstein-Barr virus (EBV) and Cytomegalovirus (CMV), which may reactivate as a result of HIV-induced immunosuppression, may also induce immune activation. Reactivation of CMV is known to occur recurrently, even in healthy HIV-uninfected individuals (Dunn *et al.*, 2002). Significant activation of both EBV- and CMV-specific CD8⁺ T-cells has been reported during HIV infection (Doisne *et al.*, 2004; Papagno *et al.*, 2004). Another indirect mechanism by which HIV can enhance immune activation is through the depletion of regulatory CD4⁺ T-cells during the course of chronic infection, as these regulatory cells are normally involved with suppression of T-cell activation and limiting inflammatory damage to tissues (Eggena *et al.*, 2005). It has been postulated that the persistent activation of the innate arm of the immune system may give rise to unrestricted production of inflammatory cytokines, which in turn activate T-cells in a non-specific manner (Matsuyama *et al.*, 1991).

1.3.3 Consequences of systemic immune activation

Irrespective of the causes of immune activation during HIV infection, it results in elevated T-cell turnover that have increased expression of the nuclear antigen Ki67 (only expressed by cycling cells), the early activation marker CD38; and the late activation marker HLA-DR

(McCune *et al.*, 2000; Hazenberg *et al.*, 2000; Lempicki *et al.*, 2000; Kovacs *et al.*, 2001; Ho *et al.*, 1993; Mahalingam *et al.*, 1993; Kestens *et al.*, 1994; Ausiello *et al.*, 1996; Orendi *et al.*, 1998; Savarino *et al.*, 2000). Measuring CD38 expression by T-cells has been used widely to assess the extent of immune activation by T-cells, but the prognostic value of this marker is improved when it is measured together with HLA-DR (Benito *et al.*, 2004).

Although blood CD4⁺ T-cell counts and viral loads are considered to be the gold standard prognostic tool for predicting rate of HIV disease progression, several studies have shown that immune hyperactivation - measured by dual expression of CD38 and HLA-DR - allows better prediction of HIV pathogenesis than either of these markers alone (Fahey *et al.*, 1998; Lawn *et al.*, 2001; Karim *et al.*, 2013). In support of this, Wilson *et al.* (2004) showed that CD8⁺CD38⁺ T-cells but not HIV load predict CD4⁺ T-cell depletion. Moreover, results from a study by Froebel and collaborators (2000) have shown that measurement of CD8⁺CD38⁺ T-cell frequencies performed better than CD4 counts for predicting HIV clinical progression. In a study in HIV+ individuals from Uganda, the extent of CD8⁺ T-cell activation prior to initiation of ART predicted lower CD4 recovery rate following the first year of therapy, independently of CD4 counts at the time of initiating ART, demonstrating the predictive value of T-cell activation in relation to CD4 reconstitution after therapy initiation. This finding confirmed that factors other than the virus itself, contribute to the ongoing stimulation of the immune system (Hunt *et al.*, 2011).

Immune hyperactivation has been directly or indirectly linked to disruption of the immune system during HIV infection. One of the unresolved paradoxes of HIV pathogenesis is the dichotomy between the extent of activation of CD4⁺ and CD8⁺ T-cell subsets. Widespread activation of T-cells during HIV infection continually depletes both the naïve and resting memory CD4⁺ T-cell pools, disrupting T-cell homeostasis and subsequently resulting in CD4⁺ T-cell depletion (Hazenberg *et al.*, 2003; Li *et al.*, 2005; Mattapallil *et al.*, 2005). In contrast, an expansion of the CD8⁺ T-cell subset occurs in HIV-infected humans (Suntharalingam *et al.*, 2006) and in mouse-models of retroviral infection (Tesselaar *et al.*, 2003). Although the fact that HIV directly infects and replicates in CD4⁺ T-cells may explain this, these findings may suggest that CD4⁺ T-cell homeostasis is more susceptible to chronic generalised immune activation than that of CD8⁺ T-cells.

Chronic immune activation of CD4⁺ T-cells also facilitates direct HIV infection by multiple mechanisms, hence fuelling further viral replication during chronic infection. Firstly, immune

activation promotes the expression of coreceptor CCR5 on HIV target cells, leading to the increased infectiousness of both T-cells and macrophages bearing this receptor (Bleul *et al.*, 1997; Wu *et al.*, 1997). Elevated trafficking of T-cells to lymphoid tissues through upregulation of adhesion molecules and chemokine production also facilitates enhanced infectivity (Shattock *et al.*, 1996). Secondly, T-cell activation causes an increase in intracellular nuclear factor kappa beta (NFκβ) expression, which in turn results in enhanced transcription of integrated HIV, thus turning activated T-cells into viral ‘superproducers’, promoting expression of HIV from latently infected cells (Kawakami *et al.*, 1988; Bellas *et al.*, 1993; Wu *et al.*, 1995). In spite of the massive depletion of CD4⁺CCR5⁺ T-cells during the acute phase of HIV infection, the aforementioned mechanisms lead to the continued recruitment of new activated T-cells to lymphoid compartments and maintenance of viral replication during the chronic phase of infection (Grossman *et al.*, 2006). This gives rise to a vicious cycle of persistent viral replication causing continued T-cell activation which in turn promotes further CD4⁺ T-cell loss through both direct viral infection and apoptosis (Figure 1.6).

T-cell activation can also impair the regenerative potential of the immune system. Several studies have shown that continued immune activation causes progressive collagen deposition and destruction of lymph node architecture (Schacker *et al.*, 2002, 2006; Estes *et al.*, 2008; Estes, 2009). Since lymph node architecture is critical for supporting normal T-cell homeostasis via interactions with antigen presenting cells (APC), the gradual depletion of normal architecture implies the shortening of resting naïve and memory T-cell half life, limiting CD4⁺ T-cell recovery after initiation of ART (Schacker *et al.*, 2005; Zeng *et al.*, 2012).

1.3.4 Effect of ART on systemic immune activation

Recently, several studies found that HIV⁺ individuals, who were successfully controlling viremia either as the result of ART or naturally, still experienced a persistent systemic inflammation and chronic immunodeficiency, which promoted continuing loss of CD4⁺ T-cells and disease progression despite controlled viremia (Hazenbergh *et al.*, 2003; Appay *et al.*, 2008). Even when ART was initiated during acute HIV infection, markers of immune activation (as measured by frequencies of CD8⁺CD38⁺HLA-DR⁺ T-cells in blood) remained substantially higher than seronegative controls (HIV⁺ individuals frequencies of

CD8⁺CD38⁺HLA-DR⁺ T-cells decrease from 72.6% to 15.6% by 96 weeks following ART initiation compared to 8.9% in HIV- women; Vinikoor *et al.*, 2013). In the SMART study, a randomised trial comparing alternate ART strategies (El-Sadr *et al.*, 2006), markers of inflammation such as interleukin-6 (IL-6) and D-dimer (involved in blood coagulation), were shown to independently predict mortality from non-HIV conditions such as cardiovascular disease in HIV+ individuals in whom viremia was controlled.

In another report from the FRAM cohort, even HIV+ individuals with a CD4⁺ T-cell counts >350 cells/mm³ and doing clinically well had higher mortality rates than the uninfected controls (Modrich *et al.*, 2010). The findings of Vinikoor *et al.* (2013) that HIV-infected individuals still have substantially greater levels of systemic activation compared to seronegative controls even long after initiating ART suggest that factors other than CD4 counts alone (such as inflammation and systemic immune activation) may impact on HIV-related morbidity and mortality. Although ART initiation substantially restores CD4⁺ T-cell numbers and reduces circulating viral loads to undetectable, ART has been shown to have less profound an impact on immune activation levels. This persistent immune activation takes a variety of forms and a better understanding of the causes and pathways involved in immune activation during HIV will be of value for the design of therapeutic strategies that could lead to a more quiescent immune system.

The next section of this review looks at systemic inflammation in the context of HIV infection in more depth.

1.3.5 Cytokines in HIV pathogenesis

During chronic HIV infection, homeostasis to restore T-cell numbers and virally-induced inflammation differentially impact on CD4⁺ and CD8⁺ T-cell immune activation. This difference in T-cell subset activation is reflected in the ways that these subsets respond to the homeostatic and inflammatory environments. Cytokines are generally classed according to the primary function they fulfil in the immune system. Historically, CD4⁺ T-cells were classified as Thelper 1 (Th1) or Th2 type, depending on the specific classes of cytokines CD4 T-cells produced (Mosmann and Coffman, 1986). Using this historical definition, Th1 cells tend to produce pro-inflammatory cytokines and stimulate cellular immunity responsible for the elimination of intracellular parasites and the perpetuation of autoimmune diseases. Th2,

on the other hand, tend to stimulate humoral immune responses, upregulating antibody production and balancing out Th1 responses through anti-inflammatory mechanisms. Overactivation of either Th pathway has been shown to cause disease, and both are able to down-regulate the activity of the other (Del Prete, 1998; Desmedt *et al.*, 1998; Romagnani, 1999; Kidd, 2003). These broad definitions have subsequently been shown to completely oversimplify a very complex network of cytokines, which includes much more diversity in helper responses than captured by these two classes alone (T regulatory cells produce IL-10, Th17 cells produce IL-17 and IL-22).

Several cytokines have been shown to play important roles on HIV replication and the ensuing pathogenesis. Therefore, it is important to measure cytokines in the context of HIV infection and immune activation as an additional means to evaluate the host immune response. The following sections discuss a subset of these cytokines, considered to be most important to this dissertation and their effects on HIV pathogenesis and disease progression in more depth. Table 1.3 summarizes some of the cytokines important to this study.

Inflammation is the immediate reaction of the immune system in response to infection and acute injury. It involves an intricate cascade of events which gives rise to migration and activation of various immune cells and release of soluble pro-inflammatory cytokines in recognition of a threat. Host genes involved in inflammatory pathways are generally switched on by cellular protein $\text{NF}\kappa\beta$, leading to newly activated macrophages to then start releasing pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6 and tumour necrosis factor-alpha (TNF- α).

Table 1. 3 Overview of the characteristics of the cytokines important to this study

Class	Cytokine	Cellular Source	Main function	Targets	Reference
Inflammatory	IL- β	Macrophages, monocytes, fibroblast, DC	Enhances inflammation, activation, fever, synthesis of acute phase proteins	Lymphocytes, endothelial cells, CNS, liver	Dunn <i>et al.</i> , 2001
	IL-6	T-cells, macrophages, epithelial cells	Involved in mediation of inflammation, activation, proliferation, apoptosis and differentiation	B-cells, liver	Shah <i>et al.</i> , 2010
	IL-12p70	Phagocytes, DC	Induction of T-cells and NK cells cytotoxicity	T-cells, NK cells	Trinchieri, 2003
	TNF- α	Monocytes, macrophages, and NK cells	Activation of NF κ β , T-cell apoptosis, fever, increases permeability and stimulates adhesion molecules	Endothelial cells, neutrophils, liver	Alfonso and Poli, 2005
Regulatory	IL-10	T-cells, DC, macrophages	Inhibition of pro-inflammatory responses	Macrophages, DC	Brockman <i>et al.</i> , 2009
Adaptive	IL-2	T-cells	Growth, differentiation and survival of antigen specific CD4 ⁺ and CD8 ⁺ T-cells	T-cells, NK cells, B-cells	Kinter <i>et al.</i> , 2000
	IFN- γ	NK cells, T-cell	Inhibition of viral replication	T-cells, B-cells, macrophages	
Hematopoietic	IL-7	DC, fibroblast, keratinocytes	Homeostasis - survival, proliferation and repertoire diversity of T-cells	CD4 ⁺ T-cells	Fry and Mackall, 2005
	GM-CSF	T-cells, macrophages, endothelial cells, fibroblasts	Monocyte differentiation into macrophages	Monocytes	Hercus <i>et al.</i> , 1994

IL-1 β

Pro-inflammatory cytokines like IL-1 β can activate NF κ B transcription factors in the cytoplasm of host cells, and these activated transcription factors then mediate NF κ B production (Crofford *et al.*, 1997). The role of IL-1 β in the enhancement of HIV replication through the direct activation of NF κ B has been shown (Osborn *et al.*, 1989; Chene *et al.*, 1999). NF κ B is known to regulate viral transcription through two NF κ B sites in the long terminal repeat region of HIV (Nabel and Baltimore, 1987). During HIV infection, IL-1 β plays an important role by increasing the expression of adhesion factors on endothelial cells which enable immune cell transmigration (Dinarello, 1994; Copeland, 2006). IL-1 β has also been shown to synergise with IL-6 to enhance HIV expression of latently infected human promonocytic cell line U1 via distinct molecular pathways, independently of NF κ B (Poli *et al.*, 1994).

HIV gp120 has also been shown to have the ability to induce production of IL-1 β directly and in a CCR5-dependent manner (Cheung *et al.*, 2008). Exogenous IL-1 β has also been shown to enhance AIDS-related Kaposi's sarcoma cell growth (Louie *et al.*, 1995) and potentially contributes to other complications in chronic HIV infection including neurological and haematological disorders (Maury *et al.*, 1990; Weiss *et al.*, 1992). In addition to its ability to directly enhance HIV replication, IL-1 β is regarded as a stimulatory haematopoietic factor, acting as an agent that induces both differentiation and maturation for a variety of cells (Neta *et al.*, 1986; Moore *et al.*, 1987).

IL-6

IL-6 has been shown to have a direct effect on immune cells, inducing their activation, proliferation, apoptosis and differentiation (especially B-cells and Kupffer cells; Nakajima *et al.*, 1989; Breen *et al.*, 1990). A number of studies have reported an increase in the expression of IL-6 in plasma during HIV infection (Breen *et al.*, 1990; Ullum *et al.*, 1996; Bastard *et al.*, 2012). IL-6, either alone or in synergy with TNF- α , has been shown to induce HIV expression in infected monocytic cells (Poli *et al.*, 1990; Spear *et al.*, 2008). Two mechanisms have been proposed for this synergy. Firstly, IL-6 may enhance levels of viral transcription and increase steady state RNA levels. Secondly, IL-6 may increase expression of HIV proteins and reverse transcriptase activity from a post-transcriptional perspective. IL-

6 and its soluble receptor, IL-6R, have also been shown to play a role in the induction of monocytes chemoattractant protein-1 (MCP-1) and recruiting neutrophils, monocytes and lymphocyte (Romano *et al.*, 1997).

TNF- α

TNF- α is one of the best characterized pro-inflammatory cytokines, which is thought to be crucial in the pathogenesis in infections like HIV, as it initiates an inflammatory cascade (Bahia and Silakari, 2010). TNF- α production by monocytes, macrophages and natural killer cells is thought to be triggered by host viral enhancing immune-regulatory responses (Alfano and Poli, 2005). Other pro-inflammatory cytokines, such as IL-6 and IL-8, can be induced by TNF- α , aiding in the upregulation of viral replication (Fernandez-Ortega *et al.*, 2004). TNF- α is regulated by a negative feedback loop, where its own expression stimulates the production of anti-inflammatory cytokine IL-10, which in turn dampens inflammation by inhibiting TNF- α production and activity (Leghmari *et al.*, 2008). The main pathogenic role of TNF- α during HIV infection is via its activation of NF κ B, which stimulates T-cell apoptosis and initiates a cascade of inflammatory responses (Duh *et al.*, 1989; Folks *et al.*, 1989).

IL-12

IL-12 is the main cytokine controlling the switch of uncommitted naïve CD4 T-cells towards Th1 cells and in the activation of natural killer cells (Villinger and Ansari, 2010). IL-12 is known to directly induce T-cell and natural killer cells cytotoxicity, and also promote macrophage ability to produce IFN- γ (Trinchieri, 2003; Egilmez *et al.*, 2011). Yang and colleagues (2010) have highlighted the protective role of IL-12 against various microbial infections, and a decrease in IL-12 concentrations has been associated with increased risk for developing opportunistic infections during HIV infection, and with a concurrent increase in IL-10 concentrations (Taoufik *et al.*, 1997; Mirani *et al.*, 2002). Finally, addition of IL-12 was shown to enhance antigen-specific CD4 and CD8 T-cell responses *ex vivo* (Villinger and Ansari, 2010).

IL-10

The regulatory cytokine IL-10 plays a role in both innate and adaptive immunity, and is produced by T-cells, dendritic cells and macrophages (Brockman *et al.*, 2009; Hedrich and Bream, 2010). IL-10 suppresses many genes that are up-regulated via toll-like receptors in both macrophages and dendritic cells, and inhibits the production and action of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-12, and TNF- α by limiting the major histocompatibility complex (MHC) class II and CD80/86 expression on monocytes and macrophages (Wang *et al.*, 2005; Kwon *et al.*, 2012). IL-10 also promotes proliferation of cytotoxic T-cells (Rowbottom *et al.*, 1999) and activation of B-cells (Trincheri, 2007). IL-10 has been suggested to have differing roles at different stages of HIV disease. During acute HIV infection, IL-10 may promote viral replication through the inhibition of effector immune response from both the innate and adaptive arms of the immune response (Naicker *et al.*, 2010). During the chronic phase of disease, however, IL-10 may have a more protective role, by reducing immune activation, and interfering with viral replication (Naicker *et al.*, 2009). In support of this, IL-10 concentrations systemically have also been shown to correlate negatively with HIV viremia and positively with better CD4⁺ T-cell recovery during chronic HIV infection (Moore *et al.*, 2001; Blackburn and Wherry, 2007).

IL-2

IL-2 is generally produced by T-cells in response to antigen binding to the T-cell receptor and this immune response also stimulates the expression of IL-2 receptors (IL-2R). This IL-2/IL-2R interaction promotes the growth, differentiation and survival of antigen-specific CD4⁺ and CD8⁺ T-cells (Cantrell and Smith, 1984; Smith, 1988) and enhances the production of IFN- γ and TNF- α by these cells (Kinter *et al.*, 2000). In a study comparing elite HIV controllers to chronically infected progressors, the highest levels of protection correlated most strongly with presence of IL-2 expressing CD8⁺ T-cells (Akinsiku *et al.*, 2011). In another study, HIV viremia was associated with the inability to develop functional HIV-specific CD4⁺ T-cells capable of IL-2 production (Younes *et al.*, 2003). However, when IL-2 was tested as a therapy for HIV in HIV-infected individuals, *in vivo* administration induced increased plasma viral loads in those that received IL-2 by stimulating HIV replication in T-cells (Davey *et al.*, 1997). Furthermore, when IL-2 was added *ex vivo* to PBMCs from HIV-infected individuals, it resulted in increased replication of HIV during cell culture (Chun *et al.*, 1998; Al-Harhi *et*

al., 1998). Pertinently, this induction of viral replication was counterbalanced by the concurrent induction of CD8⁺ T-cell mediated HIV suppression (Kinter *et al.*, 1995).

IFN- γ

During HIV infection, IFN- γ acts by inhibiting viral replication (Dhawan *et al.*, 1995; Schroder *et al.*, 2004; Pedroza-Martins *et al.*, 2006). It induces an antiviral state through the initiation of Fas-mediated cell killing and heightened sensitivity to TNF- α (Tsujimoto *et al.*, 1986; Xu *et al.*, 1998). In addition, IFN- γ acts on APCs to enhance their expression of HLA class II, hence enhancing their capacity to present antigens (Li *et al.*, 2011). IFN- γ has also been positively associated with viral replication (Koyanagi *et al.*, 1988; Vingerhoets *et al.*, 1998) and T-cell activation (Whitmire *et al.*, 2005). It is also the most commonly assayed marker in antigen-specific T-cell responses by either flow cytometry or ELISPOT, and is the gold standard measure of efficacy for HIV-specific vaccine or therapeutic trials aimed at eliciting T-cell responses (Reece *et al.*, 2004). Despite its wide use in clinical and vaccine trials, a strong IFN- γ response to vaccination is not a widely useful correlate of protection against HIV infection (Skinner *et al.*, 2003; Elias *et al.*, 2005).

IL-7

During HIV infection, production of the homeostatic cytokine IL-7 is closely linked with HIV-induced lymphopenia and CD4⁺ T-cell depletion (Llano *et al.*, 2001; Napolitano *et al.*, 2001; Albuquerque *et al.*, 2007). IL-7 plays a vital role in the homeostasis of naïve and memory T-cell by regulating survival, proliferation and repertoire diversity (Fry and Mackall, 2005; Surh and Sprent, 2008). IL-7 has been linked to the upregulation of the expression of the death receptor Fas on naïve T-cells (Fluur *et al.*, 2007; Rethi *et al.*, 2008). The depletion of CD4⁺ and CD8⁺ T-cells caused by a combination of alterations in lymphoid tissue mediated homeostasis and IL-7 exposure can be partially reversed by initiation of ART (Zeng *et al.*, 2012). Moreover, due to its potent effect on survival and expansion of T-cells, IL-7 is being targeted as a therapeutic agent for T-cell reconstitution (Snyder *et al.*, 2006; Sportès *et al.*, 2008). IL-7 therapeutic trials in humans have shown that it does facilitate better CD4 reconstitution but also increases plasma viral loads substantially, with the result being that

individuals on IL-7 therapy did clinically worse than those not taking IL-7 (Vandergeeten *et al.*, 2013).

Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF has been shown to induce monocyte differentiation into macrophages, and enhancing the effector function of mature macrophages by increasing their phagocytic activity (Smith *et al.*, 1990). GM-CSF directly enhances HIV replication through interacting with HIV long terminal repeat (LTR) (Watanabe *et al.*, 2002; Osiecki *et al.*, 2005), by down-modulating the anti-viral properties of beta-chemokines (Si *et al.*, 2002) and by inducing proliferation in monocytes and macrophages (Crowe and Lopez, 1997). Studies have demonstrated that GM-CSF, administration in tandem with ART, can be clinically beneficial (Krown *et al.*, 1992).

1.3.6 Causes of systemic cytokine production during HIV infection

The acute stage of HIV infection is characterised by a systemic cytokine storm (Stacey *et al.*, 2009) in which cytokines like IFN- α , IL-15, TNF- α , MCP-1 and inducible protein-10 (IP-10) were shown to be upregulated. Even though the production of these cytokines coincided with peak viremia during acute HIV infection, studies have shown that cytokine concentrations do not return to pre-infection levels following reduction in viremia or during the chronic phase of infection, when viral setpoint has been reached (Stacey *et al.*, 2009; McMichael *et al.*, 2010). Pathogenic SIV infection in macaques, which are not the natural hosts for SIV, is similarly characterised by a cytokine storm identical to the one described in HIV infection (Katsikis *et al.*, 2011). In contrast to studies in macaques, SIV infection in the natural primate hosts for SIV are characterised by either a complete absence or a much more attenuated cytokine storm and generally lower systemic levels of these cytokines (Kornfeld *et al.*, 2005; Jacquelin *et al.*, 2009; Campillo-Gimenez *et al.*, 2010).

Gordon and collaborators (2007) described the massive depletion of memory type CD4⁺ T-cells lining the gastrointestinal mucosa of SIV-infected macaques and HIV-infected humans. They hypothesized that translocation of microbial products across the damaged GI mucosa was a major cause of increased systemic inflammation during HIV pathogenesis. They

proposed that the CD4⁺ T-cell depletion resulted in alterations in gut epithelial integrity, which then facilitates translocation of microbial products such as lipopolysaccharides (LPS), flagellin and CpG DNA across the leaky gastrointestinal mucosa into systemic circulation. Marchetti *et al.* (2013) further suggested that binding of microbial products to Toll-like receptors (TLR) on activated host cells induced the release of pro-inflammatory cytokines which results to further immune activation. Initiation of ART did not reverse the loss of memory CD4⁺ T-cells lining the gastrointestinal mucosa and ‘leaky gut’ has been postulated to remain a significant factor in ongoing activation and inflammation detected during chronic disease even in those individuals on ART (Mehandru *et al.*, 2006).

Several studies have shown that other viral and bacterial coinfections may contribute to systemic inflammation during HIV infection. A weakening immune system related to HIV infection can lead to loss of control of other pathogens in the body. For instance, many HIV+ individuals also harbour infections with other latent viruses such as herpes simplex virus 1 and 2 (HSV-1 and HSV-2), varicella zoster and CMV (Lisco *et al.*, 2009). Studies have suggested that hepatitis C virus (HCV) co-infection of HIV+ individuals may be an important contributor to high levels of systemic inflammation, potentially place these individuals at much higher risk for the development of subsequent liver disease (Bruno *et al.*, 2008; Page *et al.*, 2011; Kushner *et al.*, 2013). Almost all HIV+ individuals are also infected with CMV (Shepp *et al.*, 1996) and these co-infections with CMV and HIV have been implicated in enhanced systemic inflammation associated with premature ageing of the immune system and development of AIDS co-morbidities (Barrett *et al.*, 2012). The South African TB/HIV coinfection rate is higher, with 55% of TB patients also testing positive for HIV, relative to the 33% prevalence globally (CDC, 2011). The immune reconstitution inflammatory syndrome (IRIS) after the initiation of ART for HIV is well documented (Elliot *et al.*, 2009; Pawlowski *et al.*, 2012).

1.3.7 Consequences of systemic cytokine production during HIV infection

The initiation of systemic inflammation and long term persistence of inflammation in blood during HIV infection has extensive and detrimental effects on health of HIV+ individuals. Systemic markers of inflammation have been linked with cardiovascular disease during HIV infection (Friis-Moller *et al.*, 2003; Palella and Phair, 2011; Duprez *et al.*, 2012). In addition, inflammation is thought to cause fibrosis of lymphatic tissue, damaging the architecture of

lymph nodes and disturbing homeostatic balance (Schacker *et al.*, 2002). Pro-inflammatory cytokines have been shown to suppress thymic function, resulting in a decline in thymic output and atrophy of the thymus (Linton and Dorshkind, 2004).

Inflammatory changes in brain tissue are common among HIV+ individuals, and these are associated with the development of a spectrum of motor, cognitive and psychological manifestations including HIV-related dementia (Harezlak *et al.*, 2011). Inflammation and a suppressed immune system contribute to cancer development during HIV infection by causing oxidative stress and DNA damage. A number of large general population studies have linked a variety of cancers during HIV infection to elevated levels of inflammation markers (Borges *et al.*, 2013). Finally, chronic inflammation in the event of HIV infection has been shown to have an effect on mortality itself (Kuller *et al.*, 2008; Boulware *et al.*, 2011).

In summary, HIV infection is characterised by immunodeficiency, which occurs in a setting of chronic immune activation and inflammation, even in those individuals on ART. A decline occurs in the CD4⁺ T-cell pool while CD8⁺ T-cell population expands during the course of HIV infection. This general and persistent cellular activation and inflammation drive the cycle of HIV replication, serving as fuel to HIV pathogenesis (Figure 1.6).

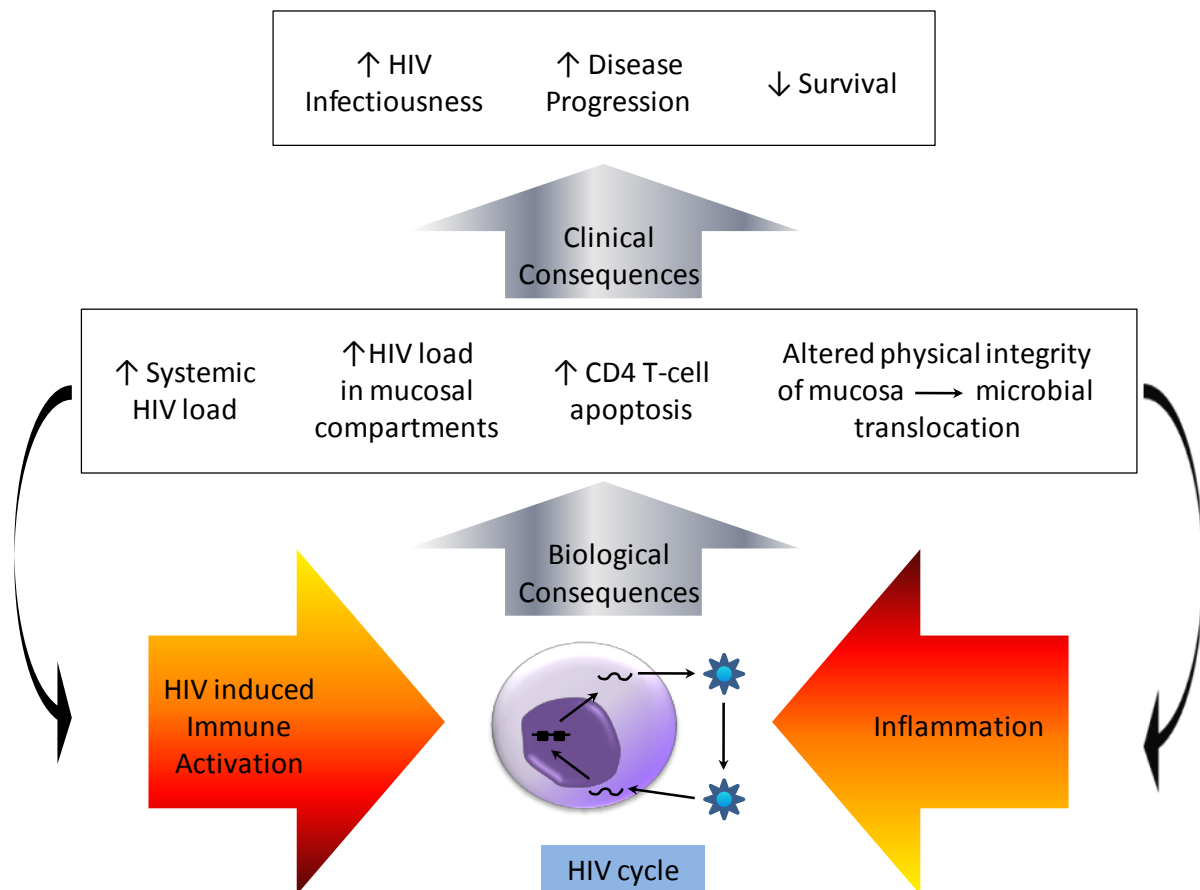


Figure 1.6 Consequences of immune activation and inflammation during chronic HIV infection *in vivo*. The schematic highlights the broad consequences of immune activation on the biology of HIV and on T-cell and associated lymphoid tissues *in vivo* and their subsequent effect on HIV transmission, pathogenesis, and survival in HIV infected individual.

1.4 Markers of HIV disease progression

1.4.1 Classical markers of HIV disease progression: Absolute blood CD4⁺ T-cell counts and HIV viral load

Because CD4⁺ T-helper cells are both the primary targets for HIV infection but also a central component of host immunity to most other infections, absolute blood CD4 counts have become a valuable indicator of the state of immune function in HIV-infected individuals. Together, measurement of absolute CD4 counts and HIV plasma loads in blood are invaluable in the staging and management of HIV infection and are the major criterion used in the WHO/CDC classification of HIV infection (Table 1.1). The WHO guidelines (2013)

now recommend that ART, consisting of a cocktail of three antiretrovirals used in combination, is prescribed for HIV-infected individuals with blood CD4 counts of <500 cells/mm³ irrespective of symptoms and for any individuals with symptoms of AIDS or an AIDS defining illness.

Several studies have compared the prognostic value of plasma HIV RNA level and/or blood CD4 counts as predictors of pathogenesis in individuals infected with HIV (Loveday *et al.*, 1995; Coombs *et al.*, 1996; O'Brien *et al.*, 1996; Welles *et al.*, 1996; O'Brien *et al.*, 1997; Chene *et al.*, 2003; Bonnet *et al.*, 2005; May *et al.*, 2006). Changes in either or both of these markers, even after initiation of ART, have been shown to have predictive value in HIV+ individuals in both early phases of disease (Katzenstein *et al.*, 1996; Langford *et al.*, 2007) and during the advanced stages of disease (Tsoukas *et al.*, 1994). Marschner and collaborators (1998) performed a meta-analysis on data available from seven AIDS Clinical Trials Group (ACTG) studies and reported that both absolute blood CD4 count and HIV plasma loads were important markers of HIV disease progression in individuals with advanced disease, with the combined use of the two markers yielding the strongest prediction power. They did report, however, that these two markers were independently prognostic.

The standard of care to monitor HIV disease progression in most settings are clinical assessment, monitoring blood CD4⁺ T-cell counts (by flow cytometry) and plasma viral loads (by RNA PCR). Although clinical assessment remains the easiest and most cost effective approach (especially in developing countries), it lacks sensitivity in predicting disease stage, and rate of disease progression.

1.4.2 Alternate markers of HIV disease progression

In addition to the classic markers for HIV disease progression, many other novel biomarkers have been studied over the past decade, including those that use cellular, viral, immunological, human leukocyte antigen (HLA) and neural markers to predict HIV disease course. Rather than measurement of absolute CD4 T-cell counts, some studies have evaluated monitoring of specific subsets of CD4 T-cell frequencies as an alternate to absolute counts. Using cox proportional hazards regression model, the significant association between percentage CD4 and disease progression was highlighted in an observational study (Hulgan *et al.*, 2007), while elevated regulatory T-cells were identified as a marker predicting worse

HIV disease prognosis (Nilsson *et al.*, 2006). Three independent cross-sectional studies evaluated host HLA (Fernandes *et al.*, 2003), serum C-reactive protein (CRP, Lau *et al.*, 2006) and soluble toll-like receptors (Heggelund *et al.*, 2004) as alternative biomarkers for monitoring HIV disease progression. In the absence of ART, some studies have documented that more rapid progression from HIV infection to AIDS is strongly associated with certain detrimental HLA types while slower progression is associated with protective HLA types (Kuniholm *et al.*, 2011; Fenandes *et al.*, 2003). Lau *et al.* (2006) demonstrated that the associations between CRP were correlated inversely with CD4 lymphocyte counts and directly with HIV RNA levels. Heggelund and collaborators (2004) reported that individuals with AIDS have lower serum levels of soluble toll-like receptor 2 compared with healthy controls and individuals with less advanced disease. Neural markers such as sphingomyelin have also been shown to predict disease progression, suggested to predict encephalic involvement (Saktor *et al.*, 2004).

Over the past decade, CD8⁺ and CD4⁺ T-cell activation status has been shown to be better biomarkers for predicting progression to AIDS and for more general immunological dysregulation associated with HIV infection than either absolute CD4 counts or plasma HIV loads. Karim *et al.* (2013) reported that coexpression of activation markers CD38 and HLA-DR were strongly predictive of worse HIV disease outcome. The percentage of CD8⁺ T-cells expressing CD38⁺ was shown to predict the development of AIDS, independently of the latest absolute CD4 counts and beta-2-microglobulin levels. Mocroft *et al.* (1997) reported that a 10% increase in frequencies of activated CD8⁺ T-cells (expressing CD38⁺) was associated with a 37% increase in the risk of AIDS. Similarly, Liu *et al.* (1997) compared CD8⁺ T-cell activation with other markers (including CD4 counts, plasma level of neopterin, beta-2-microglobulin, soluble IL-2 receptor, soluble CD8, and TNF- α) and found that elevated CD38 on CD8⁺ T-cells was the most predictive marker of those studied for development of a clinical AIDS diagnosis and death. Deeks *et al.* (2004) defined a immune activation “set point” as stable CD38 expression on both CD4⁺ and CD8⁺ T-cells during early HIV infection and suggested that this activation set point predicted subsequent CD4⁺ T-cell changes, independently of viral load. Sousa *et al.*, (2002) showed a direct causal relationship between immune activation (HLA-DR and CD38) and CD4 depletion in HIV disease and only an indirect relationship of these parameters to the virus replication rate.

Although several surrogate markers to monitor the rate of HIV disease progression have been proposed, validation of these markers has proven difficult for various reasons, with reasons

ranging from low detection limits of putative biomarkers in body fluids and wide variability in different cohorts. Evaluation of these alternative markers for HIV disease progression has enabled key insight into novel on the pathogenesis of HIV/AIDS (Moanna *et al.*, 2005). In summary, these studies showed that: (1) HIV is a key but by no means the only factor predicting disease progression; (2) disruption to the immune system function can be largely attributed to a chronic state of generalised immune activation and associated increased T-cell turnover; and (3) HIV-induced chronic immune activation provides a new alley for the targeting of disease marker and subsequently personalised therapeutic intervention.

1.5 The female genital tract

While the mucosal surfaces of the gastrointestinal, reproductive and respiratory tracts serve as a barrier to the external environment, they are also constantly exposed to the external environment and represent a major portal of entry for numerous invading microbes, which leave them exposed to foreign antigens (Ganz, 2002; Mowat, 2003; Nagler-Anderson, 2006; Shacklett *et al.*, 2009). Therefore, the mucosal immune system faces the delicate balance of safeguarding the body and combating invading pathogens through the secretion of various soluble factors of antimicrobial nature, cytokines, and the modulation of innate and adaptive immune responses involving phagocytic dendritic cells and T-cell responses (Pitman and Blumberg, 2000; Mowat, 2003; Montilla *et al.*, 2004; Shacklett *et al.*, 2009). At the same time, it needs to induce tolerance to the commensal microbes that make up the flora at the surface of these anatomic sites (Shin and Kaul, 2008). In short, the mucosal immunological compartments are unique and consist of both mechanical and cellular interactions which serve as the first line of defence against external elements.

The gastrointestinal and respiratory tracts consist of organised mucosal-associated lymphoid tissue (MALT). At the gut mucosa, this is referred to as the gut associated lymphoid tissues (GALT) and this is where adaptive immune responses take place. In GALT, Peyer's patches and the draining mesenteric lymph nodes provide a platform for the priming of T and B-cells found there, with antigens taken up and redirected from the epithelial lamina propria (Mowat and Viney, 1997; Mowat, 2003). While GALT and bronchus-associated lymphoid tissues (BALT) are characterised by the same form of organisation (with diffuse lymphoid tissues that fulfil the purpose of effector sites), the female genital tract lacks similar organisation,

with scattered populations of immune cells responsible for antigen uptake throughout the compartment (Neutra *et al.*, 1996; Hladik *et al.*, 2007; Haase, 2010). Given that sexual contacts account for the majority of HIV transmission worldwide, knowledge of the anatomy of the female genital tract is essential for the understanding events unfolding at this site prior, during and thereafter transmission (Gouws *et al.*, 2006).

1.5.1 Organisation of the cervicovaginal compartment

The lower genital compartment of women consists of three distinct regions: (1) the vagina and its associated mucosa, (2) the ectocervix and (3) the endocervix (Figure 1.7; Hladik and McElrath, 2008; Kumamoto and Iwasaki, 2012). The endocervix is lined with a single layer of columnar epithelium, while the ectocervix and vagina comprises of a non-keratinised stratified squamous epithelium (Quayle, 2002; Coombs *et al.*, 2003; Hirbod and Broliden, 2007). The presence of a multilayered squamous epithelium in the vagina and ectocervix may confer a greater mechanical protection against invading pathogens compared to the single-layered epithelium of the endocervix (Hladik and Hope, 2009). On the other hand, the larger surface area of the vaginal and ectocervical mucosa may allow greater access to pathogens (Hladik and McElrath, 2008). The transformation zone is an abrupt transition between the ectocervix and endocervix and is characterised by the presence of the largest number of lymphocytes, in particular CD4⁺ T-cells, in the lower genital compartment in women (Edward and Morris, 1985; Pudney *et al.*, 2005; Hladik and McElrath, 2008). A thick mucus plug is also present in this squamocolumnar junction. A comparable amount of dendritic cells and Langerhans cells have been characterised in the endo- and ectocervix, as well as the vaginal opening regions (Pudney *et al.*, 2005). A variety of other immune cells, such as macrophages and granulocytes, have also been identified in the cervicovaginal compartment (White *et al.*, 1997; Nkwanyana *et al.*, 2009). Because of its greater density of CD4⁺ T-cells and CD1a⁺ dendritic cells compared to the vagina, the ectocervical mucosa is considered more vulnerable to HIV transmission (Coombs *et al.*, 2003). High frequencies of CD8⁺ T-cells and APC in the ectocervix and transformation zone suggest the possibility that these sites are the predominant locations for the induction of effector CTL responses in the lower female genital tract (Pudney *et al.*, 2005).

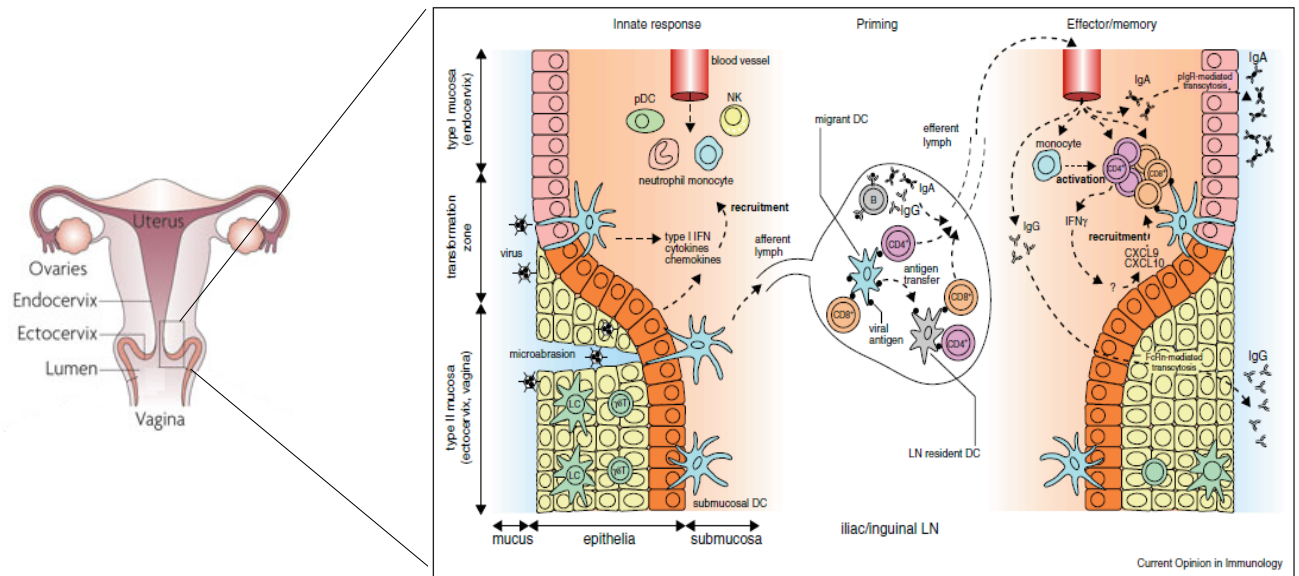


Figure 1. 7 Architecture of the lower female genital tract. The cervicovaginal compartment is made up of the vaginal mucosa, the ectocervix, the transformation zone and the endocervix. Each of these sites is defined by a specific cellular architecture and leukocytes distribution. (Adapted from Hladik and McElrath, 2008 and Kumamoto and Iwasaki, 2012).

1.5.2 Factors affecting susceptibility to heterosexual HIV acquisition

The vagina, ectocervix and endocervix have all been shown to be susceptible to HIV infection, with penetration of the virus having been demonstrated in all three sites (Hladik and Hope, 2009). An intact vaginal epithelium offers the best protection against HIV acquisition ($\approx 99\%$; to date unmatched by any prophylactic strategies tested against transmission) (Royce *et al.*, 1997), acting as a physical barrier to viral penetration (Shattock and Moore, 2003; Miller *et al.*, 2005). In addition, cervical mucus has shown to be effective in trapping HIV-infected seminal cells and free virus (Maher *et al.*, 2005; Miller *et al.*, 2005). Despite these effective barriers, HIV still gains entry across the female genital mucosa via breaches in the barrier.

Disruption in the physical integrity of the mucosal barrier (Haase, 2010), by mechanisms such as micro-trauma possibly during penetrative sex (Figure 1.7), may provide immediate access to sub-mucosal target cells - including T-cells, dendritic cells and Langerhans cells (Prakash *et al.*, 2004; Li *et al.*, 2009; Keele *et al.*, 2011). Sexually transmitted infections (STIs), especially those that cause ulcers or other disruptions in the mucosal barrier, have also been shown to increase risk for HIV infection (Johnson *et al.*, 2008; Kaushic, 2009; Ward

and Ronn, 2010; Petrova *et al.*, 2013). The natural and recurrent sloughing of the multilayered stratified epithelium of the vagina and ectocervix has been suggested to provide some measure of protection, because this process would result in rapid healing of microabrasions in the female genital tract. This process is generally thought to prevent bacterial colonisation and/or viral access to intraepithelial target cells (Quayle, 2002). This has been confirmed in studies of vaginal transmission of SIV in primates where thicker genital epithelium in the genital compartment has been shown to be protective against infection by SIV (Kaizu *et al.*, 2004; Smith *et al.*, 2004). Use of progesterone-based contraceptives, such as Depo-Provera (DMPA), which results in thinning of the genital epithelium, has been suggested to increase susceptibility to HIV infection (Baeten *et al.*, 2005; Stringer and Antonsen, 2008; Mestecky *et al.*, 2009).

Plasma HIV load of the HIV+ individuals has been reported in multiple studies to be a significant predictor of HIV transmission to HIV- individuals, with plasma viral levels of >50 000 cps/ml being associated with transmission and viral loads <1500 cps/ml being associated with a lower risk of transmission (Quinn *et al.*, 2000; Donnell *et al.*, 2010). Furthermore, a number of studies have reported the positive association between plasma HIV load and HIV shedding in both the male and female genital secretions (Hart *et al.*, 1999; Kovacs *et al.*, 2001; Gumbi *et al.*, 2008).

Two main mechanisms have been suggested by which HIV establishes infection *in vivo*: (1) cell-free HIV infection of target cells resident at the genital mucosa including CD4⁺ T-cells, macrophages and DCs; and/or (2) cell-to-cell transfer of HIV from HIV-infected donor cells (Pope and Haase, 2003). During cell-to-cell infection, the transfer of HIV can take place either from one T-cell to another T-cell, from an infected macrophage to a DC or from an infected DC to T-cells (Pope and Haase, 2003; Groot *et al.*, 2008). Resting and activated CD4⁺ T-cells were found to be the predominant targets for SIV and HIV infection in the genital tract (Zhang *et al.*, 1999; Hladik *et al.*, 2007). This was further evidenced by the large scale depletion of mucosal CD4⁺ T-cells during acute HIV infection and maintained systemically during the chronic phase of infection (Veazey *et al.*, 2003; Brenchley *et al.*, 2004). Likewise, the earliest targets of HIV in cervical tissue culture were shown to be memory CD4⁺ T-cells (Gupta *et al.*, 2002). Macrophages have, in contrast, been identified as the predominant target cells in the vaginal lamina propria (Greenhead *et al.*, 2000). The highly phagocytic Langerhans cells are common residents of the genital epithelium and express both CD4 and CCR5 and may thus internalise R5 HIV strain and transfer virus to

CD4⁺ T-cells (Hladik *et al.*, 2007). HIV can also be either endocytosed or transferred to CD4 and CCR5 receptors on DCs, leading to viral-cell membrane fusion and infection. Endocytosed HIV may be degraded or transferred to CD4⁺ T-cells or macrophages (Geijtenbeek *et al.*, 2000).

1.5.3 Characterisation of HIV-specific immune responses in the genital tract

Numerous studies have demonstrated the existence of mucosal T-cell responses in the female genital compartment and semen of HIV-infected individuals (Musey *et al.*, 1997; Kaul *et al.*, 2000; Shacklett *et al.*, 2000; Musey *et al.*, 2003) and it has been suggested that these local T-cell responses may be involved in local control of HIV infection. Activation of lymphocytes through antigen presentation or via cytokines produced by antigen-associated stimulation of the innate immune system induces a multitude of anti-viral responses by the adaptive arm of the immune system (Iwasaki, 2010). The existence of an inflammatory gradient and chemotactic signals call immune cells to migrate from lymph nodes to cervicovaginal effector tissue to exert an anti-viral response. Cervical HIV-specific CD8⁺ T-cells at the genital mucosa have been found to secrete IFN- γ , TNF- α and MIP-1 β (Bere *et al.*, 2013) and be capable of cytolytic effector function in response to HIV antigens in infected women. Studies in HEPS women have implicated HIV-reactive CTLs and genital HIV-specific neutralising antibodies in protection from HIV infection (Kaul *et al.*, 2000; Kulkarni *et al.*, 2003; Alimonti *et al.*, 2006; Hirbod and Broliden, 2007).

It is known that polyfunctional T-cells, capable of secreting multiple cytokines, having cytolytic activity and with high proliferative capacity, predict better HIV disease outcome as it became clear that IFN- γ production alone by CD8⁺ T-cells may not be sufficient for HIV control (Harari *et al.*, 2004, Pantaleo and Koup, 2004). The role for genital tract anti-HIV CTLs in controlling HIV locally therefore needs further investigation. Though detectable in acutely SIV-infected macaques, CTL responses only arise after the peak in viremia had occurred and after a productive infection was established in blood (Reynolds *et al.*, 2005). The majority of HIV-infected individuals, except for ECs and LTNP, would inevitably progress to AIDS even in the presence of anti-HIV CTLs in the absence of ART. Even if CTLs play an important role in the control of HIV, there are other factors associated with the virus itself that may drive HIV pathogenesis, rendering the immune system overwhelmed.

1.5.4 Inflammation and HIV shedding at the female genital mucosa

Many studies have attempted to investigate biological factors that may influence susceptibility to HIV infection. Many of these studies have shown that plasma HIV load is one of the most important predictors of HIV shedding in the genital tract, even among HIV-infected women on ART (Quinn *et al.*, 2000; Sheth *et al.*, 2004; Rebbapragada *et al.*, 2007). Independently of plasma viral load, inflammation in the genital tract has been identified as a central factor influencing both susceptibility to infection and the amount of HIV shed in genital secretions (Spear *et al.*, 2008).

Production of pro-inflammatory cytokines are thought to increase the risk for HIV infection (in HIV- individuals) and transmission (from HIV+ individuals) in the mucosal compartment because these cytokines are involved in immune cell recruitment, activation and differentiation (Wira *et al.*, 2005; Gabay *et al.*, 2006; Dinarello *et al.*, 2009; Nkwanyana *et al.*, 2009). Unlike DCs and Langerhans cells, whose maturation is accompanied by down-regulation of CCR5, monocyte differentiation to macrophages, which is induced by pro-inflammatory cytokines, is accompanied by up-regulation of the HIV co-receptor, CCR5 (Hariharan *et al.*, 1999; Kaufmann *et al.*, 2001; Cunningham *et al.*, 2008). Pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6 and TNF- α induce the expression of transcription factor NF κ B (Poli *et al.*, 1990; Niu *et al.*, 2004). In addition to enhancing the expression of various host cell proteins involved in inflammation (cytokines, growth factors, adhesion molecules and acute phase proteins), NK- κ B also binds to HIV LTR and directly up-regulates HIV replication (Poli *et al.*, 1990; Niu *et al.*, 2004). IL-8 and GM-CSF stimulate HIV replication in macrophages (Lane *et al.*, 2001) while IL-8 and IL-2 induce HIV replication in T-cells (Chun *et al.*, 1998; Lane *et al.*, 2001). Additionally, pro-inflammatory cytokines such as TNF- α may facilitate HIV infection by disrupting tight junctions between genital tract epithelial cells, reducing the integrity of this barrier (Nazli *et al.*, 2010). Pro-inflammatory cytokine production and associated recruitment of CD4⁺ T-cell targets for infection have been shown to be important prerequisites for establishment of a productive systemic SIV infection following vaginal infection (Li *et al.*, 2009; Haase, 2011). Furthermore, inhibition of inflammatory responses using a topically-applied anti-inflammatory agent (glycerol-monolaurate) was found to prevent SIV infection (Li *et al.*, 2009).

Many potential inducers of inflammation exist in the female genital tract. Among the most significant and well characterised are infections with STIs (including HSV-2, syphilis,

gonorrhoea, trichomoniasis and chlamydia) and perturbations in the vaginal microflora involved in bacterial vaginosis, all of which have been associated with upregulation of pro-inflammatory cytokines in the genital compartment (Agrawal *et al.*, 2007; Rebbapragada *et al.*, 2007; Libby *et al.*, 2008). Changes in oestrogen and progesterin concentrations associated with the use of hormonal contraceptive have also been shown to influence inflammation in the genital tract (Hunt *et al.*, 1997; Prakash *et al.*, 2001). Physiological concentrations of oestrogen been shown to stimulate IL-1 β production by endometrial cells, which in turn induces MCP-1 expression (Akoum *et al.*, 2000). Cytological alterations of the cervix occur as a result of hormone changes and are associated with higher cervicovaginal IL-1 β and IL-6 concentrations (Zara *et al.*, 2008). Progesterone-based injectable hormone contraceptives have been associated with increased numbers of inflammatory cells in cervicovaginal fluid, while oral contraceptive use is associated with increased CCR5 expression on CD4⁺ T-cells (Ghanem *et al.*, 2005; Prakash *et al.*, 2001).

Studies of serodiscordant couples have suggested that genital tract inflammation, due to current or prior STIs, is an important contributing risk factor for HIV transmission to the uninfected partner (Quinn *et al.*, 2000; Gray *et al.*, 2001; Freeman *et al.*, 2006; Ward & Ronn, 2010). However, a definitive causal relationship between genital tract inflammation and the frequency of activated targets for HIV replication has yet to be described at the female genital tract as well as the consequences to susceptibility to infection and local HIV pathogenesis. Our current understanding of the relationship between inflammation, immune activation and HIV risk in the genital mucosa largely comes from epidemiological studies where cause and effect are difficult to define, and from non-human primate studies which may imperfectly mirror vaginal transmission in the human female genital tract.

1.6 Study aims and objectives

The overall aims of this study were to investigate the impact of partner HIV status on HIV susceptibility and disease progression in HIV serodiscordant heterosexual couples by measuring systemic immune activation and inflammation, genital tract inflammation and HIV shedding in South African women with HIV seroconcordant or serodiscordant male partners.

Study rationale

Several studies have described biological and behavioural differences that exist between HIV-infected individuals in HIV seroconcordant and discordant relationships, focusing on plasma HIV load, blood CD4 counts and sociobehavioural factors (Freeman *et al.*, 2004; Malamba *et al.*, 2005; Rogers *et al.*, 2005; Kumarasamy *et al.*, 2010). HIV serodiscordant couples account for close to 44% of HIV seroprevalence in Sub-Saharan Africa (Were *et al.*, 2006) and heterosexual transmission of HIV is considered to be the leading cause of new HIV infection in adults from Africa. This study in HIV seroconcordant and serodiscordant couples aims to investigate whether systemic immune activation and inflammation, which are important determinants of HIV pathogenesis, have an impact on disease progression in HIV-infected individuals or protection against HIV acquisition in HIV negative individuals. One of the many biological factors determining HIV transmission efficiency that may play a role in differential spread and disease patterns is genital tract inflammation (Spear *et al.*, 2008). This study aims to further investigate whether differences in HIV status impacts on genital tract inflammation or release of HIV into genital secretions in women with stable HIV seroconcordant or serodiscordant male partners.

Specific Objective 1

To investigate the impact of sexual partner HIV status on classical markers of HIV disease progression (blood CD4 counts and plasma HIV load) in HIV+ individuals in seroconcordant and serodiscordant relationships.

Specific Objective 2

To investigate the impact of partner HIV status on systemic T-cell activation and inflammation in HIV+ individuals in serodiscordant versus seroconcordant relationships; and the value of these alternative markers in predicting rates of HIV disease progression.

Specific Objective 3

To investigate the role of inflammatory and homeostatic cytokines in the female genital tract on HIV shedding in HIV+ women in serodiscordant versus seroconcordant relationships.

Specific Objective 4

To investigate how biological markers of HIV risks (including genital and systemic inflammation and immune activation) differ in HIV negative women at risk of HIV infection (with HIV+ serodiscordant partners) compared to women with HIV- seroconcordant partners.

CHAPTER 2

Impact of partner HIV status on blood CD4 count, HIV load and systemic HIV-specific T-cell responses

2.1 Introduction

Sub-Saharan Africa remains the epicentre of the HIV/AIDS epidemics, accounting for 69% of the global HIV prevalence and 68% of new cases in 2011 (UNAIDS, 2012). A considerable fraction of HIV infections are transmitted between stable couples, especially in countries with mature epidemics (Guthrie *et al.*, 2007; Lingappa *et al.*, 2008). With an estimate of only 62% of HIV-infected individuals in sub-Saharan Africa having access to ART and the yearly 1.8 million new HIV infections in this region, it is likely that a greater number of HIV-infected individuals will be in relationships with serodiscordant partners (UNAIDS, 2012). By the end of 2008, of the 22.4 million HIV positive people living in sub-Saharan Africa (UNAIDS, 2009), approximately 44% of couples were estimated to be HIV serodiscordant (Were *et al.*, 2006).

Studies in HIV serodiscordant couples have provided useful insight into the dynamics of factors that influence risk of HIV acquisition, allowing measurement of HIV incidence per person year, rate of acquisition from one gender to another and per coital act HIV transmission risk (Quinn *et al.*, 2000; Gray *et al.*, 2001; Hollingsworth *et al.*, 2010). Many studies have focused on the biological and behavioural differences that exist between HIV+ individuals in HIV seroconcordant and serodiscordant relationships (Freeman *et al.*, 2004; Malamba *et al.*, 2005; Rogers *et al.*, 2005; Kumurasamy *et al.*, 2010). Studies in HIV seroconcordant and serodiscordant couples can lead to the development of couples-focused HIV prevention interventions that target risky sexual behaviours and control the biological correlates of HIV transmission. Moreover, HIV serodiscordant couples are increasingly seen as a critical target for the evaluation of HIV vaccines and other prevention strategies.

Transmission of HIV at an individual level is dependent on two important factors: (1) the infectiousness of the ‘HIV donor’; and (2) the susceptibility of the uninfected ‘recipient’ (Fox and Fidler, 2010). This depends on the amount of virus within the secretions of the ‘donor’, the frequency and nature of exposure, and finally the concomitant existence of co-factors that have been documented to enhance the efficiency of HIV transmission such as STIs (Nusbaum *et al.*, 2004; Johnson *et al.*, 2008; Ward *et al.*, 2010). The risk of HIV acquisition is estimated to be quite low following a single sexual exposure (Royce *et al.*, 1997; Quinn *et al.*, 2000) and one of the main determinants of this is the level of HIV in the inoculum deposited during sex (Chakraborty *et al.*, 2001; Coombs *et al.*, 2003). It is well documented that HIV loads in

blood significantly predict the amount of virus being shed in genital secretions, although other local factors are likely also important (Hart *et al.*, 1999; Garcia-Bujalance *et al.*, 2004; Vettore *et al.*, 2006; Chan *et al.*, 2008; Cu-Uvin *et al.*, 2010).

Immunosuppression after HIV infection leads to the lysis and depletion of CD4⁺ T-lymphocytes, making CD4 count in blood a reliable indicator of immune function (McCune *et al.*, 2001). Blood CD4 counts have hence been used as a major criterion on which the Centres for Disease Control and Prevention/World Health Organisation (CDC/WHO) classification of HIV infection and AIDS staging for clinical management is based (WHO, 2012). In addition to absolute CD4⁺ T-cell counts, the prognostic significance of HIV RNA loads in plasma as a predictor of clinical progression in infected subjects is well documented (Mellors *et al.*, 1996; Phillips *et al.*, 2001; Badri *et al.*, 2008; Ding *et al.*, 2009). Mellors *et al.* (1997) described the relationship between higher plasma HIV loads and more rapid clinical progression, which lead to plasma HIV load being linked to increased risk of rapid progression, and thus became a widely used indicator for initiation of ART.

While direct cellular killing of infected target cells is an important correlate of protection during HIV-infection, CTLs also secrete a number of different soluble factors (mainly cytokines and chemokines; Cocchi *et al.*, 2000), which have also been implicated either directly or indirectly in protection. It is now known that virus-specific CD8⁺ T-cells with a "polyfunctional" profile, defined by the capacity to secrete multiple cytokines or chemokines, are most competent in controlling viral replication in chronic HIV infection. Some studies have evaluated CD4⁺ and CD8⁺ T-cell HIV-specific polyfunctional responses on exposure to specific HIV immunogen (Lichterfeld *et al.*, 2004; Precopio *et al.*, 2007). These studies showed that long-term enhancement of HIV-specific CD4⁺ T-cells and polyfunctional CD8⁺ T-cell responses are associated with immune control.

Blood HIV-specific CTLs have been shown to secrete the antiviral cytokine IFN- γ and display cytolytic function in response to various HIV epitopes in HIV+ individuals (Fan *et al.*, 1993; McMicheal *et al.*, 2001). Harari *et al.* (2004) described a correlation between IL-2 production by CD4⁺ T-cells and HIV load. The primary function of IL-2 is to trigger T-cell division and also acts as a co-stimulator for resting naïve CD8⁺ T-cells and enhances activation and proliferation of CD8⁺ T-cell, B-cells and natural killer cells (Goldsby *et al.*, 2000; Li *et al.*, 2007). β -chemokines RANTES, MIP-1 α and MIP-1 β have been associated with better control of HIV and slower disease progression in LTNP (Zagury *et al.*, 1998;

Ullum *et al.*, 1998; Xiang *et al.*, 2004). These chemokines have also been shown to suppress HIV activity through competition for the HIV coreceptor CCR5 (Cocchi *et al.*, 2000).

A number of studies have reported that partner status impacts on plasma viral loads in HIV+ individuals (Malamba *et al.*, 2004; Rogers *et al.*, 2005; Kumarasamy *et al.*, 2010), although the consequence of these differences on clinical disease course in individuals has not been thoroughly investigated. This chapter aims to investigate the impact of partner HIV status on (1) the rate of HIV clinical disease progression (measured by plasma viral loads and absolute blood CD4 counts) and (2) systemic HIV-specific T-cell responses in HIV-infected individuals.

2.2 Materials and Methods

2.2.1 Description of study participants

One hundred and ninety five (195) HIV-infected individuals in stable HIV seroconcordant relationships and 142 HIV-infected individuals in HIV serodiscordant relationships were included for this part of the study. These couples in stable long-term relationship with their partners were recruited from the Empilisweni Clinic in Gugulethu, Cape Town. They generally attended the clinic as couples, although interviews regarding demography and sexual behaviours were conducted individually with each participant. A history of symptomatic STIs, genital discharge, ulceration and genital warts was obtained during clinical examination. Absolute blood CD4 cell counts and HIV viral loads were measured for each HIV+ individual at enrolment into the study (baseline), and then at months 12 and 24. All HIV+ individuals were naïve to ART. All aspects of the study were approved by the Research Ethics Committee of the University of Cape Town (UCT REC# 258/2006) and informed written consent was obtained from all individuals before initiation of the study.

2.2.2 Peripheral blood mononuclear cells (PBMCs) collection and processing

Blood (16ml) was collected from each study participant by standard venipuncture into sterile ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK) and processed within 4 hours of collection. PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation using Leucosep® tubes. Ficoll, wash buffer and medium used during PBMC isolation were warmed to room temperature prior to use. Three millilitres of Ficoll-Hypaque density gradient was poured into a 15ml Leucosep® tube fitted with filter disc at its 3ml mark and centrifuged at 2500rpm (1215 x g) for one minute in a bench top centrifuge (Megafuge 1.0, Heraeus Instrument) to allow the Ficoll to move below the disc. Whole anti-coagulated blood was gently poured onto the Leucosep gradient and centrifuged at 2500rpm for 15 minutes. Plasma was aspirated using a disposable Pasteur pipette to within 0.5–1 cm from the PBMCs “disc” or “buffy” layer and was transferred to 1.8 ml cryovials (Greiner Bio-one, Frickenhausen, Germany) and stored at -80°C. The buffy layer enriched with PBMCs was removed using a disposable Pasteur pipette and transferred to a 15ml Sterilin tube. The PBMCs were washed twice in 15ml of 1% FCS PBS (phosphate

buffered saline (PBS, Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% fetal calf serum (FCS, Life Technologies Corporation, Carlsbad, CA, USA)), with centrifugation at 1200rpm (280 x g) for 10 minutes. After the second wash, PBMCs were resuspended in 10ml RPMI1640 (Life Technologies Corporation, Carlsbad, CA, USA) medium supplemented with 5mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mg/ml fungin and 10% FCS. Cells were counted using an automated Guava cell counter.

2.2.3 Counting of PBMCs using the automated Guava cell counter

Ten microlitres of the resuspended PBMCs was added to 190µl Guava Viacount reagent (Guava Technologies, Hayward, CA, USA) and mixed thoroughly using a vortex mixer. Cells were incubated at room temperature for 8 minutes, acquired using the automated Guava cell counter and analysed using the Cytosoft® software.

2.2.4 Ex-vivo stimulation, intracellular cytokine staining and flow cytometry

Fresh PBMCs were used for this part of the study. Peripheral blood mononuclear cells (2 x 10⁶ cells/ml) were investigated for their functional capacity to produce either of IFN-γ, IL-2 or MIP-1β following antigenic stimulation with (i) a single pool of 121 HIV-1 subtype C (Du422) Gag overlapping peptides (final concentration of 1 µg/ml; peptides kindly provided by the NIH AIDS Reagent Repository), (ii) PMA/ionomycin (final concentration 10 µg/ml each; Sigma-Aldrich, St Louis, MO, USA), or (iii) left untreated for 6 hours at 37°C and 5% CO₂. Brefaldin A (10 µg/ml; Sigma-Aldrich, St Louis, MO, USA) was added after the first hour of stimulation. After stimulation, the cells were washed once in 2ml of 10% FCS PBS containing 0.01% NaN₃ for 5 minutes at 1500rpm (437 x g) at room temperature. The pelleted cells were resuspended in the dead volume and stained with APC-labelled anti-CD3 (Becton-Dickinson, San Jose, CA, USA) and PE-Cy5-labelled anti-CD8 (Becton-Dickinson, San Jose, CA, USA) for 30 minutes on ice. Cells were then washed in 2ml of 10% FCS PBS containing 0.01% NaN₃ for 5 minutes at 1500rpm (437 x g) at room temperature followed by fixation and permeabilisation with 0.5ml of BD Cytotfix/Cytoperm (Becton-Dickinson, San Jose, CA, USA) for 20 minutes at room temperature. Cells were washed in 2ml of BD Perm/Wash buffer (Becton-Dickinson, San Jose, CA, USA) by centrifugation at 1500rpm at room temperature for 5 minutes. The supernatant was discarded, the cell pellet resuspended and

stained with Phycoerythrin (PE) labelled anti-IFN- γ and Fluorescein Isothiocyanate (FITC) labelled anti-IL-2 or FITC labelled anti-MIP-1 β for 30 minutes on ice. Finally, cells were washed in 2ml of Perm/Wash buffer and fixed with BD Cell Fix (Becton-Dickinson, San Jose, CA, USA). Cell fluorescence was assessed using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Compensation and analysis of data were done using FlowJo software (Tree Star, Ashland, OR, USA).

2.2.5 Determination of HIV viral load in blood plasma

HIV viral loads were measured in blood plasma using Nuclisens Easyq HIV Version 1.2 (National Health Laboratory Services Diagnostic Virology Laboratory, Groote Schuur Hospital, Cape Town, South Africa). The detection limit of the assay was 50 HIV RNA copies/ml. Participants with blood plasma ≤ 50 HIV RNA copies/ml were considered to be aviremic.

2.2.6 Statistical analysis

Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Comparison of unpaired non-parametric data was done using the Mann-Whitney U test. The Spearman's rank test was applied to test for correlation between non-parametric data. Statistical inferences on binary sets of data were performed using the Fisher's exact test and odds ratios calculated. Statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA). All tests were two-tailed and p-values of ≤ 0.05 were considered significant.

2.3 Results

2.3.1 Clinical and socio-behavioural characteristics

One hundred and ninety-five individuals in chronic HIV seroconcordant relationship and 142 chronically HIV-infected individuals in HIV serodiscordant relationship were included for this study. All individuals were naïve to anti-retroviral therapy at the time of study. The baseline socio-demographic and clinical characteristics of participants included in this Chapter are reported in Table 2.1. In this cohort, women were more likely to be the HIV+ partner in a serodiscordant couple than men, with 77% of HIV+ serodiscordant individuals being women compared to 23% men ($p < 0.0001$ compared to almost equal men:women distribution in HIV seroconcordant couples). The median age of the HIV+ individuals in seroconcordant relationships was 33 years (IQR 28-40) while those in serodiscordant relationships had a median age of 34 (IQR 28-40). There was no significant difference in the ages when comparing individuals in those two groups. There was no difference between HIV seroconcordant and serodiscordant participants in sociodemographic and sexual behavioural parameters like cohabitation, lifetime sexual exposure duration and monthly frequency of sex act. However, HIV+ serodiscordant individuals reported a significantly higher condom usage over the last month than did HIV seroconcordant individuals (75% versus 62%; $p = 0.01$). Clinical manifestations of genital disease as defined by the presence of ulceration and/or discharge over the past 6 months were not different when comparing HIV seroconcordant individuals to serodiscordant individuals. All men in the study were circumcised.

Table 2. 1 Clinical and socio-behavioural characteristics of participants included in the study

Characteristics	n	Concordant	n	Discordant	P-value
n		195		142	
Male [%] †		48		23	P<0.0001
Age [yr; median (IQR)] *	195	33 (28-40)	139	34 (28-40)	1.0
Living together with partner [%] †	194	70	140	62	0.2
Sexual exposure [median years of sex (IQR)] *	194	16 (11-22.)	139	16 (11-24)	0.9
Sex acts in the last month [median (IQR)] *	190	4 (2-10)	131	4 (2-8)	0.4
Condom usage [%] †	191	62	137	75	0.01
Genital ulceration in the last 6 months [%] †	100	13	107	12	0.7
Vaginal discharge in the last 6 months [%] †	100	30	107	29	1.0

† p-value calculated using Fischer's exact test

* p-value calculated using Mann-Whitney U-test

IQR: Interquartile range

2.3.2 Impact of partner HIV status on individual plasma viral loads

The level of HIV in plasma was compared in HIV-infected individuals in seroconcordant and serodiscordant relationships (Figure 2.1). HIV seroconcordant participants had 0.5 Log higher plasma viral load than HIV serodiscordant participants, with a median Log 4.4 (IQR 3.7-5.0) for seroconcordant individuals versus Log 3.9 (IQR 3.3-4.5) for serodiscordant individuals. This 0.5 Log difference was equivalent to a difference of 14700 HIV RNA copies/ml in individuals in seroconcordant versus serodiscordant relationships. The difference in viremia observed between seroconcordant and serodiscordant participants persisted over time (month12 and month24; Table 2.2).

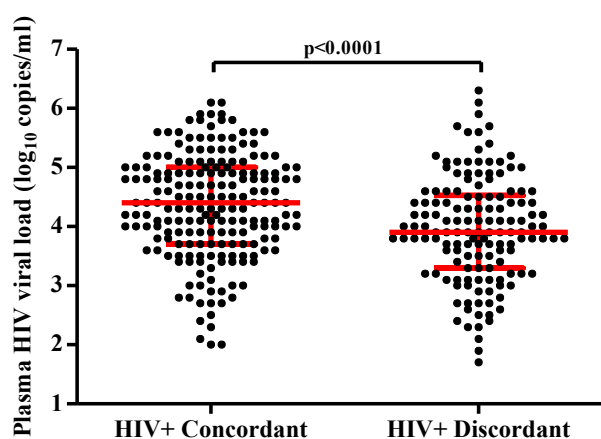


Figure 2. 1 Comparison between plasma HIV viral load in seroconcordant versus serodiscordant HIV+ individuals. Each data point represents an individual's plasma viral load. The red line indicates the median viral load and associated IQR for each group. Mann-Whitney U test was used to compare the plasma viral load between HIV seroconcordant and HIV serodiscordant individuals.

Table 2. 2 Longitudinal plasma HIV load in seroconcordant and serodiscordant individuals

Time point	n	Median (IQR) HIV Plasma Viral Load cps/ml	
		Concordant	Discordant
Baseline	195, 142	23000 (5600-92000)	8300 (2125-35250)
12month	76, 65	21500 (5000-100000)	7900 (2200-35500)
24month	29, 34	25100 (2500-112200)	11200 (1200-27000)

Gender did not impact on this difference in viral load between HIV seroconcordant and serodiscordant individuals, with HIV+ women and men in seroconcordant relationships having similar plasma viral loads, and those in serodiscordant relationships having similar viral loads (Figure 2.2A and B, respectively).

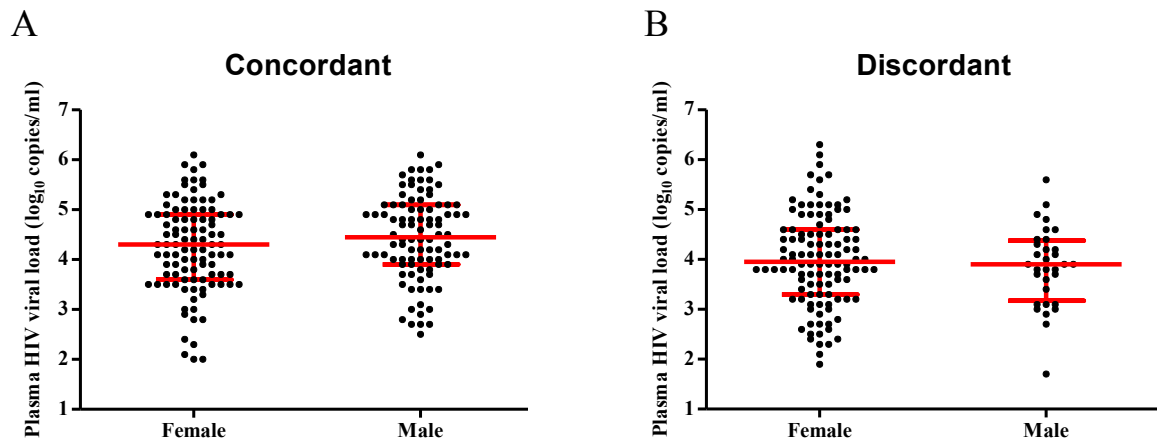


Figure 2. 2 Comparison between plasma HIV viral load in women versus men, in seroconcordant (A) and serodiscordant (B) relationship. Each data point represents an individual’s plasma viral load. The red line indicates the median viral load and associated IQR for each group. Mann-Whitney U test was used to compare the plasma viral load between men and women in HIV seroconcordant or HIV serodiscordant individuals.

To test whether there was evidence for sharing of viruses with similar characteristics (HIV set-point similarities) within HIV+ seroconcordant couples, the relationship between male and female partners’ viral loads was investigated (Figure 2.3). No relationship was found between partners’ plasma HIV load, possibly suggesting that either properties of virus were not influencing viral loads in each partner in a couple (ie, host genetics may have been confounding this analysis) or that the phenotype of the virus was not similar between individuals in a couple.

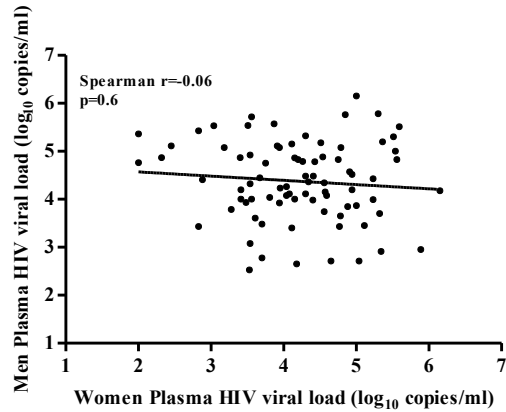


Figure 2. 3 Correlation between seroconcordant male partner HIV plasma viral load and matching female partner HIV plasma viral load. Matching HIV plasma viral loads of partners within HIV seroconcordant couples were correlated using Spearman rank test.

2.3.3 Relationship between HIV plasma viral load and absolute blood CD4 count

In these individuals, plasma viral loads correlated significantly with absolute CD4 counts (Figure 2.4; HIV seroconcordant individuals (Rho=-0.44, p<0.0001) and serodiscordant individuals (Rho=-0.47, p<0.0001).

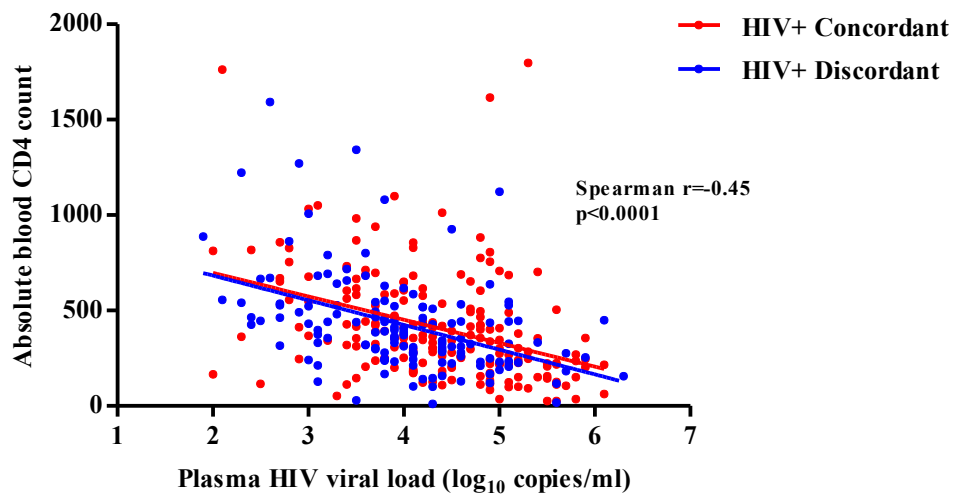


Figure 2. 4 Correlation between HIV plasma viral load and absolute blood CD4 count. Matching HIV plasma viral load and absolute blood CD4 count of HIV seroconcordant and HIV serodiscordant individuals combined were correlated using Spearman rank test.

2.3.4 Impact of partner HIV status on absolute blood CD4 counts during HIV infection

The impact of differences in plasma viral loads in HIV seroconcordant and serodiscordant individuals was investigated on whether there were stellar differences between the absolute CD4 count in the two groups (Figure 2.5). Despite a 0.5 Log difference in viral loads between serodiscordant versus seroconcordant couples (Figure 2.1) and a significant correlation between CD4 counts and plasma viral loads in both groups (Figure 2.4), HIV+ individuals in seroconcordant relationship had a very moderate reduction in median absolute CD4 count compared to their serodiscordant counterparts [354 CD4 cells/ml (IQR 217-561) in seroconcordant versus 387 cells/ml (IQR 250-526) in serodiscordant, Δ 23 CD4 cells/ml; $p=0.3$]. This trend in absolute CD4 counts was maintained over 2 years of study (month12 and month24; Table 2.3). Previous studies in serodiscordant couples have shown variable outcomes with relation to impact of partner status on absolute CD4 count as a clinical marker of disease status. While some have reported no significant difference in absolute CD4 count when comparing participants according to couple status (Malamba *et al.*, 2005; Kumarasamy *et al.*, 2010), others reported lower CD4 counts in concert with higher plasma viral loads in seroconcordant versus serodiscordant couples (Chatterjee Rogers *et al.*, 2005; Kumarasamy *et al.*, 2010).

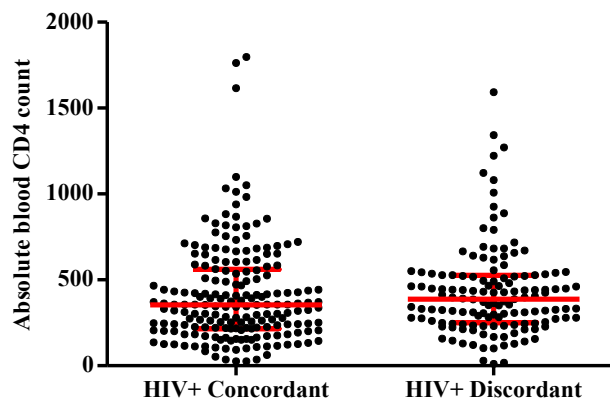


Figure 2. 5 Comparison between absolute CD4 count in seroconcordant versus serodiscordant HIV+ individuals. Each data point represents an individual's absolute CD4 count. The red line indicates the median absolute CD4 count and associated IQR for each group. Mann-Whitney U test was used to compare the absolute CD4 count between HIV seroconcordant and HIV serodiscordant individuals.

Table 2. 3 Longitudinal absolute blood CD4 count in seroconcordant and serodiscordant individuals

Time point	n	Median (IQR) CD4+ T-cell counts (cell/μl)	
		Concordant	Discordant
Baseline	192, 136	354 (217-561)	387 (250-526)
12month	64, 60	352 (238-489)	379 (242-493)
24month	27, 26	275 (208-429)	296 (181-440)

2.3.5 Relationship between changes in HIV plasma load and blood CD4 count

In light of the finding that HIV seroconcordant and serodiscordant individuals have similar absolute blood CD4 counts despite a 0.5 log₁₀ difference in plasma HIV load, the relationship between changes in these two markers and their respective level at baseline were determined. HIV+ individuals were classified according to whether they experienced an increase or a decrease in absolute blood CD4 count or plasma HIV load from baseline visit to month 12 (Table 2.4).

Only 83 individuals had matching data available for these two time points, of which 53 experienced a decrease in CD4 count from baseline to month 12. This decrease in CD4 count was associated with a corresponding increase in HIV plasma burden for the same period. This increase in plasma HIV load accounted for 35% of the CD4⁺ T-cell depletion in blood and was statistically significant in univariate analysis. A high CD4 absolute count in this group of 53 individuals at baseline was highly predictive of the eventual decrease over the one year period ($R^2 = 0.77$, $p < 0.0001$). The same trends were observed when separating the 53 individuals according to their partner's HIV status. None of the other three groups (increased CD4 count, increased HIV plasma load, decreased HIV plasma load) showed any association with either of the two markers. Exploratory analyses by gender, sexual practices, clinical manifestations (ulceration and genital discharge) and stratifying HIV plasma load or CD4 absolute count did not reveal any characteristic resulting in a greater predictive ability to determine fluctuations in either of the two disease markers in peripheral blood. This analysis suggest that HIV+ individuals who experienced the greater CD4 decline over the first year of study were those that started with the highest CD4 counts at baseline; and that viral load was a major contributor to this decline. This analysis also suggests that a possible contributor to the apparent discrepancy between (1) correlation between VL and CD4 counts generally; (2) higher VL in seroconcordant versus serodiscordant individuals; but (3) no difference in CD4⁺

T-cells counts at the same time point or longitudinally may be the fact that individuals enrolled in this study already had relatively low CD4 counts at baseline (354 cells/ml for seroconcordant vs 387 cells/ml for serodiscordant at baseline, Table 2.3).

Table 2. 4 Longitudinal analysis of changes in blood CD4 count and HIV load

	Spearman rank correlation coefficient				
	n	Δ CD4	Δ HIV load	m0 CD4	m0 HIV load
Decreased CD4[†]	53	-	-0.34*	0.77***	-0.19
Increased CD4[‡]	30	-	0	0.12	0.06
Decreased HIV load[†]	38	-0.09	-	-0.08	-0.20
Increased HIV load[‡]	45	0.14	-	-0.03	-0.35*

m0: baseline; m12: month12; Δ CD4: change in CD4 count from m0 to m12; Δ HIV load: change in HIV load from m0 to m12

†: decrease in parameter from m0 to m12; ‡: increase in parameter from m0 to m12

P-values<0.05 are denoted by *, <0.001 by ***

2.3.6 Impact of partner status on magnitude of HIV-specific T-cell responses

The magnitude of *ex vivo* HIV Gag-specific cytokine producing T-cell responses in the blood of HIV+ individuals in seroconcordant and serodiscordant relationships was investigated. Representative plots of the gating strategy used and cytokine (IFN- γ , IL-2, MIP-1 β) production by CD4⁺ and CD8⁺ T-cells isolated from blood are shown in Figure 2.6.

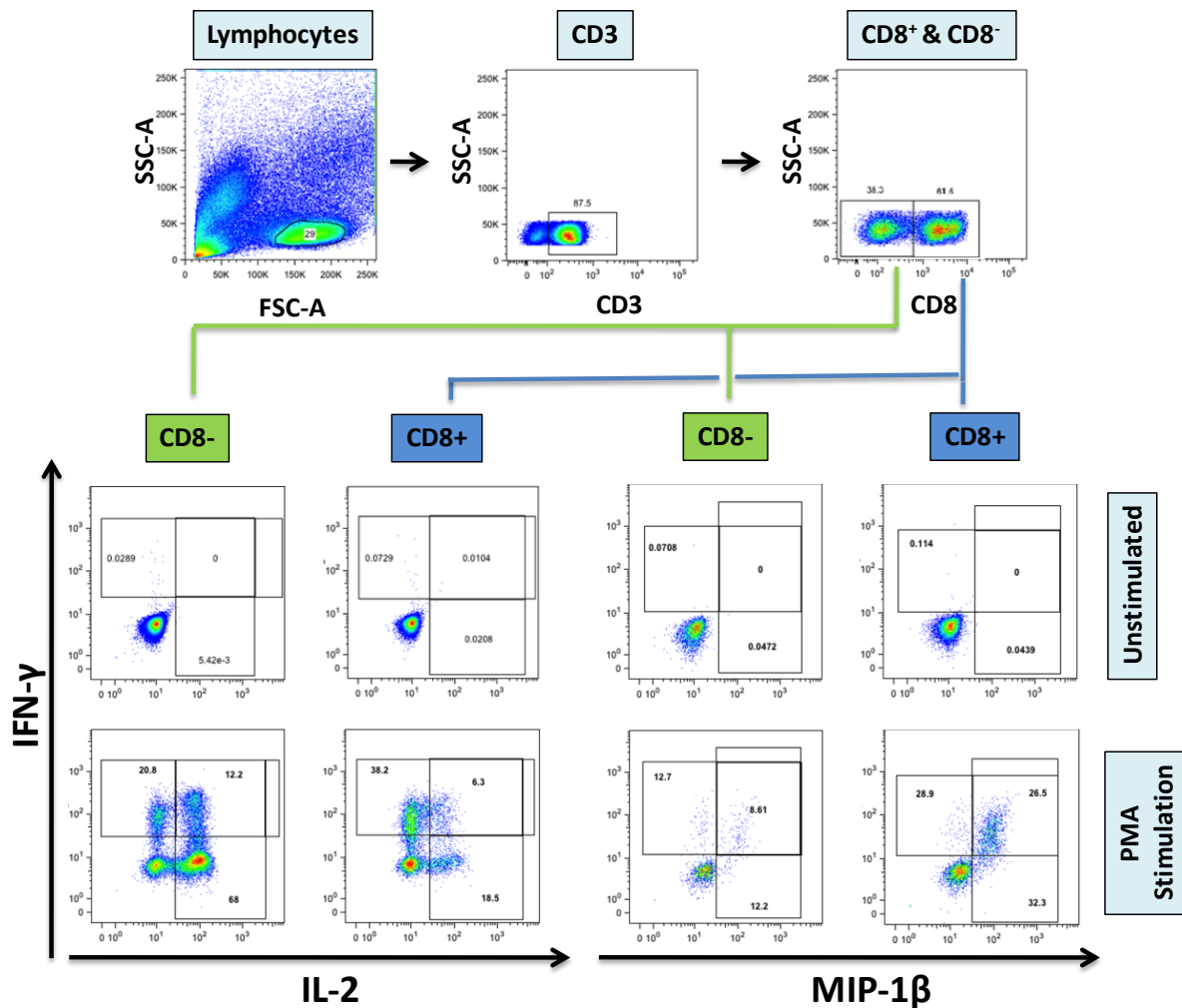


Figure 2. 6 Representative plots showing gating strategy for *ex vivo* intracellular cytokine production by peripheral blood monuclear cells (PBMCs) in absence of, or response to stimulation with PMA/ionomycin. A. PBMCs were stained with fluorochromes-labelled monoclonal antibodies to define CD3 (APC), CD8 (PE-Cy5), IFN- γ (PE) and IL-2 or MIP-1 β (FITC) interchangeably. CD3⁺ T-cell populations were distinguished into CD8⁻ and CD8⁺ T-cell populations. B. Cells were either left unstimulated (top panels) or stimulated *ex vivo* with PMA/ionomycin for 6 hours (bottom panels). Cell fluorescence was measured using an LSRII flow cytometer, gates set according to fluorescence minus-one (FMO) staining, while compensation and analysis was performed using FlowJo software.

Overall, more HIV+ seroconcordant individuals had HIV-specific immune responses (IFN- γ and MIP-1 β) and at higher frequencies than HIV+ serodiscordant individuals. Of the 195 HIV+ seroconcordant individuals, 30/195 had PBMC available and were included in this part of the study. Of these, 21/30 (70%) had IFN- γ responses above background for the CD8⁻ T-cell subset. Of the 142 HIV+ serodiscordant individuals, 27/142 were studied and 11/27 (41%) had IFN- γ responses above background (Fisher's exact test revealed a significant

difference, $p=0.04$). As for the $CD8^+$ T-cell subset, of the 30 HIV seroconcordant individuals, 25/30 (83%) had $IFN-\gamma$ responses above background while only 14/27 (52%) serodiscordant individuals showed $IFN-\gamma$ responses above background (Fisher's exact test revealed a significant difference, $p=0.02$). Similarly, a higher number of HIV+ seroconcordant individuals had Gag-specific $IFN-\gamma$ responses above background than serodiscordant individuals for $CD8^-$ T-cells 23/30 [(77%) compared to 6/27 (23%), respectively, $p=0.02$]. A similar pattern was observed for $CD8^+$ T-cells, although this was not significant. In comparison to $IFN-\gamma$ and $MIP-1\beta$ responses, IL-2 response frequencies were generally lower following Gag-stimulation and no difference in IL-2 responsiveness was observed between serodiscordant or seroconcordant individuals by either $CD8^-$ or $CD8^+$ T-cells.

Figure 2.7 shows the net Gag responses between HIV+ seroconcordant and serodiscordant individuals, showing $CD8^-$ and $CD8^+$ T-cell subsets separately. The frequencies of Gag-specific $IFN-\gamma$ producing T-cells were elevated in HIV+ seroconcordant compared to serodiscordant individuals in both T-cell subsets. Similarly, frequencies of $MIP-1\beta$ producing cells were elevated in HIV+ seroconcordant compared to serodiscordant individuals, significantly so for $CD8^-$ T-cells ($p=0.006$ for $CD8^-$ and $p=0.06$ for $CD8^+$). However, no difference was found when comparing the frequencies of IL-2 production by T-cells between the two groups. The lack of difference in response to PMA stimulation when comparing cytokine production of $CD8^-$ and $CD8^+$ T-cells of seroconcordant individuals to serodiscordant ones suggest that there was no difference on the potential functionality of the T-cell subsets between the two groups.

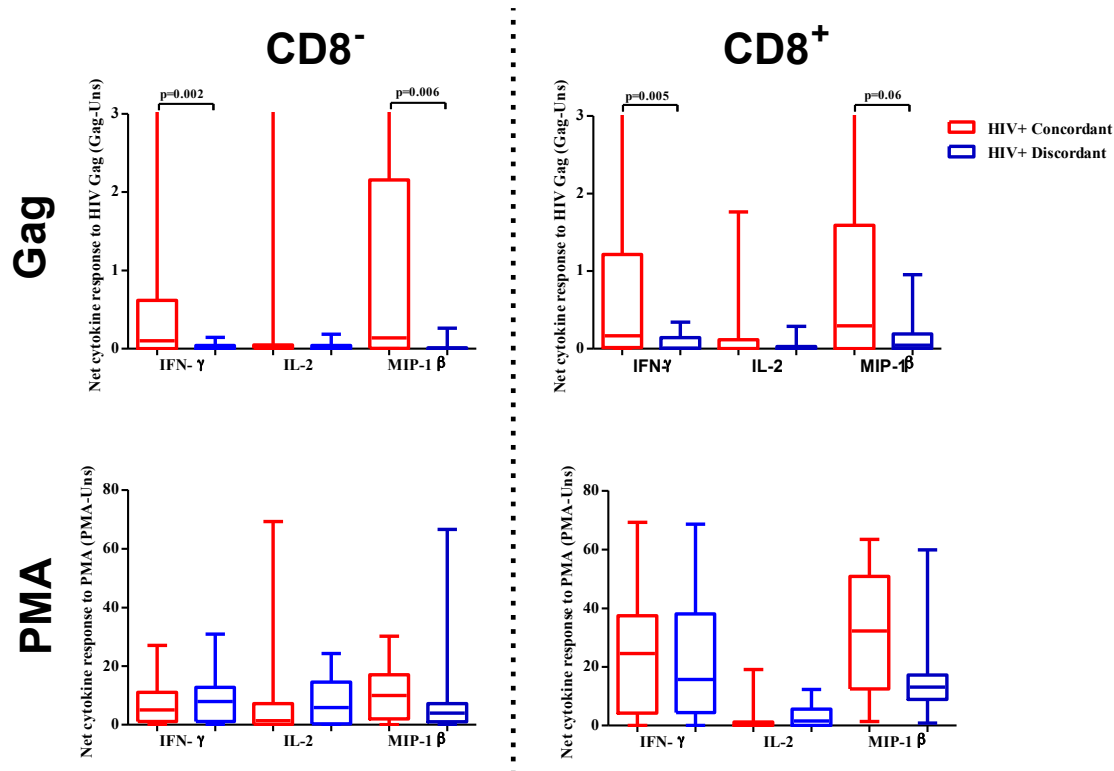


Figure 2. 7 Comparison between *ex vivo* cytokine production by PBMCs from HIV seroconcordant and HIV serodiscordant individuals. Top panel. Frequencies of IFN- γ ⁺CD3⁺, IL-2⁺CD3⁺ and MIP-1 β ⁺CD3⁺ for the CD8⁻ and CD8⁺ T-cells were assessed in response to Gag-stimulation. Background frequencies (measured as unstimulated PBMCs) of these subsets were subtracted from the Gag stimulated specimen. Bottom panel. Frequencies of IFN- γ ⁺CD3⁺, IL-2⁺CD3⁺ and MIP-1 β ⁺CD3⁺ for the CD8⁻ and CD8⁺ T-cells were assessed in response to PMA-stimulation. Background frequencies (measured as unstimulated PBMCs) of these subsets were subtracted from the PMA stimulated specimen.

Others have suggested that polyfunctional CD8⁺ T-cell responses are associated with immune control (Chakrabarti, 2010). When looking at dual production of cytokines (IFN- γ ⁺IL-2⁺ & IFN- γ ⁺MIP-1 β ⁺) no difference was found between concordant and discordant individuals (Figure 2.8).

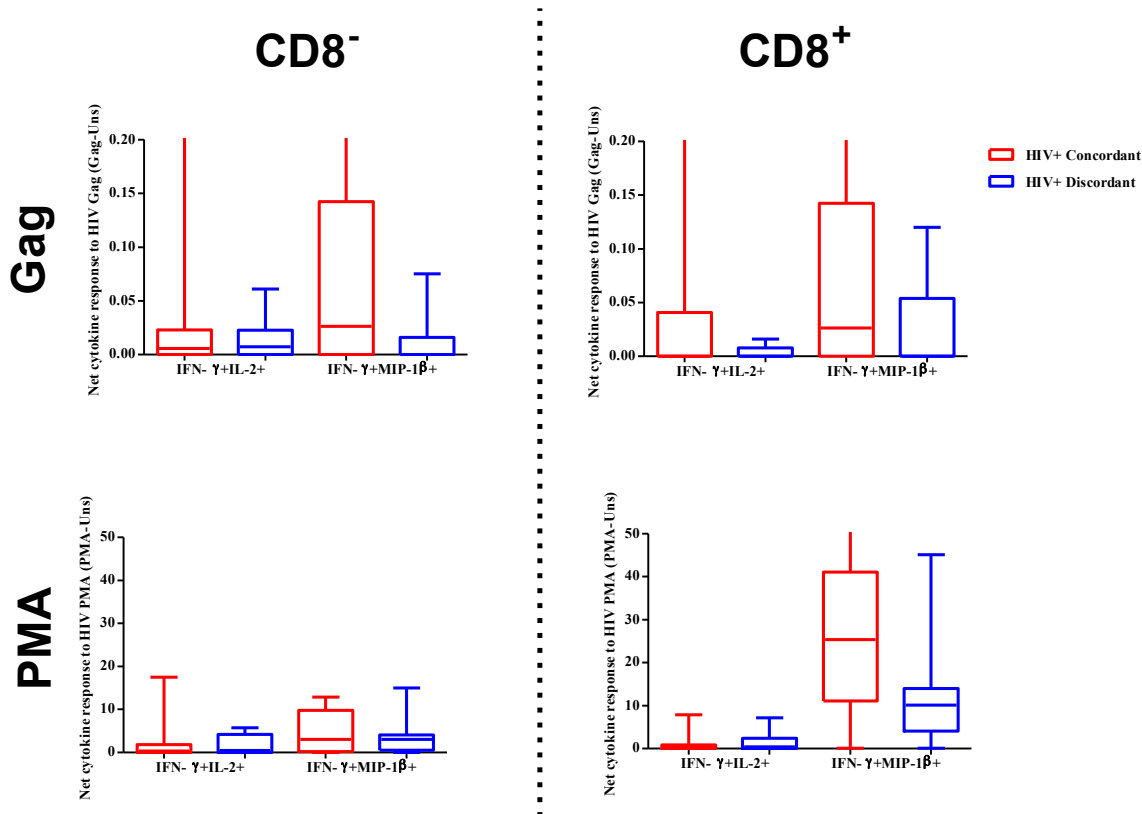


Figure 2. 8 Comparison between *ex vivo* cytokine production by PBMCs from HIV seroconcordant and HIV serodiscordant individuals. Top panel. Frequencies of IFN- γ ⁺IL-2⁺CD3⁺ and IFN- γ ⁺MIP-1 β ⁺CD3⁺ for the CD8⁻ and CD8⁺ T-cells were assessed in response to Gag-stimulation. Background frequencies (measured as unstimulated PBMCs) of these subsets were subtracted from the Gag stimulated specimen. Bottom panel. Frequencies of IFN- γ ⁺IL-2⁺CD3⁺ and IFN- γ ⁺MIP-1 β ⁺CD3⁺ for the CD8⁻ and CD8⁺ T cells were assessed in response to PMA-stimulation. Background frequencies (measured as unstimulated PBMCs) of these subsets were subtracted from the PMA stimulated specimen.

The relationship between the function of HIV-specific CD8⁺ T-cell responses and viral load has been defined previously. While certain studies have shown that the relationship between CD8⁺ T-cell response and viral load is not universally consistent throughout the chronic phase of infection (Addo *et al.*, 2003; Cao *et al.*, 2003; Jiao *et al.*, 2006; Kiepiela *et al.*, 2007), analysis of HIV-specific CD8⁺ T-cell responses in other studies were shown to positively correlate with HIV viral load (Ogg *et al.*, 1998; Betts *et al.*, 2001; Edwards *et al.*, 2002). A significant association was observed between the HIV-specific IFN- γ responses by CD8⁺ T-cell and HIV plasma load in 32 individuals for which both measurements were characterised (Figure 2.9; R=0.51, p=0.007). The association remained strong and significant when performed for HIV seroconcordant individuals alone (n=17; R=0.51, p=0.04). Though a

positive correlation was observed between IFN- γ specific responses in CD8⁺ T-cells of serodiscordant individuals and their plasma HIV load, this association was not significant (n=15; R=0.4, p=0.14). No association was observed between any of the levels of the three cytokines assayed and CD4%.

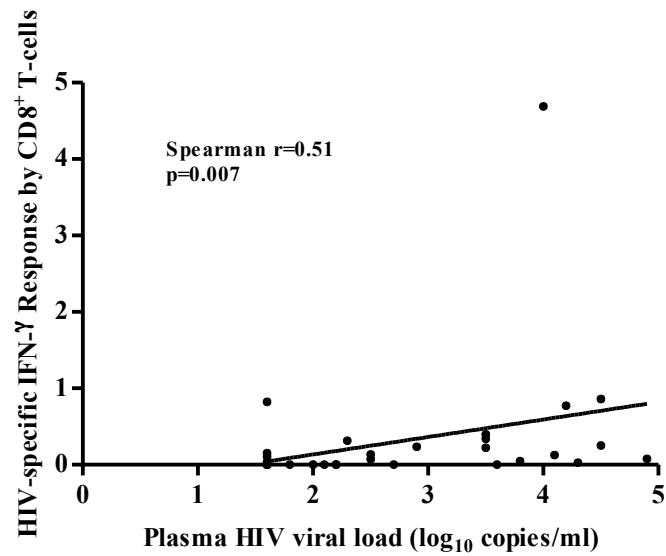


Figure 2. 9 Correlation between HIV plasma viral load and HIV-specific IFN- γ Response by CD8⁺ T-cells. Matching HIV plasma viral load and HIV-specific IFN- γ Response by CD8⁺ T-cells of 17 HIV seroconcordant and 15 HIV serodiscordant individuals combined were correlated using Spearman rank test.

2.4 Discussion

This chapter explores potential risks associated with being an HIV+ individual in a relationship with a stable partner who is either HIV seroconcordant or serodiscordant, using objective biomarkers of HIV disease progression, such as plasma HIV load and absolute blood CD4 count. HIV seroconcordant individuals in this study had a 0.5 log₁₀ higher plasma HIV load than their serodiscordant counterparts, which was equivalent to a difference in viral load of 14700 copies viral RNA/ml. Even though HIV loads in plasma were found to be negatively associated with absolute CD4 counts, this 0.5 log₁₀ difference in viremia between groups did not predict a worse clinical outcome as indicated by lower absolute CD4 counts at future visits over a 2 year period of follow-up.

There have been several studies in couples which have yielded conflicting findings about the relationship between viral load and CD4 counts in HIV seroconcordant and serodiscordant couples (Malamba *et al.*, 2005; Chatterjee Rogers *et al.*, 2005; Kumarasamy *et al.*, 2010). Malamba *et al.* (2005) reported that HIV+ seroconcordant individuals had an average of 0.34 Log₁₀ higher plasma viral load than those in HIV serodiscordant relationships. In their study, a 0.34 Log₁₀ difference in viral load translated to a difference of 71 422 RNA copies/ml, and this was associated with greater CD4 decline in the seroconcordant than the serodiscordant individuals. It could be that the 5-fold greater difference in plasma viral load in the Malamba *et al.* (2005) study compared to the current study accounted for the difference in clinical outcome and that a 71 422 RNA copies/ml difference was sufficient to drive greater absolute blood CD4 decline between their two groups. Interestingly, the median CD4 count reported in seroconcordant participants in the Malamba study was much lower than the baseline median CD4 count in the seroconcordant individuals in this current study (250 cells/ml versus 354 cells/ml) while an opposite trend was found between median CD4 count of serodiscordant individuals in the Malamba study (467 cells/ μ l) and the same marker in the current study (387 cells/ μ l). As longitudinal analysis in this study revealed that a higher CD4 count at previous visit was associated with a greater CD4 decline as measured at the following visit, it could be that the individuals included in this study had already reached set-point in their chronic stage of HIV infection, characterised only by slight changes in both blood CD4 count and viral load over time. Similarly, Chatterjee Rogers *et al.* (2005) reported that HIV-infected men in seroconcordant relationship had significantly lower CD4 counts and higher viral load than men in serodiscordant relationship. However, the very low CD4 count

(97 in seroconcordant men and 222 in serodiscordant men) and higher median plasma viral load (360 000 cps/ml for seroconcordant men and 291 000 cps/ml for serodiscordant men) in their study could be indicative of these participants being in AIDS stages. No mention is made of the length of time for which those participants have been infected. The participants included in the present study are all in the chronic phase of infection. Moreover, the consistent level of viremia maintained in both seroconcordant and serodiscordant individuals from baseline through month 12 and up to month 24 reflects the fact that these individuals have probably reached viral set-point already.

The finding in this Chapter that a higher plasma HIV load in HIV+ seroconcordant individuals compared to individuals in serodiscordant relationships did not predict lower absolute blood CD4 counts in seroconcordant individuals is unexpected. It may be that the difference in plasma viral load between HIV+ individuals in seroconcordant versus serodiscordant relationships is central to whether these individuals experience worse HIV clinical course based on CD4 decline. For instance, if the difference in plasma viral load is high in seroconcordant versus serodiscordant individuals, then the impact of CD4 decline will be more evident. Similarly, if the cohort is chronic and average CD4 counts are relatively low at baseline, then decline in CD4 counts will be more difficult to quantify because the study is starting off an already low base.

Many factors have been shown to affect blood CD4 count and HIV load during infection. It is essential that all these factors are controlled for if one is to dissect the intricacies governing over the differences in CD4 count and plasma HIV load. As an example, in a cross-sectional analysis of married couples in seroconcordant and serodiscordant heterosexual relationships, Kumarasamy *et al.* (2010) reported completely contrasting findings to our studies. They reported seroconcordant individuals having a significantly higher CD4 count (205 cells/ μ l) and lower viral load (89 000 cps/ml) than serodiscordant individuals (139 cells/ μ l and 100 000 cps/ml respectively). However, it is also important to point out that more seroconcordant individuals were on ART compared to serodiscordant individuals (14.1% versus 8.5%; $p=0.004$), which would influence both plasma viral loads and CD4 counts. In the present study, ART was controlled for by only enrolling participants naïve to therapy. Moreover, in the study conducted by Malamba *et al.* (2005), they reported that women in HIV seroconcordant relationship were 6.5-fold more likely to have reported an STI in the 6 months prior to enrolment than those in serodiscordant relationship. In contrast, HIV+ individuals in the study presented in this Chapter reported an equal manifestation of STDs

across the two groups, which suggests that concomitant STIs did not impact on differences seen in plasma viral load in this cohort. Of note, Malamba *et al.* (2005) also suggested that circumcision status of male partners impacted on viral load differences in the context of couples, with a significantly of higher report of circumcision in serodiscordant males (24%) compared to seroconcordant males (6%). There is strong epidemiological evidence that uncircumcised men are at an increased risk of HIV transmission than circumcised men (Moses *et al.*, 1998; Bailey *et al.*, 1999; Quinn *et al.*, 2000; Gray *et al.*, 2007). Since all of the men in this study were circumcized, this potential factor would not be considered a confounder. While Chatterjee Rogers *et al.* (2005) documented a lower CD4 count associated with the elevated viremia in seroconcordant participants compared to serodiscordant participants, the couples enrolled in their study were recruited from men having sex with HIV+ commercial sex workers. Since the individuals in this current study were in stable long-term heterosexual relationships, the nature of the partnership when interpreting results from seroconcordant versus serodiscordant individuals is important to consider.

There is broad consensus about the prognostic value of CD4 counts in predicting rate of disease progression (Lepri *et al.*, 1998; Williams *et al.*, 2006) and that clinical HIV disease progression is preceded in all untreated individuals by an increase in HIV RNA in plasma (Henrard *et al.*, 1995). However, there is substantial debate about the utility of using CD4 absolute cell counts alone as a valid surrogate for virological outcome in plasma, and whether variability in CD4 cell counts adequately reflect variability in viral load (Badri *et al.*, 2008). While some studies have shown that this may have limited predictive value (Grabar *et al.*, 2000; Florence *et al.*, 2003; Jevtovic *et al.*, 2005), others suggest that these parameters are predictive of one another, where in one such study, it was determined that an average of 1 Log₁₀ increase in viral load was associated with a 55 cell/ μ l decrease in CD4 count (Lima *et al.*, 2009). Rodriguez *et al.* (2006) reported that even though elevated plasma HIV loads were associated with greater rates of CD4 decline overall, viral burden had minimal value for predicting the rate of CD4 cell decline in individual patients, with only 4-6% of the variability in CD4 numbers being explained by changes in plasma VL. Mellors *et al.* (2007) subsequently confirmed the poor association between these two markers. In a selected group of participants for whom CD4 count and plasma HIV load were available for two consecutive time points in this present study, 35% of the variability in CD4 depletion in blood was attributable to changes in HIV plasma load. Collectively, all these studies suggest a limited capacity of CD4 cell counts alone to explain variability in viral load measurement at an

individual level in untreated patients. CD4 T-cell counts can be influenced by sex, age, race, concomitant infections, and even geography (Maini *et al.*, 1996). However, all of these factors have been controlled for in our study. If differences in plasma viral loads only partially account for changes in absolute blood CD4 level, a key question to address is what are the other determinants accounting for this residual variability in CD4 cell decay? Improvements in the treatment of HIV infection and AIDS may result from an improved understanding of this 65% of CD4 cell decline that remains enigmatic.

One of the most important questions raised by the finding in this Chapter that HIV+ seroconcordant individuals sustain a higher plasma viral load over time than serodiscordant individuals is about the potential influence of each of the seroconcordant partner's HIV load and viral characteristics on properties of viremia in their similarly HIV+ partners. Several studies have shown that qualitative aspects of the virus can be additional risk factors associated with HIV progression and transmission (Baeten *et al.*, 2007; Kiwanuka *et al.*, 2008; Alizon *et al.*, 2010; Shirreff *et al.*, 2011). HIV is characteristically highly genetically diverse (Hoelscher *et al.*, 2002; Kiwanuka *et al.*, 2008). Previous studies have reported that HIV infection with multiple types simultaneously is an important factor that might influence subsequent viral load setpoint and HIV disease progression, with those individuals infected with >1 type having higher setpoints (Kanki *et al.*, 1999; Grobler *et al.*, 2004; Campbell *et al.*, 2009; Redd *et al.*, 2011). In this Chapter, it was impossible to determine who had donated the virus compared to who received the virus in HIV+ seroconcordant couples, whether these couples in fact infected each other or came into the relationship already infected. No association was observed between plasma viral loads within a couple, suggesting that either properties of virus were not influencing viral loads in each partner in a couple (ie, host genetics may have been confounding this analysis) or that the phenotype of the virus was not similar between individuals in a couple. Since one of the hypotheses of this study was that HIV+ seroconcordant couples would share HIV that was phenotypically similar, this finding was unexpected and suggested that other non-viral factors were possibly influencing viral setpoint. Recently, the genomic determinants of susceptibility to HIV infection, control of viral replication and pathogenesis have been investigated in depth (Telenti, 2006). Telenti (2006) argued that there was a great deal of inter-individual variability in HIV viral setpoint after seroconversion, part of which is known to be due to genetic differences among infected individuals. Rotger and collaborators (2010) have shown a significant contribution of variable host gene expression in the control of HIV, related to host DNA polymorphisms. In their

genome-wide search, they observed two distinct profiles with 260 genes differentially expressed depending on HIV viral load. While it has been shown that host variants identified in multiple genome-wide analyses (Dalmasso *et al.*, 2008; Limou *et al.*, 2009; Fellay *et al.*, 2009) explain only 13% of the variation in viral setpoint, the remaining 87% of variation unaccounted for, indicating that other biological determinants of control are yet to be identified.

Despite this finding, ongoing HIV superinfection resulting from intracouple transmission could be occurring (Piantadosi *et al.*, 2007; Redd *et al.*, 2011) and could be a molecular mechanism influencing viral load within seroconcordant couples. Hecht *et al.* (2010) recently observed a strong correlation between HIV RNA levels within HIV transmission pairs that they followed up from the time of transmission through to the chronic stage of infection and concluded that virus characteristics are an important determinant of viral load in early HIV infection. The cohort studied in this dissertation consisted of infected individuals who have reached set-point and are already in the chronic phase of infection. Although not measured in this study, the lack of association in viremia between partners could be driven by host immune factors such as HLA genotype. Moreover, strong CTL responses have been reported to select for escape mutations in HIV, particularly those in Gag, which could substantially impact on fitness and therefore viral loads (Friedrich *et al.*, 2004; Martinez-Picado *et al.*, 2006). These mutations can persist after transmission and influence viral load in recipients after transmission and during early HIV infection (Chopera *et al.*, 2008; Goepfert *et al.*, 2008).

Possibly linked to higher plasma HIV load in HIV+ seroconcordant compared to serodiscordant individuals, HIV+ seroconcordant individuals also had substantially higher frequencies of IFN- γ producing CD8⁻ and CD8⁺ T-cells. The observation of a strong positive correlation between Gag-specific IFN- γ responses by CD8⁺ T-cells and plasma HIV load confirms that a higher antigenic exposure in immune compartments can immunologically translate into elevated HIV-specific antiviral responses. IFN- γ has been shown to be an important antiviral cytokine, commonly produced in response to HIV infection (Fan *et al.*, 1993; McMichael *et al.*, 2001). It is a useful predictive tool for how effectively the host immune system is fighting the infection and has been shown to inhibit viral replication in infected cells (Dhawan *et al.*, 1994; Guidotti *et al.*, 1999). IFN- γ has also been shown to promote protective T helper type 1 (Th1) immune responses (Mosmann *et al.*, 1989) which include both cell-mediated immunity and antibody mediated immunity.

In addition to IFN- γ production as a means of identifying HIV-specific T-cell responses, IL-2 and MIP-1 β were also assayed in this study to get a broader measurement of HIV-specific effector function. While no difference was observed between seroconcordant and serodiscordant individuals for IL-2 expression in CD8⁺ and CD8⁻ T-cells, it was evident that higher frequencies of T-cells from HIV⁺ seroconcordant individuals produced MIP-1 β than serodiscordant individuals. MIP-1 β was interesting to include in this study because it is a chemokine that directly binds to and can therefore potentially modulate expression of the HIV co-receptor CCR5 (Trumpfheller *et al.*, 1998). MIP-1 β has also been shown to be associated with asymptomatic HIV infections (Cocchi *et al.*, 2000) and with a decreased risk of HIV disease progression (Ullum *et al.*, 1997), because it acts as antagonists to HIV cell entry. Collectively, these findings suggest that antiviral immunity might be afforded through the release of HIV-suppressive chemokines from activated CD8⁺ T-cells, and as these chemokines down-regulate CCR5 expression on cell surface, it leads to a lower proportion of CD4⁺ T-cell being infected and targeted for killing. This higher production of MIP-1 β could be indirectly driving the similitude in CD4 count in seroconcordant and serodiscordant individuals.

This first chapter provides evidence that differences exist between HIV⁺ individuals in seroconcordant and serodiscordant relationships. A difference of 0.5 Log₁₀ or 14 700 copies/ml was observed in plasma HIV load between HIV⁺ seroconcordant versus serodiscordant individuals. It appears that 0.5 Log₁₀ difference in plasma HIV load (14 700 copies/ml) between HIV seroconcordant and serodiscordant individuals did not significantly influence subsequent rates of disease progression in this cohort since CD4 counts remained comparable over the 24 months of follow-up. If this 0.5 Log₁₀ difference in plasma HIV load (14 700 copies/ml) was not great enough to impact on pathogenesis as measured by absolute CD4 count, what could be the implication of this difference during the course of chronic HIV infection? Pedraza *et al.* (1999) have shown that a 15 000 copies/ml difference in plasma viral load was enough to differentiate successful heterosexual HIV transmission from non-transmission. We can therefore hypothesise that a higher HIV load could increase the risk of HIV acquisition in uninfected partners and possibly also the risk of superinfection within seroconcordant couples. While it seems intuitive that plasma HIV RNA levels are an important determinant in therapeutic decision making, and recommended as one of the elements on which timing of initiation of ART is based (BHIVA, 2005; DHHS, 2006; Hammer *et al.*, 2006), the clinical impact of the relatively modest difference from this study

on long term CD4 loss was not evident. Chapter 3 explores the role of systemic immune activation and inflammation as potential mediator driving differences observed in plasma viral load between HIV+ individuals in HIV seroconcordant and serodiscordant relationships.

CHAPTER 3

Impact of partner HIV status on systemic immune activation and inflammation during chronic HIV infection

3.1 Introduction

In response to pathogen challenge, humans generally experience persistent, low-grade, periodic immune activation and inflammation, which reflects that anti-microbial immunity has been mounted and should be beneficial (Deeks and Walker, 2004). In contrast, our non-human primates ancestors do not experience similar persistent immune activation (Silvestri, 2008). During HIV-infection, however, studies have demonstrated that the level of systemic T-cell activation predicted worse disease course for those infected (Liu *et al.*, 1998; Giorgi *et al.*, 1999; Hazenberg *et al.*, 2003; Deeks *et al.*, 2004; Wilson *et al.*, 2004). Immune activation in the context of HIV infection is a term that refers to a wide range of biological processes, such as cell activation, proliferation and apoptosis, and their associated consequences (Appay *et al.*, 2008). Although these immune processes are generally considered to be protective to the host during most other infections, this paradoxically also exacerbates pathogenesis during HIV infections by resulting in a constant supply of activated HIV target cells (Lawn *et al.*, 2001).

Zhang and colleagues (1998) showed that $CD4^+CCR5^+$ T-cells are the target cells preferentially infected by HIV. The infection and depletion of $CD4^+$ T-cells characterize the most influential event in the pathogenesis of HIV infection, with the main cellular target during the established infection being activated $CCR5^+CD4^+$ T-cells. Low density of the HIV co-receptor CCR5 expressed on T-cells was found to be a determining factor in resistance to HIV infection (Reynes *et al.*, 2003). Hazenberg *et al.* (2003) reported that these persistently elevated levels of systemic immune activation represent the main driver of disease progression in HIV pathogenesis and plays a bigger role than the actual $CD4^+$ T-cell depletion in determining disease course. Studies of SIV disease course in macaques, which are not considered to be the natural hosts for SIV and experience similarly high viral loads as humans, have shown that systemic $CD4^+$ T-cell loss and persistent but low levels of immune activation point to immune activation being the most important driver of disease progression (Milush *et al.*, 2007).

A network of pro-inflammatory, regulatory and haematopoietic cytokines has been shown to affect virtually every step of the life cycle of HIV, from entry into new cells to budding of new progeny (Catalfamo *et al.*, 2012). These systemic cytokines are also thought to be responsible for localised and systemic inflammatory responses, including the recruitment of

potential HIV target cells at point of infection. IL-1 β , IL-6, and TNF- α , either alone or synergistically, have been shown to act on HIV-infected cells to up-regulate HIV replication (Fauci *et al.*, 1996; Poli *et al.*, 1999). Initiation of systemic inflammation during HIV infection and chronic establishment of this state, driven by the replication of HIV in blood and mucosal compartments, have detrimental and far-reaching consequences to human health during chronic infection.

The aim of this Chapter was to evaluate the impact of stable sexual partner status on the extent of systemic immune activation and inflammatory cytokine production in HIV+ individuals during the chronic stages of HIV infection. A statistical model of HIV pathogenesis is proposed, which incorporates the four important factors possibly impacting on higher plasma viral loads in HIV+ seroconcordant compared to serodiscordant individuals and rate of disease progression depending on partner status. These include the role of (1) CD4⁺ T-cell frequencies, (2) immune activation of systemic T-cells, (3) production of inflammatory and regulatory cytokines in plasma, and (4) plasma viral load. Use is made of differences, both biological and behavioural, between HIV+ individuals in stable relationships with either HIV+ seroconcordant or HIV- serodiscordant partners, and to try understand mechanisms predicting the establishment and exacerbation of HIV immunodeficiency.

3.2 Materials and Methods

3.2.1 Description of study participants

A total of 262 HIV+ individuals were included for this part of the study, of which 144/262 were in HIV seroconcordant relationship and 118/262 were in serodiscordant relationship. Chapter 2 (Section 2.2.1) provides a full description of these individuals. HIV+ individuals were followed longitudinally during chronic infection, with immune activation and inflammatory cytokine production in blood being measured at 6, 18 or 30 months into the study. Although HIV plasma loads were available for all 262 individuals at enrolment into the study (month 0), viral loads were only available for 77/262 participants at the subsequent longitudinal time points included in this Chapter.

3.2.2 PBMCs collection and processing

Blood (16ml) was collected from all study participants using standard venipuncture into sterile ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK). Blood was processed within 4 hours of collection; PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation as described in Chapter 2 (Section 2.2.2).

3.2.3 Cryopreservation of PBMCs

All PBMCs used in this study were cryo-preserved and subsequently stored in liquid nitrogen. Briefly, fresh freezing solution (20% DMSO in FCS) was prepared prior to cryo-preservation of cells and reduced to 0°C on ice prior to addition to reduce its cellular toxicity. PBMCs were pelleted by centrifugation at 1200rpm (280 x g) for 10 minutes and re-suspended in cold 100% FCS at 2×10^7 cells/ml. PBMCs were then aliquoted into pre-cooled 2ml cryovials at 500 µl (1×10^7 cells) per cryovial. To each cryovial, 500 µl of the pre-cooled 20% DMSO FCS freezing solution was added dropwise, with constant swirling, to bring the final concentration of cells to 1×10^7 cells/ml per cryovial and the final concentration of DMSO to 10%. The sealed cryovials were transferred immediately to a pre-cooled “Mr. Frosty” (Nalgene) freezing container which facilitated slow controlled cooling at -

1°C/minute. The “Mr. Frosty” container was transferred immediately to -80°C for 24 hours after which the cells were transferred to liquid nitrogen for long term storage.

3.2.4 Thawing of cryo-preserved PBMCs

Frozen PBMCs were thawed rapidly in a 37°C water bath until a small piece of the ice crystal was still visible in the thawed cryovial. Pre-warmed (37°C) R1 (RPMI1640 [Life Technologies Corporation, Carlsbad, CA, USA] medium supplemented with 5mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mg/ml fungin and 1% FCS) was added dropwise, with continuous gentle shaking. Once the thawed cryovial was filled with R1, the content of the vial was transferred to a 50 ml Sterilin tube which was then topped up to the 25 ml mark with R1. This cell suspension was carefully mixed by inverting the tube 3 times and centrifuged at 1500rpm (437 x g) for 10 minutes. The resulting supernatant was decanted, the cell pellet resuspended and the wash step repeated. After the second wash, the supernatant was decanted and cell pellet resuspended in 10 ml of R20 (RPMI1640 (Life Technologies Corporation, Carlsbad, CA, USA) medium supplemented with 5mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mg/ml fungin and 20% FCS) and rested overnight at 37°C and 5% CO₂. The cell concentration and viability of the thawed PBMCs was measured using the Guava cell counter and viacount reagent as previously described (Section 2.3.3).

3.2.5 Counting of PBMCs using the automated Guava cell counter

Thawed PBMCs were diluted 20-fold in Guava Viacount reagent and counted using a Guava Automated cell counter as previously described in Chapter 2 (Section 2.2.3).

3.2.6 Staining for markers of T-cell activation by flow cytometry

PBMCs were stained in BD Falcon Round-Bottom tubes (BD Biosciences, San Jose, CA, USA) and 1 million PBMCs were used per staining reaction per participant. Prior to staining with antibodies, PBMCs were incubated with LIVE/DEAD[®] Fixable Violet Dead Cell Stain for 20 minutes at room temperature and then washed with 1% FCS PBS. The pelleted cells were resuspended in a dead volume and stained for 30 minutes at room temperature with

phenotypic marker Peridin Chlorophyll Protein-Cyanine 5.5 (PerCP-Cy5.5) labeled anti-CD4 (BD Pharmingen, San Jose, CA, USA), QDot605 labelled anti-CD8 (Invitrogen, Carlsbad, CA, USA), Allophycocyanin (APC) labelled anti-CD195/CCR5 (BD Biosciences, San Jose, CA, USA), Phycoerythrin Cyanine 7 (PE-Cy7) labelled anti-CD38 (BD-Biosciences, San Jose, CA, USA), Phycoerythrin (PE) labelled anti-HLA-DR (BD Biosciences, San Jose, CA, USA), and finally Pacific Blue (Pac Blue) labelled anti-CD14 (BD Pharmingen, San Jose, CA, USA) and anti-CD19 (Invitrogen, Carlsbad, San Jose, CA, USA). Anti-CD14 and anti-CD19 antibodies were included as dump markers to exclude monocytes and B-cells from analysis, respectively. Cells were then washed twice with 1% FCS PBS, centrifuged for 5 minutes at 1500rpm at room temperature, fixed and permeabilised with BD CytoPerm/CytoFix (BD Biosciences, San Jose, CA, USA) for 20 minutes at room temperature. Cells were then washed with Perm/Wash buffer (BD Biosciences, San Jose, CA, USA) for 5 minutes at 1500rpm, room temperature. The intracellular staining then ensued, with Allophycocyanin-Hilite (APC-H7) labelled anti-CD3 (BD Biosciences, San Jose, CA, USA) and Fluorescein Isothiocyanate (FITC) labelled anti-Ki67 (BD Biosciences, San Jose, CA, USA). Cells were washed in 2ml of BD Perm/Wash buffer by centrifugation at 1500rpm at room temperature for 5 minutes and fixed with BD Cell Fix (Becton-Dickinson, San Jose, CA, USA). Cell fluorescence was assessed using a BD LSR Fortessa flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Fluorescence minus one (FMO) was used to distinguish continuous population. Compensation and analysis of data were done using FlowJo software (Tree Star, Ashland, OR, USA).

3.2.7 Measurement of cytokine concentrations in blood plasma

The concentrations of IL-1 β , IL-6, IL-12p70, TNF- α , IL-10, IL-2, IFN- γ , IL-7, and GM-CSF were measured in blood plasma using High Sensitivity Human Cytokine LINCOplex Premixed kits (LINCO Research, MO, USA). The sensitivity of the kits ranged between 0.01 and 0.48 pg/ml for each of the 9 cytokines measured. Data was collected using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories Inc) and a 5 PL regression formula was used to calculate cytokine concentrations from the standard curves. Data was analysed using BIOplex manager software (version 4; Bio-Rad Laboratories Inc). Cytokine concentrations that were below the detection limit of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero.

3.2.8 Determination of HIV viral load in blood plasma

HIV viral load in plasma was assayed using the NuclisensEasyq HIV Version 1.2 by the NHLS Diagnostic Virology Laboratory (Groote Schuur Hospital, Cape Town, South Africa). The detection limit of the assay was 50 HIV RNA copies/ml.

3.2.9 Statistical analysis

Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Comparison of unpaired non-parametric data was done using the Mann-Whitney U test. Statistical inferences on binary sets of data were performed using the Fisher's exact test and odds ratios calculated. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons. Quantile regression analyses were used to estimate the median value of the independent (response) variable for given levels of the dependent (predictor) variables. Adjustment for multiple comparisons was performed using a false discovery rate step-down approach. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 11 (College Station, TX, USA). All tests were two-tailed and p-values of ≤ 0.05 were considered significant.

3.3 Results

3.3.1 Characteristics of HIV seroconcordant and serodiscordant participants

Two hundred and sixty two black South African HIV+ individuals, from 194 heterosexual couples, were included to study the role of sexual partner status on systemic immune activation and inflammation, as potential drivers of HIV disease progression (Table 3.1). The participants were classified into HIV seroconcordant or serodiscordant relationships, based on the HIV status of their current stable partners. Of the 194 couples, 76/194 (39%) were HIV seroconcordant (both partners HIV+), and 118/131 (61%) were HIV serodiscordant (one partner HIV+ and the other HIV-). In this cohort, women were more likely to be the HIV seropositive partners in HIV serodiscordant couples than men (80/118 couples, 68%). No difference in age, age at sexual debut or whether couples cohabited was observed when comparing HIV+ individuals in serodiscordant and seroconcordant relationships (Table 3.1). All HIV+ individuals were naïve to HAART and all men were circumcised.

Although absolute CD4 counts were performed at enrolment into this study (considered to be month 0; Chapter 2), they were not performed at subsequent time points studied in this Chapter. As a result, CD4 percentages (of CD3⁺ T-cells) were used in this study as a marker for absolute CD4⁺ T-cell counts. Hulgán *et al.* (2007) and Pirzada *et al.* (2006) have reported that CD4 percentages are a reliable biomarker for absolute CD4. In this Chapter, CD4 percentages were similar in HIV+ individuals in seroconcordant or serodiscordant relationships (Table 3.1).

The median plasma HIV load in HIV+ individuals was 22000 copies/ml (IQR: 50-100000). A higher proportion of individuals in HIV seroconcordant relationships had plasma viral loads of >1500 cps/ml than those in HIV serodiscordant relationships (89% versus 58%, p=0.003), suggesting that HIV seroconcordance is associated with higher viral loads. Possibly as a result of perceived risk of transmitting to their HIV- partners, a higher frequency of condom use was self reported by HIV+ individuals in serodiscordant relationship when compared to their seroconcordant counterparts (p=0.009). Despite this, 42% of participants in serodiscordant relationship who reported condom usage also reported recent genital

ulceration or/and vaginal discharge. This suggests that self-reported condom use in this cohort may be over-reported.

Table 3. 1 Clinical and socio-behavioural characteristics of HIV+ individuals

Characteristics	n	HIV Positive		P-value
		Concordant	Discordant	
N		144	118	
Male [%] †		53	32	0.004
Age [yr; median (IQR)] *	141, 116	37 (32-42)	38 (32-45)	0.4
Living together with partner [%] †	142, 117	70	66	0.5
Age at first sex [median (IQR)] *	141, 117	17 (16-18)	16 (16-18)	0.9
Sexual exposure [median years of sex (IQR)] *	140, 116	21 (15-25)	20 (14-29)	0.5
Blood CD4% [median (IQR)] *	40, 36	45 (24-65)	38 (21-64)	0.5
Plasma HIV load >1500 cps/ml [%] † #	38, 39	89	58	0.003
Sex acts in the last month [median (IQR)] *	137, 116	3 (1.5-6)	3 (1.25-5)	0.3
Condom usage [%] †	123, 112	58	74	0.009
Genital ulceration in the last 6 months [%] †	61, 79	8	6	0.6
Vaginal discharge in the last 6 months [%] †	61, 79	12	15	0.5

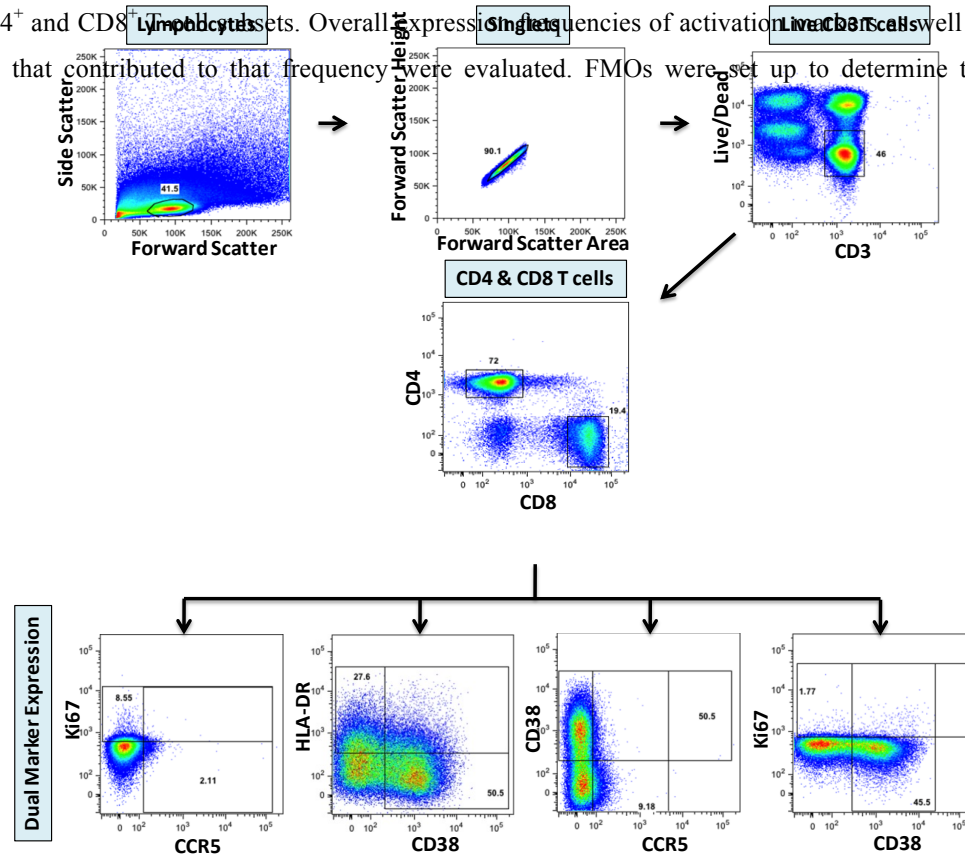
† p-value calculated using Fischer's exact or *Mann-Whitney U-test; IQR: Interquartile range; #A cut-off of 1500 cps/ml plasma was selected as this has been reported to be the lowest plasma level of HIV that was associated with heterosexual transmission of HIV (Quinn *et al.*, 2000).

3.3.2 Impact of partner HIV status on T-cell activation in blood

The expression of activation markers CD38, HLA-DR, CCR5 and the nuclear proliferation marker Ki67 by T-cells in blood was measured to investigate the impact of partner status on activation of T-cells in HIV+ individuals. In specific combinations, these markers allowed assessment of the frequency of (1) highly activated T-cells (defined by dual expression of HLA-DR and CD38), (2) activated proliferating T-cells (defined by dual expression of Ki67 and CD38); and (3) CCR5+ T-cells that represent the preferred target cells for HIV infection [activated CCR5+ cells (defined by dual expression of CCR5 and CD38) or proliferating CCR5+ cells (dual expression of CCR5 and Ki67)]. The overall level of activation (total activation) was defined as the cumulative expression of any permutation of the markers. Figure 3.1 shows representative plots of the gating strategy used to define each of these populations.

Figure 3. 1 Representative plots showing the gating strategy used to define activated T-cell populations by flow cytometry.

Peripheral blood mononuclear cells were stained with fluorochrome-labelled monoclonal antibodies to define CD3 (APC-H7), CD4 (PerCPCy5.5), CD8 (QDot605), CCR5 (APC), CD38 (PE-CY7), HLA-DR (PE), Ki67 (FITC) and CD14/CD19/LIVE/DEAD Fixable Violet Dead Cell Stain (Pac Blue). Cell doublets/aggregates were removed by gating on singlets. Live CD3⁺ T-cell populations were differentiated into CD4⁺ and CD8⁺ lymphocytes. Overall expression frequencies of activation markers as well as that of the permutations of expression that contributed to that frequency were evaluated. FMOs were set up to determine the positioning of the specific gates.



General activation status (total activation) was calculated for each T-cell population and was defined as the cumulative expression of any permutation of the markers (Figure 3.2). HIV+ individuals with HIV seroconcordant partners had higher frequencies of activated CD4⁺ and CD8⁺ T-cells than those in HIV serodiscordant relationships, significantly so for CD4⁺ T-cells (p=0.01 for CD4⁺ and p=0.11 for CD8⁺ T-cells; Figure 3.2).

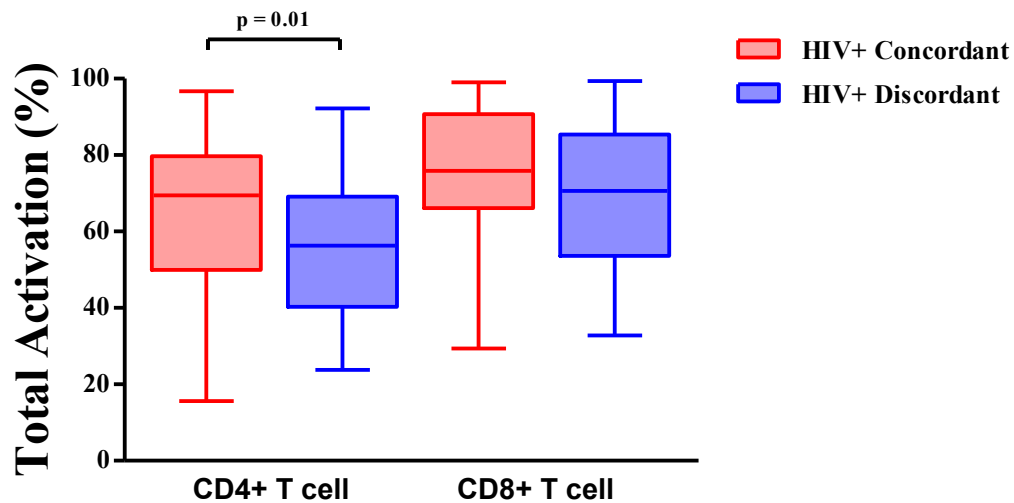


Figure 3. 2 Impact of partner HIV status on total activation of CD4⁺ and CD8⁺ T-cells in the blood from HIV+ individuals in HIV seroconcordant (red) versus serodiscordant (blue) relationships. Frequencies of immune activation markers on CD4⁺ and CD8⁺ T-cells derived from the blood of HIV+ individuals in serodiscordant and seroconcordant relationships were assessed for expression of CCR5, Ki67, HLA-DR, and CD38. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Mann-Whitney U tests were applied to compare blood T-cell activation frequencies between seroconcordant and serodiscordant groups.

CCR5, CD38, HLA-DR and Ki67 expression by CD4⁺ and CD8⁺ T-cells were compared individually or in biologically important combinations [HLA-DR/CD38 (representing highly activated T-cells), Ki67/CCR5 (representing proliferating T-cells which may be susceptible to HIV infection), CD38/CCR5 (representing susceptible, activated T-cells) and CD38/Ki67 (representing activated, proliferating T-cells)]. HIV+ individuals in HIV seroconcordant relationship had significantly higher frequencies of CD4⁺ T-cells expressing the HIV co-receptor CCR5 than those in serodiscordant relationships (p=0.03; Figure 3.3A). In contrast, expression of CD38, HLA-DR or Ki67 by CD4⁺ T-cells did not differ between groups. While

no causative relationship can be established between seroconcordant individuals' higher expression of CCR5 and their potentially higher susceptibility to HIV infection prior to seroconversion (since this is a cross sectional study and cause and effect can only be inferred in longitudinal studies where biological markers are measured pre- and post-infection), we can speculate that continuous exposure to HIV in a concordant couple situation may lead to increased immune activation (compared to sex with an uninfected partner in a discordant couple situation), and also potentially increase the likelihood of superinfection.

Similarly, significantly higher frequencies of HIV+ individuals in HIV seroconcordant relationships expressed CCR5 on the surface of their CD8⁺ T-cells than their HIV serodiscordant counterparts (Figure 3.3B). In contrast, HIV seroconcordant individuals had lower frequencies of activated CD8⁺ T-cells than their serodiscordant counterparts as measured by the expression of either CD38 (p=0.03; Figure 3.3B) or HLA-DR (p=0.05; Figure 3.3B). This was surprising since total activation of CD8⁺ T-cells was similar in HIV+ individuals in seroconcordant and serodiscordant relationships (Figure 3.2). Elevated CCR5 but lower CD38 and HLA-DR in seroconcordant versus serodiscordant individuals (Figure 3.3) may account for the overall reduced difference between groups noted previously in Figure 3.2.

In addition to significant differences in CCR5 expression by both T-cell subsets between groups, HIV+ seroconcordant individuals had the significantly higher dual-expression of CD38/CCR5 by both T-cell subsets compared to serodiscordant ones (p=0.03 for CD4⁺; Figure 3.3C and p=0.02 for CD8⁺; Figure 3.3D).

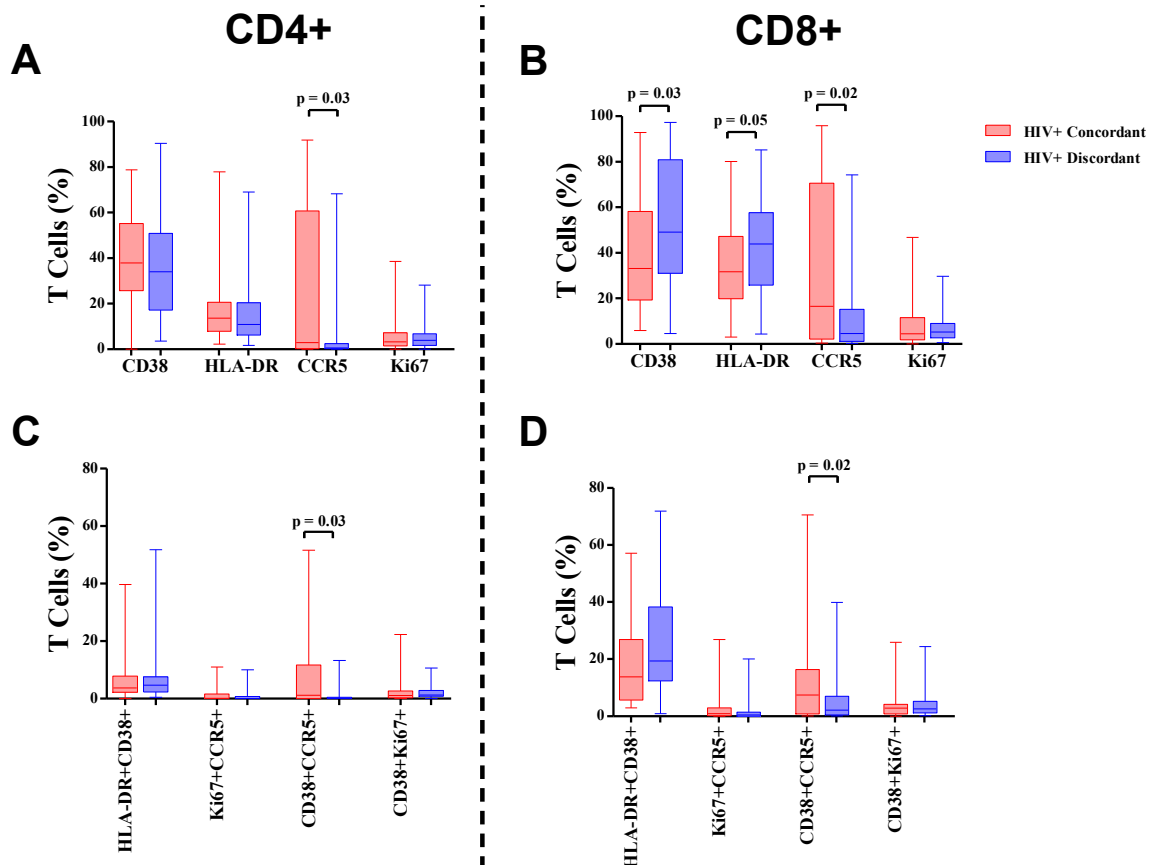


Figure 3. 3 Impact of partner HIV status on systemic T-cell activation (defined by expression of CD38, HLA-DR, CCR5, or Ki67) in HIV+ individuals in HIV seroconcordant (red) versus serodiscordant (blue) relationships. Frequency of specific activation marker expression (CCR5, Ki67, HLA-DR, CD38) on CD4⁺ (Panel A & C) and CD8⁺ (Panel B & D) T-cells derived from the blood of HIV+ individuals in HIV seroconcordant and serodiscordant relationships was assayed. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Mann-Whitney U tests were applied to compare blood T-cell activation frequencies between seroconcordant and serodiscordant groups.

3.3.3 Impact of gender on T-cell activation in blood

The levels of the individual markers for activation, proliferation and HIV co-receptor expression were investigated in men and women to determine if gender played a role in level of systemic activation. Generally, frequencies of all markers were similar in men and women for both CD4⁺ and CD8⁺ T-cells except for CD4⁺CD38⁺ which was significantly elevated in women compared to men (p=0.009; Figure 3.4).

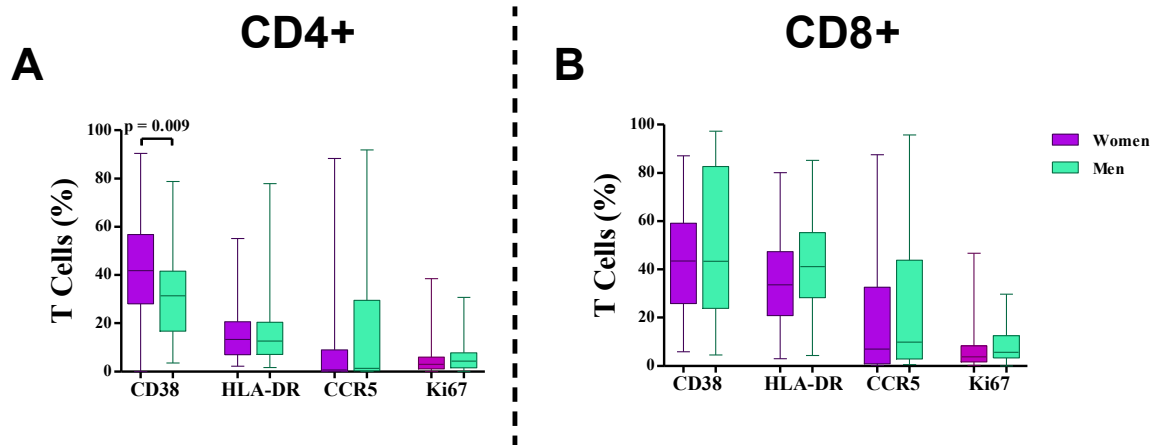


Figure 3. 4 Impact of gender on systemic T-cell activation (defined by expression of CD38, HLA-DR, CCR5, or Ki67) in HIV+ women (purple) versus men (green). Frequency of specific activation marker expression (CCR5, Ki67, HLA-DR, CD38) on CD4⁺ (Panel A) and CD8⁺ (Panel B) T-cells derived from the blood of HIV+ women and men was assayed. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Mann-Whitney U tests were applied to compare blood T-cell activation frequencies between men and women.

3.3.4 Relationship between HIV plasma load and systemic T-cell activation

Because significantly more HIV+ individuals with seroconcordant positive partners had HIV plasma loads >1500 copies/ml than those with serodiscordant negative partners (Table 3.1), the impact of differences in plasma viremia on T-cell activation in HIV+ individuals was investigated (Figure 3.5). The cut-off of > or <1500 cps/ml was used in this Chapter because of the published finding by Quinn *et al.* (2000) that showed, from 415 serodiscordant heterosexual couples in rural Uganda, that no HIV transmission occurred within couples where the HIV+ partner had HIV viral loads <1500 cps/ml. Total activation by both CD4⁺ and CD8⁺ T-cells was significantly higher in HIV+ individuals with plasma viral loads of >1500 cps/ml than those with <1500 cps/ml (p=0.01 for CD8⁺ and p=0.04 for CD4⁺ T-cells).

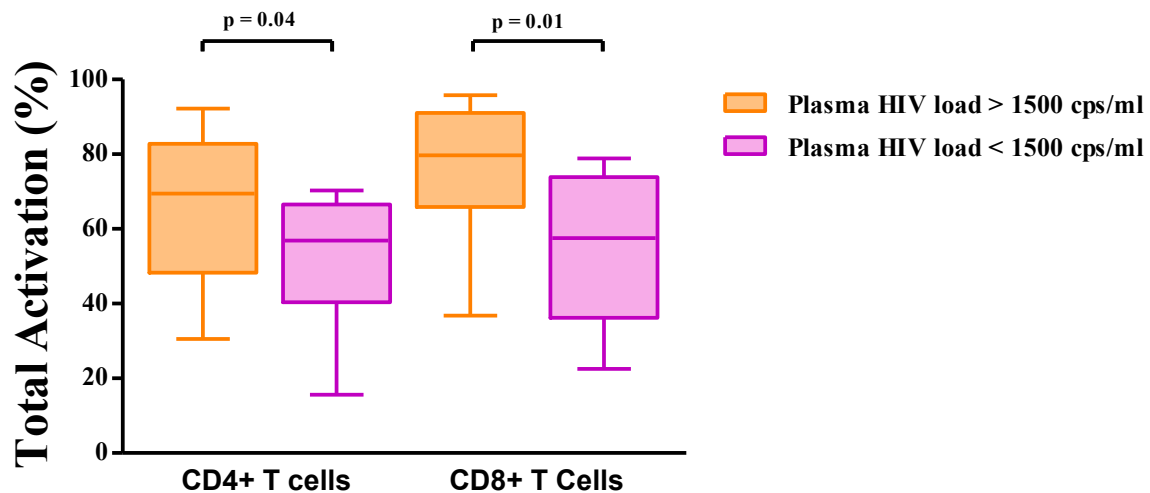


Figure 3. 5 Comparison of total activation of CD4⁺ and CD8⁺ T-cells in the blood of HIV-infected individuals with plasma viral loads of >1500 cps/ml (orange) or <1500 cps/ml (purple). Frequencies of immune activation markers on CD4⁺ and CD8⁺ T-cells derived from the blood of HIV+ individuals with HIV plasma viremia <1500 cps/ml and >1500 cps/ml were assessed for expression of CCR5, Ki67, HLA-DR, CD38, and combinations of these markers. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Mann-Whitney U tests were applied to compare blood T-cell activation frequencies between the two groups.

At the level of individual and defined combinations of activation marker expression, HIV+ individuals with plasma viral loads >1500 cps/ml had significantly higher frequencies of CD4⁺ T-cells expressing CCR5 than those with viral loads <1500 cps/ml (p=0.007; Figure 3.6A). In contrast, expression of CD38, HLA-DR and Ki67 by CD4⁺ T-cells did not similarly track with plasma viral loads. CD8⁺ T-cells from those with viral loads >1500 copies/ml did not express elevated levels of CCR5 compared to those individuals with viral loads <1500 copies/ml (Figure 3.6B), although significantly greater frequencies of CD8⁺ T-cells from these individuals did express CD38 and CD38 in combination with HLA-DR (p=0.01 for both; Figure 3.6B and D) compared to those with lower viral loads.

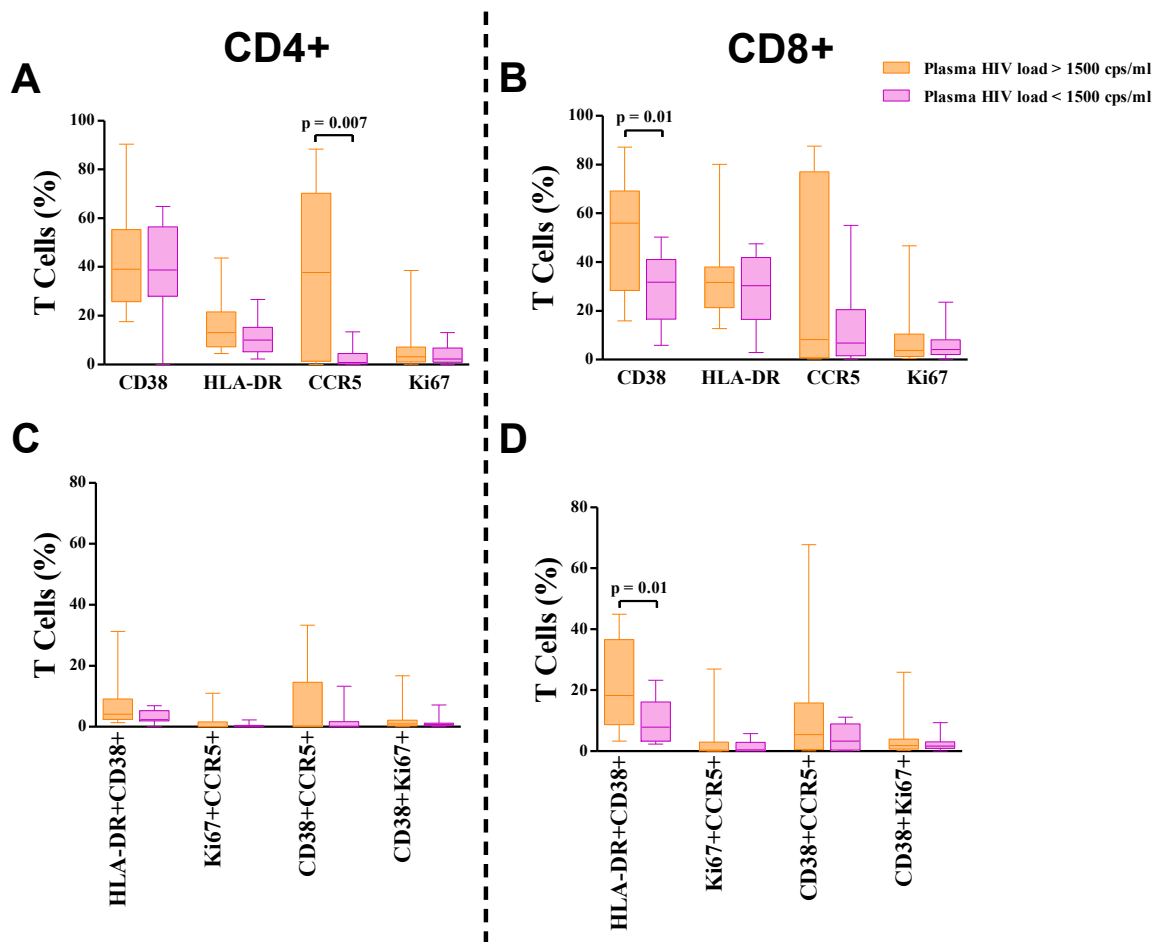


Figure 3. 6 Impact of plasma viral loads on the frequency of activation and proliferation marker expression by CD4⁺ and CD8⁺ T-cells in the blood. Frequencies of activation and proliferation marker expression (A-B: CCR5, Ki67, HLA-DR, CD38; and C-D: combinations of these) on CD4⁺ and CD8⁺ T-cells derived from the blood of HIV+ individuals with plasma viral loads <1500 cps/ml (purple) and >1500 cps/ml (orange). The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Mann-Whitney U tests were applied to compare blood T-cell activation frequencies between the two groups.

To further investigate whether plasma viral load was related to systemic T-cell activation in these HIV+ individuals, a univariate quantile regression model was used to test the relationship between plasma HIV load and frequency for each activation marker, alone or in combination (Figure 3.7). Overall, HLA-DR expression by CD4⁺ T-cells was generally positively associated with plasma viral loads either alone (in both seroconcordant and

serodiscordant) or in association with CD38 (seroconcordant; β -coefficient 1.74, $p=0.02$), irrespective of partner status, with every 1% increase in HLA-DR/CD38 expression by CD4⁺ T-cells predicting a similar 1.74% increase in plasma viral load. In contrast to CD4⁺ T-cells, expression of activation markers by CD8⁺ T-cells did not significantly positively predict changes in plasma viral loads (Figure 3.7B, D and F).

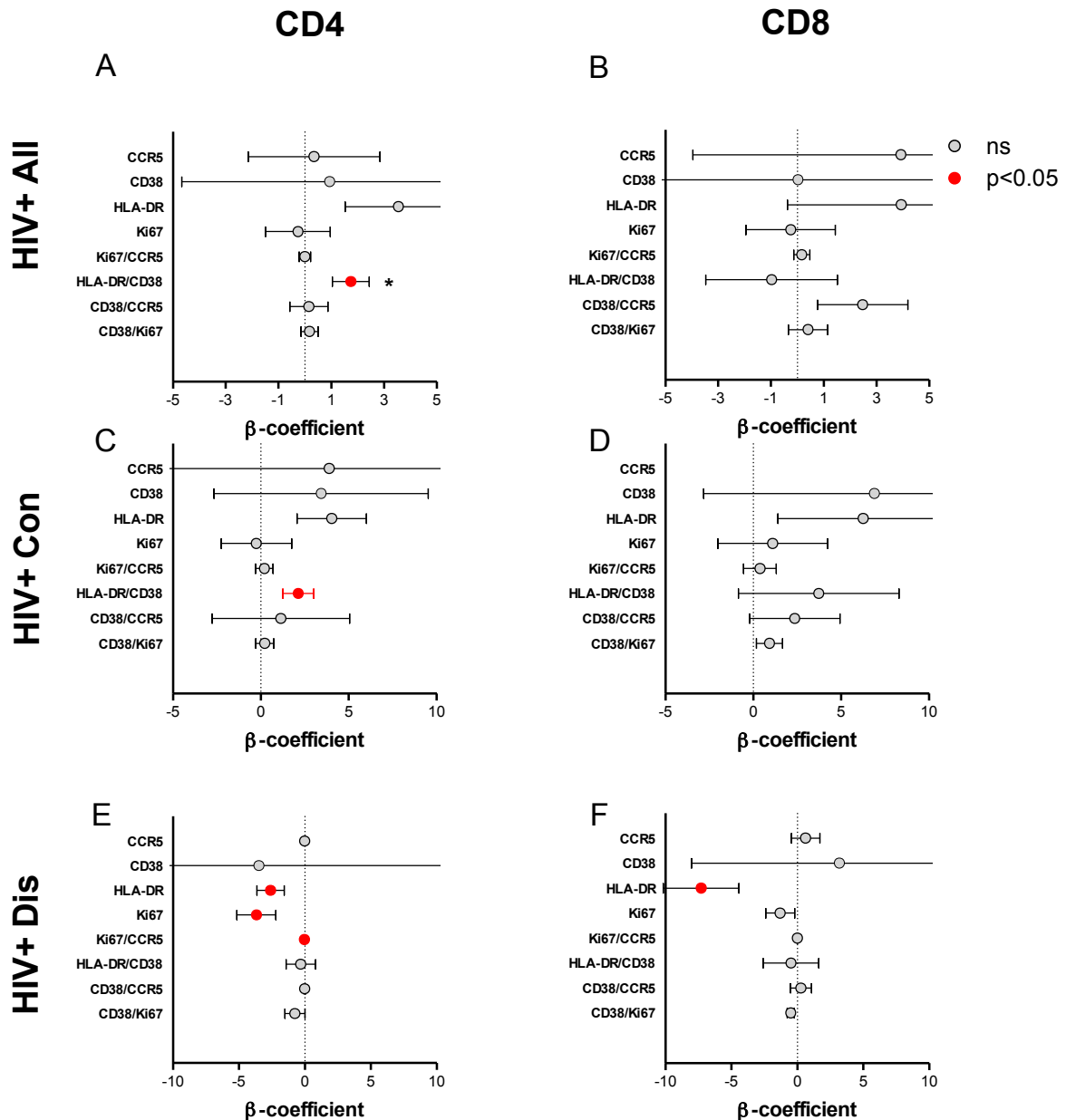


Figure 3. 7 Relationship between T-cell activation in blood and plasma HIV load. The association between plasma HIV load and blood CD4⁺ (left panels) or CD8⁺ (right panels) T-cells expressing immune activation markers CCR5, CD38, HLA-DR, Ki67, or their combination was assessed in all HIV+ individuals (A,B), in HIV+ seroconcordant individuals (C,D), and in HIV+ individuals whose partners were negative (serodiscordant; n=8; E,F) by univariate quantile regression analyses. Regression β -coefficients depicted in red circles represent significant associations (p -values ≤ 0.05) before adjusting for multiple comparisons. Bars represent standard error. All p -values were adjusted for multiple comparisons using the false discovery rate step-down approach (Columb and Sagadai, 2006). * denotes associations that stayed significant after adjusting for multiple

comparisons. β -coefficients are interpreted as the amount by which plasma viral load (HIV RNA copies/ml) increases with every percent increase in activation marker frequency.

3.3.4 Impact of clinical status ($CD4^+$ T-cell frequencies) on T-cell activation

To determine the relationship between $CD4^+$ T-cell frequencies (as an indicator of absolute $CD4^+$ T-cell counts) and T-cell activation, a univariate quantile regression model was used with CD4 percentages as the independent variable (Figure 3.8). In both $CD4^+$ and $CD8^+$ T-cells subsets, irrespective of partner HIV status, expression of HLA-DR (either alone or in combination with CD38) was consistently predictive of lower $CD4^+$ T-cell frequencies in blood, after adjusting for multiple comparisons and HIV plasma load. Unlike plasma viral loads, this finding suggests that T-cell activation (defined as HLA-DR/CD38) broadly predicted lower $CD4^+$ T-cell frequencies in blood, and further suggested that partner status did not impact on this relationship. This may partially explain why the 0.5 log₁₀ difference between plasma viral loads in HIV+ individuals in seroconcordant and serodiscordant relationships did not predict more severe subsequent CD4 decline.

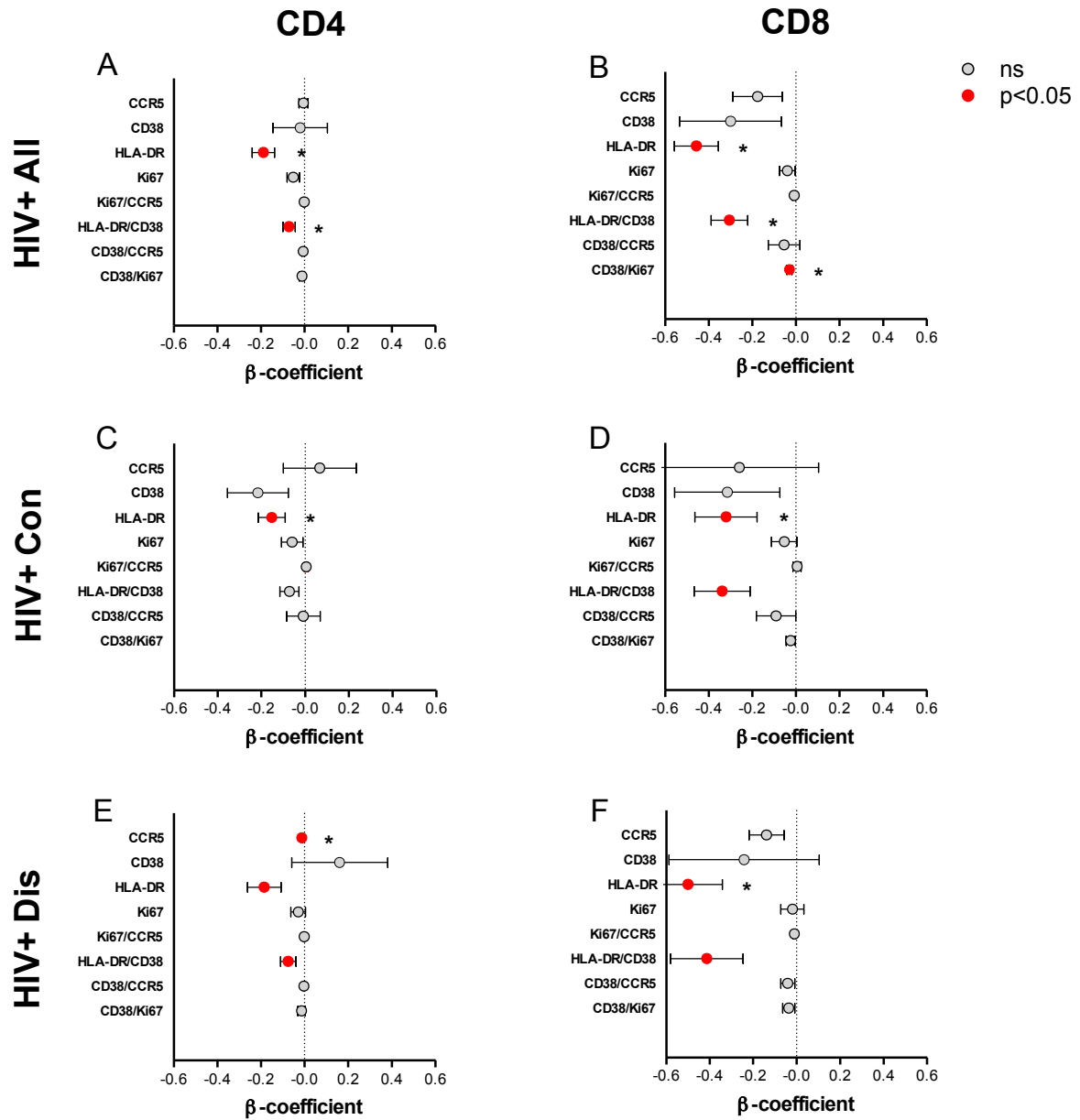


Figure 3. 8 Relationship between T-cell activation and CD4⁺ T-cell frequencies in blood of HIV+ individuals. The association between blood CD4 frequencies and CD4⁺ (left panels) or CD8⁺ (right panels) T-cells expressing immune activation markers CCR5, CD38, HLA-DR, Ki67, or their combination was assessed in HIV+ seroconcordant (C,D), HIV+ serodiscordant (E,F), and their combination (A,B) by univariate quantile regression analyses. Regression β -coefficients depicted in red circles represent significant associations (p -values ≤ 0.05). Bars represent standard error. All p -values were adjusted for multiple comparisons using the false discovery rate step-down approach (Columb and Sagadai, 2006). * denotes associations that stayed significant after adjusting for multiple comparisons. β -coefficients are interpreted as the amount by which plasma viral load (HIV RNA copies/ml) increases with every percent increase in activation marker frequency.

3.3.5 Cytokine concentrations in blood from HIV+ individuals

The concentrations of IL- β , IL-6, IL-12p70, TNF- α , IL-10, IL-2, IFN- γ , IL-7 and GM-CSF were measured in plasma from HIV+ individuals in either serodiscordant or seroconcordant relationships (Table 3.2). Concentrations of cytokines were generally elevated in seroconcordant individuals compared to serodiscordant individuals, significantly so for IL-1 β (p=0.04), TNF- α (p=0.03), IFN- γ (p=0.05) and IL-10 (p=0.02). None of the differences stayed significant after adjustment for multiple comparisons.

Table 3. 2 Impact of couple HIV status on systemic cytokine levels in HIV+ individuals

Function	Cytokine	Median cytokine conc (IQR; pg/ml)		P-value*
		HIV Positive		
		Concordant	Discordant	
Inflammatory	IL- β	0.72 (0.075-2.85)	0.38 (0.04-1.47)	0.04
	IL-6	5.26 (2.83-11.10)	4.48 (2.57-8.62)	0.2
	IL-12p70	0.005 (0.005-0.42)	0.005 (0.005-0.005)	0.5
	TNF- α	9.45 (5.61-15.94)	7.64 (4.94-13.07)	0.03
Regulatory	IL-10	12.60 (7.75-23.44)	10.53 (6.47-15.74)	0.02
Adaptive	IL-2	0.11 (0.005-0.70)	0.14 (0.005-0.87)	0.6
	IFN- γ	1.39 (0.27-6.33)	1.09 (0.14-2.80)	0.05
Hematopoietic	IL-7	1.58 (0.61-3.09)	1.45 (0.72-3.25)	0.9
	GM-CSF	0.19 (0.005-0.64)	0.30 (0.005-0.86)	0.1

*Mann-Whitney U tests were applied to compare cytokine concentrations between the seroconcordant and serodiscordant groups.

3.3.6 Relationship between plasma cytokines and markers of HIV disease progression

The relationship between cytokine concentrations in plasma and markers of HIV disease progression (plasma viral load and frequencies of CD4⁺ T-cells in blood) was investigated using quantile regression analysis (with viral load or CD4 frequencies as the predictor variables). In HIV+ individuals in seroconcordant relationships, TNF- α was positively associated with plasma viral load, after adjustments for multiple comparisons (β -coefficient 3.79; or a 3.79% increase in viral load for every 1% increase in TNF- α concentrations; Figure 3.9A). In contrast to the positive effect of TNF- α on plasma viral loads, a significant negative relationship was observed between IL-2 concentrations and viral load in this group, after

adjustment for multiple comparisons (β -coefficient of -0.31; or a decrease of 0.31% in plasma viral load for every 1% increase in plasma IL-2 concentrations). In HIV+ individuals in serodiscordant relationships, TNF- α and IL-2 were not similarly associated with changes in plasma viral load (Figure 3.9B), although plasma concentrations of TNF- α were significantly lower in this group than in seroconcordant HIV+ individuals (Table 3.2). Concentrations of IL-12p70 in plasma negatively predicted plasma viral loads in serodiscordant HIV+ individuals, although this was not significant after adjusting for multiple comparisons. In contrast to their relationship with plasma viral loads, none of the cytokines measured correlated with the frequency of CD4⁺ T-cells in blood in any of the groups evaluated in this study (data not shown).

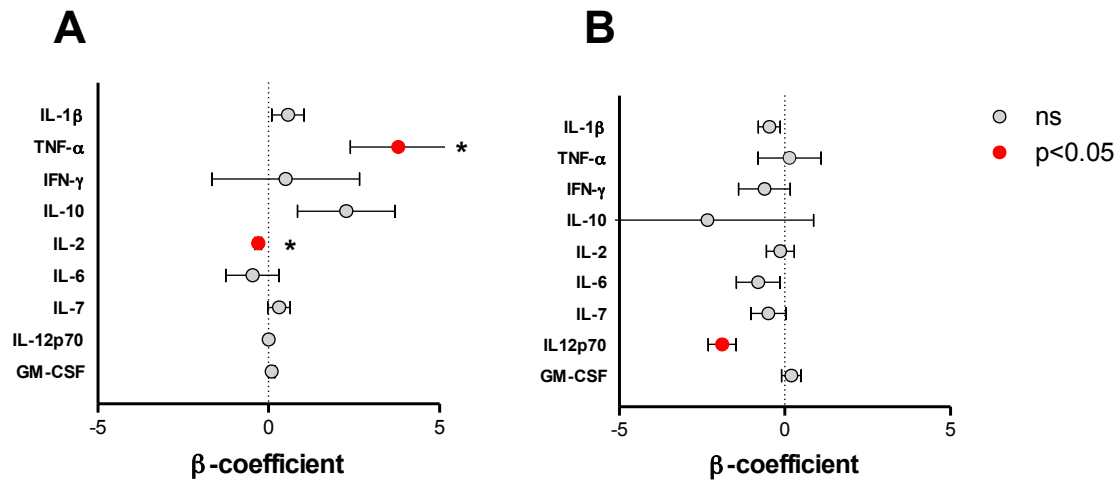


Figure 3. 9 Relationship between plasma cytokine concentrations and viral loads. The association between blood HIV load and levels of different cytokines in plasma was assessed in HIV+ seroconcordant (A) and HIV+ serodiscordant (B) by univariate quantile regression analyses. Regression β -coefficients depicted in red circles represent significant associations (p -values ≤ 0.05). Bars represent standard error. All p -values were adjusted for multiple comparisons using the false discovery rate step-down approach (Columb and Sagadai, 2006). * denotes associations that stayed significant after adjusting for multiple comparisons. β -coefficients are interpreted as the amount by which plasma viral load (HIV RNA copies/ml) increases with every percentage increase in activation marker frequency.

3.3.7 Modeling the impact of systemic immune activation on plasma HIV loads and CD4 counts

In the light of the various independent associations observed in this study between systemic immune activation or inflammation and plasma HIV loads, multivariate linear regression was used to determine whether there was a combined effect of these two markers in shaping HIV viremia while adjusting for blood CD4%. The linear regression model included log transformed plasma HIV load as the independent variable and the predictor variables included known to influence plasma viremia: (1) T-cell activation (both CD4⁺ and CD8⁺), (2) plasma cytokines level and (3) CD4⁺ T-cell frequency.

In this model (Table 3.3), plasma HIV load was positively associated with CD4⁺ T-cell activation (defined by the co-expression of HLA-DR and CD38), with a 1.45% increase in plasma HIV load for every 1% increase in the frequency of CD4⁺HLA-DR⁺CD38⁺ in blood. Similarly, plasma HIV load was significantly positively associated with plasma TNF- α concentrations, with a 2.5% increase in plasma HIV load for every 1% increase in plasma TNF- α concentrations. Both these associations were shown to be independent of blood CD4%.

Table 3. 3 Contributing factors to plasma HIV level

Predictor variables	β-coefficient[‡]	CI95%[†]	P-value[*]
CD4 ⁺ HLA-DR ⁺ CD38 ⁺ (log transformed %)	1.45	0.21 - 2.69	0.023
TNF- α (log transformed pg/ml)	2.50	1.06 - 3.95	0.001
CD4% (log transformed %)	0.49	-0.77 - 1.76	0.43

‡ β -coefficients for multiple linear regression analyses represent the change in mean log of plasma HIV copies/ml with every unit increase of the predictor variable, adjusting for all other parameters. In this model, for example, a β -coefficient of 2.50 for TNF- α represents a 2.5% increase in the mean log of plasma HIV copies/ml with every 1% increase in log transformed plasma level of TNF- α .

† CI95% = 95% Confidence Interval.

*P-values ≤ 0.05 were considered significant.

In Chapter 2, using a selected group of individuals for whom CD4 counts and plasma HIV loads were available for two consecutive time points, 35% of the variability in CD4 depletion in blood was attributable to changes in HIV plasma loads. To establish what other factors may be predicting the remaining 65% of CD4 decline that remained unaccounted for, a multivariate linear regression model was developed to elucidate the effect of systemic

immune activation and inflammation on blood CD4 decline. All data included in the model were log transformed and the selection of the predictor variables for the model was based on the strength of their univariate association with CD4% (Sections 3.3.4 and 3.3.6).

In this model (Table 3.4), CD4 frequencies were negatively associated with CD4 and CD8 T-cell activation (defined by the expression of HLA-DR on both subsets), with a 1.09% and 0.56% decrease in CD4% for a respective 1% increase in CD4⁺HLA-DR⁺ and CD8⁺HLA-DR⁺ respectively. These associations remained significant after adjustment for plasma HIV loads. In the overall model, systemic immune activation, as defined by the frequency of CD4⁺HLA-DR⁺ and CD8⁺HLA-DR⁺, after adjusting for plasma HIV load accounted for 51% of the changes in CD4%, an improvement of 16% on the univariate model which only included plasma viremia as predictor variable.

Table 3. 4 Contributing factors to blood CD4%

Predictor variables	β-coefficient[‡]	CI95%[†]	P-value[*]
CD4 ⁺ HLA-DR ⁺ (log transformed %)	-1.09	-1.95 - -0.22	0.016
CD8 ⁺ HLA-DR ⁺ (log transformed %)	-0.56	-1.04 - -0.80	0.024
Plasma HIV load (log transformed cps/ml)	2.32	-2.14 - 6.38	0.25

‡β-coefficients for multiple linear regression analyses represent the change in mean log of blood CD4% with every unit increase of the predictor variable, adjusting for all other parameters. In this model, for example, a β-coefficient of -1.09 for CD4⁺HLA-DR⁺ represents a 1.09% decrease in the mean log of CD4% with every 1% increase in log transformed blood frequency of CD4⁺HLA-DR⁺.

† CI95% = 95% Confidence Interval.

*P-values ≤0.05 were considered significant.

3.4 Discussion

It has been established that HIV infection is characterized by chronic systemic immune activation, and that this hallmark of HIV infection can lead to accelerated T-cell apoptosis (Grossman *et al.*, 2002), which in turn contributes to further CD4 decline (Sousa *et al.*, 2002), and is strongly predictive of the rate of HIV disease progression (Hazenberg *et al.*, 2003). Persistent immune activation is primarily manifested by an increase turnover of T-cells and high levels of CD4⁺ and CD8⁺ T-cell apoptosis. In addition, immune activation is also defined by hyperactivation of T-cells, as measured most commonly by expression of CD38 and HLA-DR. While CD4⁺ T-cells are the primary targets for HIV infection and resultant death, it is important to also evaluate the activation status of CD8⁺ T-cells which are chronically activated by their function as cytotoxic cells against HIV-infected targets. Recent studies have also shown that biomarkers associated with inflammation like IL-6, D-dimer and changes in CRP predict mortality in HIV+ individuals (Kuller *et al.*, 2008; Ledwaba *et al.*, 2012).

Significantly higher plasma HIV loads were found in HIV+ individuals with seroconcordant partners compared to individuals in serodiscordant relationships in Chapter 2. The underlying mechanism accounting for this difference in viremia or the impact of viral load differences on rate of disease progression between HIV+ seroconcordant and serodiscordant individuals remained unclear. This Chapter investigated the influence of systemic immune activation and inflammation in this cohort on the rate of HIV disease progression. Measurement of total activation (defined by the cumulative expression of the four markers) in this cohort of chronically HIV+ individuals showed that both CD4⁺ and CD8⁺ T-cells were similarly activated, and that CD4⁺ T-cells (but not CD8⁺ T-cells) from HIV+ seroconcordant individuals were more activated than CD4⁺ T-cells from serodiscordant individuals. The differences seen in total activation between HIV+ seroconcordant and serodiscordant participants was mainly attributed to CCR5 expression. The higher expression of this HIV co-receptor on T-cells of HIV seroconcordant individuals than serodiscordant individuals provides a plausible explanation or underlying mechanism that could account for the significant differences in plasma viremia in HIV seroconcordant versus serodiscordant individuals. The level of expression of CCR5 has previously been shown to be an important correlate of HIV infectability of target cells (Wu *et al.*, 1997).

Binding of HIV to CCR5 on CD4⁺ T-cells is known to activate CD4⁺ T-cells, by an HIV Env-mediated signalling pathway, promoting the (1) expression of CD38 and HLA-DR; and (2) release of soluble factors including cytokines (Holm and Gabuzda, 2005). These released factors may in turn activate bystander CD8⁺ T-cells. T-cell activation is a precursor to clonal expansion, which during a normal immune response would end in death of a large number of T-cells through apoptosis after the infection was cleared. This dynamics of activation, clonal expansion and apoptosis differ for CD8⁺ and CD4⁺ T-cells (Catalfamo *et al.*, 2008, 2011). The dynamics of immune activation is principally governed by the inflammatory response to HIV infection and the homeostatic response to CD4 T-cell depletion (Catalfamo *et al.*, 2008). The greater proportion of activated CD8⁺ T-cells compared to activated CD4⁺ T-cells in either of the HIV seroconcordant or serodiscordant groups could be explained by the greater longevity of the expanded CD8⁺ T-cell pool upon activation to give rise to a stable resting pool of memory cells, whereas activated CD4 cells would also be infected with HIV and therefore die more quickly than their activated CD8 counterparts (Homann *et al.*, 2001; Foulds *et al.*, 2002).

Expression of HLA-DR alone (without CD38) by CD8⁺ T-cells has been suggested in several studies to be a surrogate marker for long-term non-progression and survival (Giorgi *et al.*, 1994, 1999; Karim *et al.*, 2013). These studies have suggested that CD8⁺HLA-DR⁺ T-cells have enhanced cytotoxic capacity and ability of rapid expansion to control HIV infection and counter HIV infected cells in tissue reservoirs than cells expressing other marker combinations. Despite observed significant differences in plasma viral loads between HIV+ seroconcordant and serodiscordant individuals, this Chapter showed that higher frequencies of CD8⁺ T-cells from HIV serodiscordant individuals were activated (defined by CD38⁺ expression) compared to seroconcordant individuals. Other studies have reported that a higher frequency of CD38 expression by CD8⁺ T-cells was associated with faster systemic CD4⁺ T-cell depletion and worse prognosis in terms of AIDS defining events (Tuailleon *et al.*, 2009; Karim *et al.*, 2013). While the HIV+ serodiscordant individuals in this study had signatures of elevated immune activation, they did not experience worse disease outcome when compared to their seroconcordant counterparts using CD4 decline as a marker and also sustained lower viral loads over time than their seroconcordant counterparts. Although CD38 expression as a marker of immune activation is a useful marker to predict HIV disease course, the value of this marker alone in this current study was undermined by the important differences between CCR5 expression by the HIV-target cells. CD4⁺CD38⁺CCR5⁺ T-cells

represent the perfect target cells for HIV to infect because of their expression of the HIV co-receptor and their activated state implies rapid HIV turnover.

When HIV+ individuals in this Chapter were categorized according to their HIV plasma load, those individuals with >1500 cps/ml HIV load had higher frequencies of both total CD4⁺ and CD8⁺ T-cell activation than individuals with <1500 cps/ml. CCR5 expression in particular was significantly higher in participants with >1500 cps/ml HIV load than those with <1500 copies/ml, which could account for the difference in viral loads (higher propensity of CD4⁺ T-cells to generate more virus). HIV+ individuals with a higher plasma HIV load (>1500 cps/ml) had significantly higher frequencies of activation markers on their CD8⁺ T-cells (CD8⁺CD38⁺ and CD8⁺HLA-DR⁺CD38⁺). This is in line with previous studies reporting a positive correlation between activation marker expression on CD8⁺ T-cells and plasma HIV loads (Bouscarat *et al.*, 1996; Eggena *et al.*, 2005; Resino *et al.*, 2004). Regression analyses repeatedly revealed the association of HLA-DR expression on T-cells (with or without the co-expression of CD38) with plasma HIV load.

Systemic CD4⁺ T-cell frequencies (indicating CD4⁺ T-cell counts) were negatively associated with HLA-DR expression on both CD4⁺ and CD8⁺ T-cells (both with or without co-expression of CD38), irrespective of partner HIV status. In terms of predictive capacity, previous studies have shown that a higher level of systemic activation predicts a faster decrease of CD4⁺ T-cells (Hunt *et al.*, 2003; Wilson *et al.*, 2004; Deeks and Walker, 2004). Analyses presented in this Chapter on the other hand suggest that activation marker expression by CD4⁺ T-cells and the inflammatory cytokine TNF- α were better predictors of HIV plasma load than CD4 frequencies. A possible explanation for this could be that T-cell decline is restricted to CD4⁺ T-cells only, whereas HIV-induced immune activation involves both CD4⁺ and CD8⁺ T-cells. Total CD8⁺ T-cell numbers have been reported to typically remain elevated until late in infection and this can be partly attributed to direct cytopathic effects of HIV, as the subset of productively infected CD4⁺ T-cells is too low to account for all of the CD4⁺ T-cell loss (Chun *et al.*, 1997; Clark *et al.*, 1999). A study in mice showed that following clearance of viral infection, the number of antigen-specific CD4⁺ memory T-cells declined readily whereas the number of CD8⁺ memory and effector T-cells remained elevated for life (Homann *et al.*, 2001). From this finding, we can postulate different rates of CD4⁺ and CD8⁺ T-cell expansion in the event of viral infection. Taken together, these data are suggestive of considerably different lifespan of expanded CD4⁺ and CD8⁺ T-cells in the course of HIV infection and the accompanying condition of chronic immune activation. In

turn, this could provide the key to the different kinetics and death rates of memory and effector T-cells, whether CD4⁺ or CD8⁺.

The higher concentrations of IL-1 β , IFN- γ , and TNF- in plasma in HIV+ seroconcordant individuals compared to serodiscordant individuals is associated with higher plasma viral loads detected in seroconcordant versus serodiscordant individuals. Moreover, as we expect an inflammatory response to be counterbalanced by a more or less equal and opposing response, it is not surprising to observe concurrently higher level of regulatory cytokine IL-10 in seroconcordant individuals compared to serodiscordant ones. Soluble markers of inflammation and coagulation such as IL-6, sCD14 and D-dimer have been shown to be useful biomarkers for systemic inflammation and its adverse effects in the context of HIV infection recently (Nixon and Landay, 2010; Kamat *et al.*, 2012; Taiwo *et al.*, 2013). These studies indicate that systemic inflammation could be playing a pivotal role in the setting of immune activation and represents a critical component of HIV disease pathogenesis as supported by the opposite trend seen in the associations of the different cytokines and plasma HIV load in seroconcordant and serodiscordant individuals.

Multivariate regression modelling revealed that CD4⁺HLA-DR⁺CD38⁺ T-cell frequency and TNF- α level in systemic circulation were significant determinants of plasma HIV load. As for predictors of CD4%, the overall model, which included CD4⁺HLA-DR⁺ and CD8⁺HLA-DR⁺ frequencies, after adjusting for plasma viral load, accounted for 51% of the variation in blood CD4%, an improvement of 16% on the previous model which only included plasma viral load as predictor. This confirms that biological markers of systemic immune activation could prove helpful in the determination of when to start therapy, when used in conjuncture with CD4 count and HIV plasma load.

The finding that the majority (68%) of the serodiscordant couples in our study had the female partner as the HIV+ one is in line with recent studies in South Africa, which report a gradual feminization of the index partner in HIV serodiscordant couples from a 29% in 2003 (Lurie *et al.*, 2003) to 67% in 2012 (de Bruyn *et al.*, 2012). Similarly, recent statistics available for female HIV+ serodiscordancy in the sub-Saharan region indicate that 77% of the index partners in HIV serodiscordant couples are female (Guthrie *et al.*, 2009). Factors that could be responsible for women being predominantly the HIV+ partner in serodiscordant couples are intergenerational sex (younger females having sex with older males), where unequal power dynamics leads to lower power of the females negotiating for condom use. Moreover,

the relatively high median age of the participants enrolled in our study coupled with a greater risk of heterosexual HIV acquisition in women and the lower risk of male heterosexual HIV acquisition as opposed to male-to-female transmission (Leynaert *et al.*, 1998; Varghese *et al.*, 2002) could be a determinant in the maintenance of the ‘serodiscordant’ status of the couple. Although biological factors are likely also to contribute to the disproportionate number of females in this study, it is also likely that this reflects unintended enrolment or recruitment bias when the study was conducted.

One of the most important findings from this chapter was the different pattern of expression of the chemokine receptor CCR5, used as coreceptor for HIV entry into CD4⁺ T-cells, between HIV⁺ discordant versus concordant individuals (Moore *et al.*, 1997). The elevated expression of this co-receptor on CD4⁺ T-cells of seroconcordant compared to serodiscordant individuals has important implication for HIV pathogenesis and lymphocyte recirculation. The significantly elevated frequencies of CCR5 expressing CD4⁺ T cells in seroconcordant individuals implies distinct susceptibility of T-cell subsets in these individuals to viral entry. This in turn may translate into a greater viral turnover, and as observed in this Chapter, HIV viral load is positively associated with inflammation. The initiation and long term persistence of inflammation in blood during HIV infection leads to the manifestation of a multiplicity of morbidities such as cardiovascular diseases (Friis-Moller *et al.*, 2003; Palella and Phair, 2011; Duprez *et al.*, 2012), fibrosis of the lymphatic tissues (Schacker *et al.*, 2002) and thymosuppressive effects (Linton and Dorshkind, 2004). Other than CCR5 expression, HIV susceptibility could be promoted by factors which enhance the number of potential target cells. In this Chapter, CCR5⁺ T-cells from seroconcordant individuals were found to be more activated than CCR5⁺ T-cells from serodiscordant individuals. This is an important finding because HIV has been shown to preferentially replicate in activated CD4⁺ T-cells (Zhang *et al.*, 1999).

Regulation of CCR5 expression is dynamic and it has been previously shown that several factors can mediate the induction of CCR5. For example, IFN- α was demonstrated to increase CCR5 expression on CD4 T-cells in thymic organ culture and neutralisation of IFN- α in R5 HIV-infected SCID-hu mice using monoclonal antibodies inhibited both CCR5 upregulation and infection of the T-cell progenitors (Stoddart *et al.*, 2010). Interleukin-2 has also been shown to upregulate CCR5 expression on macrophages *in vitro* (Weissman *et al.*, 2000). While it is clear that an association exists between the level of CCR5 expression and HIV load in blood and other anatomical compartments, with greater levels of CCR5 expression

being associated with higher HIV RNA levels (Stewart *et al.*, 1997; de Roda Husman *et al.*, 1999; Reynes *et al.*, 2000), the causal relationship between these two biological markers is best confirmed in longitudinal studies. Finally, host genetic predisposition with regard to CCR5 expression level warrants further attention in the elucidation of the cyclic nature of its relationship with HIV burden.

Chemokines and their respective receptors have been postulated to direct migration of specific immune cells to sites of inflammation (Springer, 1994; Mackay, 1996), and virtually all T-cell chemotactants studied to date selectively attract memory/activated T-cells (Schall *et al.*, 1993; Qin *et al.*, 1996, 1998). CCR5 has been characterised as a receptor responding functionally to a group of CC-chemokines which includes MIP-1 β (Samson *et al.*, 1996; Blanpain *et al.*, 1999). In the previous Chapter, a significantly greater frequency of MIP-1 β was observed when intracellular cytokine staining assays were performed, in both CD4⁺ and CD8⁺ T-cell subsets of seroconcordant individuals when compared to serodiscordant individuals. The expression of chemokine receptor CCR5, and potentially its ligands MIP-1 β , are widely regarded as central to the pathogenesis of HIV infection as their regulation is thought to influence leukocyte migration, as well as HIV infection.

In conclusion, this Chapter suggests that continuous immune hyperactivation in the event of chronic HIV infection may lead to continuous activation and differentiation of T-cells in a cycle involving systemic immune activation, inflammation and constant replication of HIV. This could be accelerating the depletion of the naïve T-cell pool, and this could be exacerbated by the limited regenerative capacity in replacing lost naïve T-cells in adults (Haase, 1999; Hazenberg *et al.*, 2000). The findings above suggest that a number of these factors could be independently used in the assessment of ‘immune profile’ of individuals with asymptomatic HIV infection. As suggested by previous studies (Ahuja *et al.*, 2008; Brass *et al.*, 2008), assessment of an ‘immune score’ combined with other factors such as co-morbidities, age and genetic profile, could help in the design of individualized therapeutic interventions prior to clinical manifestations of HIV immunodeficiency.

CHAPTER 4

Impact of partner HIV status on female genital tract inflammation and cervicovaginal HIV shedding during chronic HIV infection

4.1 Introduction

With heterosexual transmission of HIV accounting for the majority of new infection worldwide (Gouws *et al.*, 2006), the female genital tract plays an important role in HIV acquisition and transmission. Recent statistics suggest that women may be at a greater risk for infection than men, with 30%-40% of the yearly global infection being estimated to occur across mucosal surfaces of the female genital tract via exposure to HIV-containing semen (Hladik and Hope, 2009). While plasma levels of HIV remain a strong predictor of transmission rates, studies have shown that the local mucosal factors like genital inflammation and concurrent STIs are also important independent determinants of HIV shedding at the genital tract and thus predict greater susceptibility to infection via the genital mucosa (LeGoff *et al.*, 2007; Johnson *et al.*, 2008; Blish *et al.*, 2012; Mitchell *et al.*, 2011, 2013). Immune activation in the female genital tract, measured at the cellular level and as soluble innate mediators, in response to HIV infection has previously been reported by others from our group (Jaspan *et al.*, 2011). Although such responses generally are a vital part of the protective response following infection, this response may also contribute to HIV susceptibility and/or pathogenesis through a steady supply of susceptible target cells to the virus (Lawn *et al.*, 2001).

In this Chapter, the impact of partner's HIV status and interplay between inflammation and local HIV shedding in the genital tract was investigated. The concentrations of eotaxin, fractalkine, IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β , IL-1 α , IL- β , IL-6, IL-12p40, IL-10, G-CSF and IL-15 were compared in genital secretions from HIV+ women in seroconcordant and serodiscordant relationships and their relative impact compared to plasma viral loads on HIV shedding into genital secretions was evaluated.

4.2 Materials and Methods

4.2.1 Description of study participants

A total of 132 HIV+ women were included for this part of the study, of which 78/132 were in seroconcordant relationships and 54/132 were in serodiscordant relationships. Section 2.2.1 provides a relevant description of the full cohort. Women who were menstruating at the study visit, who were post-menopausal or had undergone a hysterectomy were excluded from the study. The samples used in this Chapter were collected at months 6, 18 and 30 after enrollment.

4.2.2 Cytobrush collection and processing

Cervical mononuclear cells (CMCs) were collected using a Digene cervical sampler (Digene Corporation, Gaithersburg, MD, USA) as described by Nkwanyana *et al.* (2009). The cervical sampler was inserted into the endocervical os under speculum examination and rotated 360°, sampling from the transformation zone. Cytobrushes were immediately transferred into transport tubes containing 3ml of cold transport medium – R10 [RPMI1640 (Life Technologies Corporation, Carlsbad, CA, USA) medium supplemented with 5mM glutamine, 100 units/ml penicillin, 100 µg streptomycin, 10 mg/ml fungin and 10% fetal calf serum (FCS)] (Life Technologies Corporation, Carlsbad, CA, USA) and immediately transferred to a 4°C Nalgene Benchtop cooler (Sigma-Aldrich, MO, USA) for transport to the laboratory. Cervical samples that were visibly contaminated with blood were excluded from further study. Cytobrushes were processed within 4 hours of collection.

Each cytobrush was flushed 30 times with R10 in the collection tube using a sterile Pasteur pipette. The cell suspension was then transferred to a 15ml Sterilin tube and centrifuged at 1200 rpm (250g) for 10 minutes. The supernatant containing soluble factors from the cervical cytobrush and associated cervical mucus was aliquoted into three equal fractions and stored in cryovials at -80°C for future assessment of cervical cytokine levels and HIV shedding. Pelleted cells were used for studies outside of this dissertation (Gumbi *et al.*, 2011, Jaspan *et al.*, 2011).

4.2.3 PBMCs collection and processing

Blood (16ml) was collected from all study participants using standard venipuncture into sterile ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK). Blood was processed within 4 hours of collection; PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation as described in Chapter 2 (Section 2.2.2).

4.2.4 Measurement of cytokine concentrations in cervical supernatant

The concentrations of Eotaxin, Fractalkine, IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β , IL-1 α , IL- β , IL-6, IL-12p40, IL-10, G-CSF and IL-15 in cervical supernatants were measured using Human Cytokine LINCOplex Premixed kits (LINCO Research, MO, USA). The sensitivity of the kits ranged between 0.19 and 27.7 pg/ml for the cytokines measured. Data was collected using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories Inc) and a 5 PL regression formula was used to calculate cytokine concentrations from the standard curves. Data was analysed using BIO-plex manager software (version 4; Bio-Rad Laboratories Inc). Cytokine concentrations that were below the detection limit of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero.

4.2.5 Determination of HIV viral load in blood plasma and cervical supernatant

HIV viral load in blood plasma and cervical supernatant was assayed using the NuclisensEasyq HIV Version 1.2 by the NHLS Diagnostic Virology Laboratory (Groote Schuur Hospital, Cape Town, South Africa). The detection limit of the assay was 50 HIV RNA copies/ml.

4.2.6 Statistical analysis

Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Comparison of unpaired non-parametric data was done using the Mann-Whitney U test. Statistical inferences on binary sets of data were performed using the Fisher's exact test and odds ratios calculated. Non-parametric assessments of variation

between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons. Spearman Rank tests were used for correlations. The associations between cervical viral load and cervical cytokine concentration were examined using a zero-inflated negative binomial regression to take into account the considerable number of zero viral loads that skewed data for individual measurement. This analysis models the probability of no HIV shedding into the genital tract (zero response) and the mean of the HIV load in those shedding virus (non-zero responses) simultaneously, using a logistic model for binary response (zero and non-zero) and a negative binomial regression model for the continuous component of the response (HIV level). Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 11 (College Station, TX, USA). All tests were two-tailed and p-values of ≤ 0.05 were considered significant.

4.3 Results

4.3.1 Characteristics of HIV+ women in this study

One hundred and thirty two HIV+ women were included to study the impact of partner HIV status on HIV-specific responses in the female genital tract, inflammation and HIV shedding in genital secretions. Of these 132 participants, 59% (78/132) were in a HIV seroconcordant relationship and 41% (54/132) were in a HIV serodiscordant relationship. No difference in age, cohabitation and duration of sexual activity was observed between groups. All the women included in this study were naïve to anti-retroviral therapy. While women from both groups reported a similar median number of sex acts in the last month (Table 4.1), HIV+ women with seroconcordant partners reported lower frequency of condom usage (60%) compared to HIV+ women with serodiscordant partners (74%; $p=0.05$). No differences were found in the incidence of vaginal discharge and genital ulceration in the last 6 months when comparing seroconcordant women to their serodiscordant counterparts, although considerably more women with seroconcordant partners (78%) were shedding HIV in their genital secretions than women with serodiscordant partners (41%; $p=0.005$). HIV+ seroconcordant women had significantly higher plasma viral loads than serodiscordant women (66000 cps/ml compared to 25000 cps/ml, respectively, $p=0.01$).

Table 4. 1 Clinical and socio-behavioural characteristics of participants included in this study

Characteristics	n	Concordant	n	Discordant	P-value
n		78		54	
Age [yr; median (IQR)] *	78	31 (25-35)	54	32 (27-38)	1.0
Living together with partner [%] †	76	72	53	65	0.2
Sexual exposure [median years of sex (IQR)] *	77	16 (10-21)	53	16 (10-22)	0.9
Sex acts in the last month [median (IQR)] *	76	4 (2-8)	52	4 (3-10)	0.4
Condom usage [%] †	73	60	49	74	0.05
Genital ulceration in the last 6 months [%] †	78	11	53	9	0.8
Vaginal discharge in the last 6 months [%] †	78	25	53	23	1.0
Plasma HIV RNA level [cps/ml] *	36	66000	19	25000	0.01
Genital HIV shedding [%] †	36	78	22	41	0.005

† p-value calculated using Fischer's exact test

* p-value calculated using Mann-Whitney U-test

IQR: Interquartile range

4.3.2 Impact of partner HIV status on HIV shedding in genital secretions

Of the 58 HIV+ women included in this part of the study for whom measurement of genital tract HIV loads were performed, 37/58 (64%) were shedding HIV in their genital secretions. A significant positive correlation was observed between plasma and cervical HIV load (Figure 4.1), with 89% of HIV+ women with HIV in their genital secretions having plasma viral loads >1500 copies/ml. HIV+ seroconcordant women were found to be shedding 1.6 Log₁₀ more HIV in their genital secretions than serodiscordant women (corresponding to a difference of 1450 cps/ml; p=0.001; Figure 4.2).

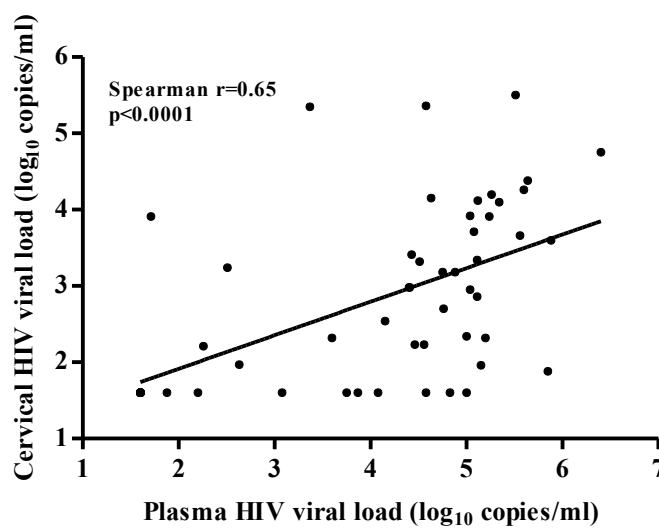


Figure 4. 1 Relationship between viral loads in plasma and genital secretions of HIV+ women. Each data point represents an individual woman's plasma and cervical viral load. Spearman Rank test was used to test the association between HIV load in plasma and cervix, and a p-value<0.05 was considered significant.

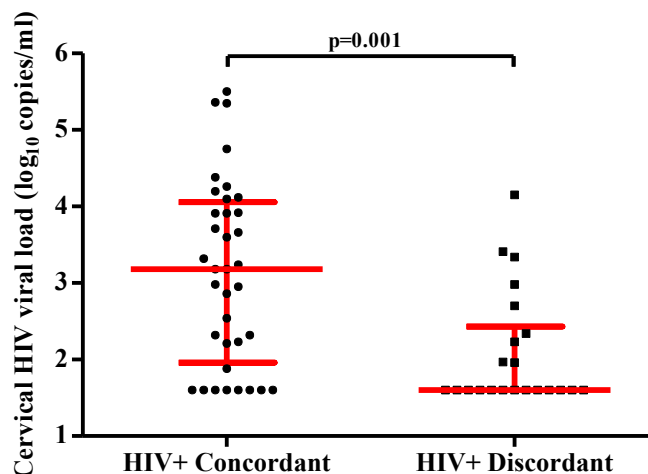


Figure 4. 2 Comparison between HIV viral loads in genital secretions from HIV+ women with HIV seroconcordant or serodiscordant partners. Each data point represents an individual women’s cervical viral load. The red line indicates the median viral load and associated IQR for each group. Mann-Whitney U test was used to compare the cervical viral load between HIV seroconcordant and HIV serodiscordant women, and p-values of <0.05 were considered significant.

4.3.3 Impact of partner HIV status on genital tract cytokines

The concentrations of IL-1 α , IL- β , IL-6, IL-8, IL-12p40, IL-10, Eotaxin, Fractalkine, IP-10, MCP-1, MIP-1 α , MIP-1 β , IL-15, and G-CSF were measured in genital tract secretions from HIV+ women with serodiscordant or seroconcordant partners (Table 4.2). Overall, no significant differences were found between HIV+ women with seroconcordant versus serodiscordant partners, although IP-10, IL-6 and G-CSF concentrations tended to be higher in HIV+ seroconcordant compared to serodiscordant women (Table 4.2).

Table 4. 2 Impact of couple HIV status on cervical cytokine levels in HIV positive women

Cytokine	Median cytokine conc (IQR; pg/ml)		P-value*
	HIV Positive		
	Concordant	Discordant	
Eotaxin	15.5 (3.5-21.22)	12.75 (5.17-25.52)	0.9
Fractalkine	77.41 (29.66-144.8)	56.44 (10.97-149.8)	0.5
IL-8	153.6 (56.67-344.5)	123.3 (61.67-424.3)	0.8
IP-10	441.1 (172.7-1002)	336.8 (100.2-867.7)	0.3
MCP-1	42.83 (16.41-122.6)	43.26 (15.97-96.65)	0.7
MIP-1α	13.64 (1.36-38.16)	15 (1.36-40.28)	0.6
MIP-1β	29.26 (10.10-43.71)	30.24 (3.71-54.74)	0.8
IL-1α	34.96 (15.52-99.38)	39.92 (17.62-122.1)	0.8
IL-1β	4.07 (0.69-7.14)	3.21 (1.1-12.8)	0.3
IL-6	18.39 (4.9-55.6)	11.46 (4.63-33.18)	0.07
IL-12p40	34.51 (5.64-67.33)	29.93 (3.55-80.7)	0.8
IL-10	2.6 (1.26-5.65)	2.91 (1.05-6.29)	1
G-CSF	759.8 (200.7-1847)	403.4 (117.5-1522)	0.1
IL-15	2.07 (1.24-4.1)	1.78 (0.88-4.55)	0.9

*Mann-Whitney U tests were applied to compare cytokine concentrations between the seroconcordant and serodiscordant groups.

4.3.4 Relationship between genital tract cytokine concentrations and HIV shedding

The relationship between cytokine concentrations and the concentration of HIV in genital secretions was investigated using linear regression analysis (Figure 4.3). Genital tract concentrations of IL-1 α and IL-1 β of HIV+ women (irrespective of partner's HIV status) were positively associated with genital HIV load, after adjusting for plasma viral loads (IL-1 α : β -coefficient 0.68 for seroconcordant and 0.50 for serodiscordant women; IL-1 β : β -coefficient 0.50 for seroconcordant and 0.52 for serodiscordant women). A β -coefficient of 0.68 for IL-1 α is equivalent to a 0.68% increase in genital tract viral load for every 1% increase in IL-1 α concentration. Additionally, levels of IL-8 (β -coefficient 0.67) and IL-10 (β -coefficient 0.51) were positively associated with genital tract HIV load in HIV+ serodiscordant women, independently of plasma HIV load.

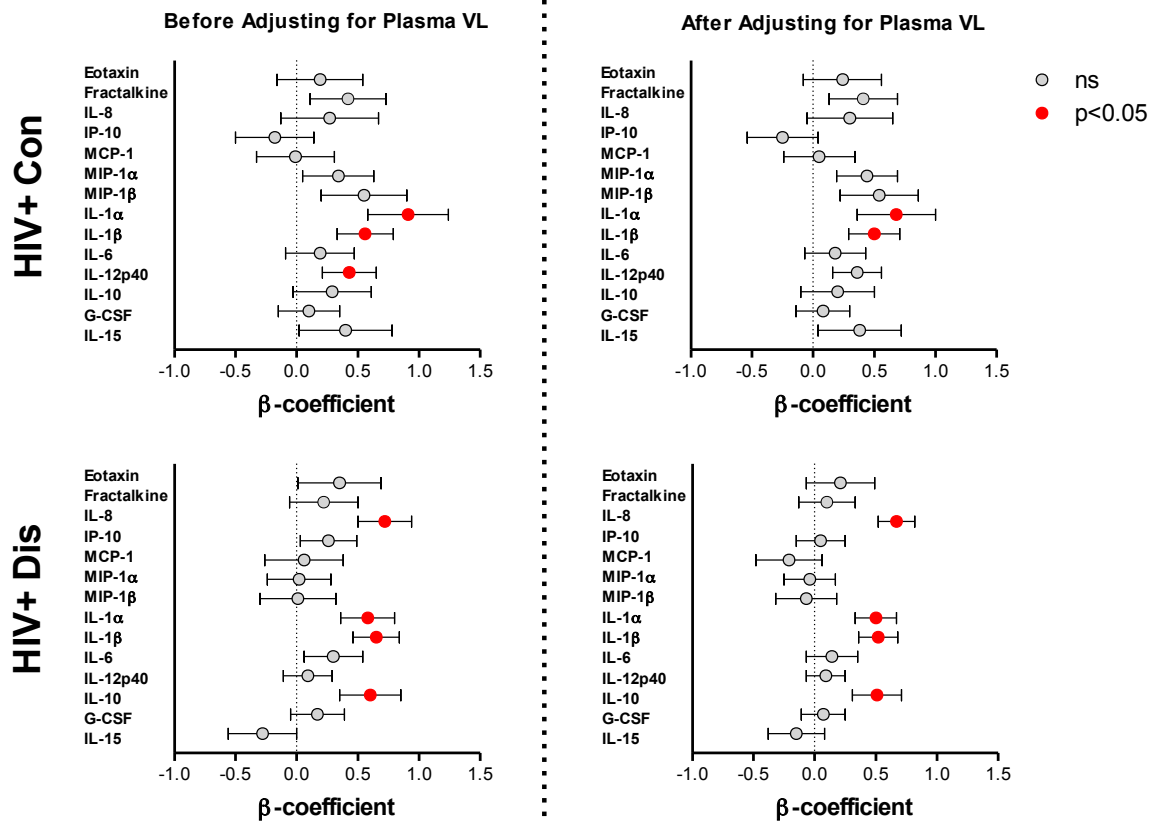


Figure 4.3 Relationship between genital tract cytokine concentrations and mucosal viral loads. The association between genital tract HIV loads and levels of different cytokines was assessed in HIV+ seroconcordant or HIV+ serodiscordant by linear regression analyses. Left panel shows the level of association before adjusting for plasma HIV load while right panel shows the level of association after adjusting for plasma HIV load. Regression β -coefficients depicted in red circles represent significant associations (p -values ≤ 0.05). Bars represent standard error. All p -values were adjusted for multiple comparisons using the false discovery rate step-down approach (Columb and Sagadai, 2006). β -coefficients are interpreted as the amount by which cervical viral load (HIV RNA copies/ml) increases with every percentage increase in cytokine concentration.

To further demonstrate whether HIV shedding in the female genital tract was associated with genital inflammation, an alternative analysis comparing concentrations of cytokines in women who were found to be shedding virus in their genital secretions with those who were not was performed (Figure 4.4). HIV+ women shedding HIV at their genital secretions had higher concentrations of the majority of cytokines measured [including IL-8 ($p=0.008$), IP-10 ($p=0.002$), IL-1 α ($p=0.007$), IL-1 β ($p=0.004$), IL-6 ($p=0.005$), MCP-1 ($p=0.03$), MIP-1 β ($p=0.01$), IL-10 ($p=0.01$) and G-CSF ($p=0.002$)].

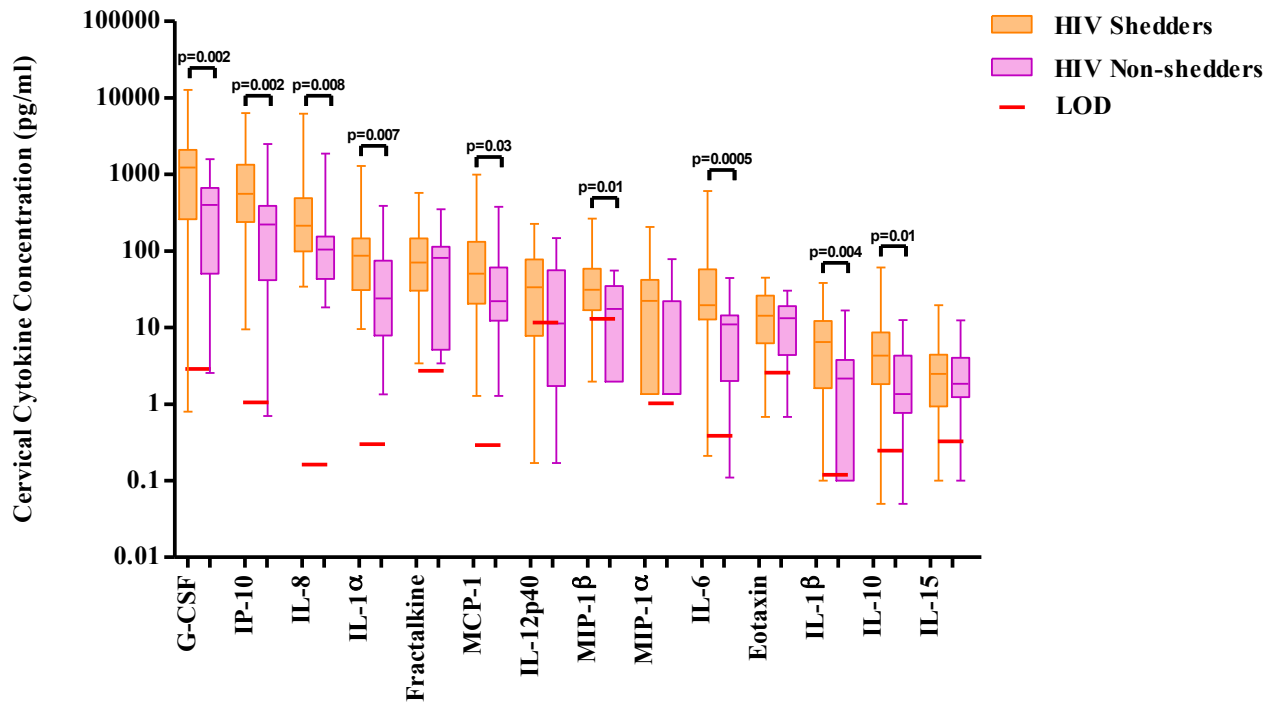


Figure 4. 4 Concentrations of cytokines in genital fluid collected from HIV+ women shedding HIV (orange) versus those not shedding HIV (pink) in their genital secretions. The concentration of cytokines in each group of women is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the measured concentration of each cytokine. Mann-Whitney U tests were applied to compare cervical cytokine concentration between seroconcordant and serodiscordant women and $p < 0.05$ were considered significant. (—) LOD: Limit Of Detection (for each individual cytokine).

4.3.5 Relationship between clinical status and cytokine concentrations in the genital tract

The relationship between clinical status (as indicated by plasma HIV loads) and cytokine concentrations in genital secretions was investigated using linear regression analysis (Figure 4.5). Overall, plasma HIV load was not associated with changes in genital cytokine concentrations.

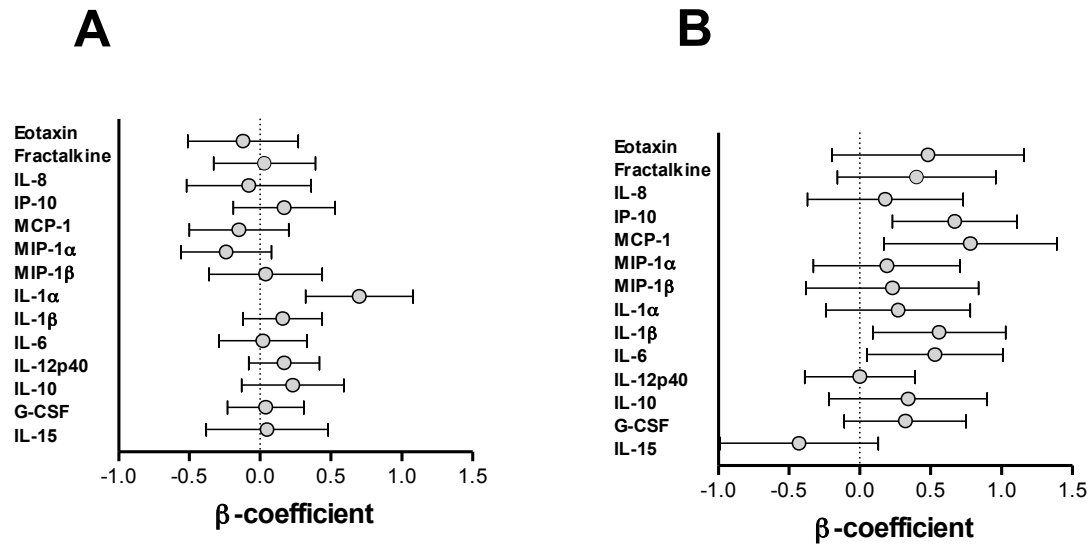


Figure 4. 5 Relationship between plasma viral loads and genital cytokine concentrations. The association between plasma HIV load and levels of different cytokines in the cervix was assessed in HIV+ seroconcordant (A; n=36) or HIV+ serodiscordant (B; n=19) by linear regression analyses. Bars represent standard error and $p < 0.05$ were considered significant.

4.3.6 Predictors of HIV shedding in the female genital tract

Since plasma HIV load and markers of genital tract inflammation (including IL-1 α , IL-1 β , IL-8 and IL-10) both predicted having detectable HIV in genital secretions in the women included in this study, the relative predictive value of each of these factors was compared in a zero-inflated negative binomial regression model. In HIV+ women who were shedding virus in their genital secretions, the linear model (the mean of the HIV load in those shedding virus [non-zero responses]) did not identify any one factor that predicted the magnitude of genital tract shedding (Table 3.3). In the inflated model (models the probability of no HIV shedding into the genital tract [zero response]), IL-1 β and plasma HIV load predicted shedding HIV at the cervix. For IL-1 β , an OR of 1.40 indicated a 40% increased odds of shedding HIV in genital secretions with every 1% increase in the concentration of IL-1 β in the genital tract. This data suggests a positive relationship between genital inflammation and HIV genital shedding, with IL-1 β concentrations independently predicting HIV shedding in that compartment (after adjusting for plasma viral load). It is important to note, however, that none of the biological parameters measured in this study predicted HIV genital shedding as strongly as plasma viral load.

Table 4. 3 Modeling predictors of genital tract HIV shedding

Linear Model	Incidence-rate Ratio¹	95% CI²	P-value
IL-1 β (log pg/ml)	1.0	0.98-1.01	0.6
IL-8 (log pg/ml)	1.0	1.00-1.00	0.9
Plasma HIV load (log cps/ml)	2.1	0.76-1.14	0.5
Inflated Model	Odds Ratio³	95% CI⁴	P-value
IL-1 β (log pg/ml)	1.4	1.01-1.93	0.04
IL-8 (log pg/ml)	1.08	0.96-1.21	0.2
Plasma HIV load (log cps/ml)	5.75	1.61-20.5	0.007

¹Incidence-rate Ratio for the linear part of the zero-inflated negative binomial regression model represents the ratio of the mean genital tract viral load associated with one unit increase in the parameter in women who were shedding HIV into the genital tract (ie. Non-zero values for cervical viral load). ²Confidence Intervals. ³Odds Ratio for the inflated or logistic part of the zero-inflated negative binomial regression model represents the odds of a genital HIV RNA level of zero i.e. the odds of not shedding HIV in the genital tract. ⁴Logistic part of the model adjusted for plasma viral load.

4.4 Discussion

The micro-environment in the female genital tract plays a significant role for both acquisition and transmission of HIV during heterosexual contact. A number of studies have shown that plasma viral loads are the best predictors of both the amount of HIV being shed in genital secretions (Iversen *et al.*, 1997; Hart *et al.*, 1999; Shepard *et al.*, 2000; Kovacs *et al.*, 2001; Vettore *et al.*, 2006; Taton *et al.*, 2011) and risk for HIV transmission to new partners (Quinn *et al.*, 2000; Attia *et al.*, 2009). Results from this Chapter confirm that plasma viral load is the strongest predictor of genital tract shedding of HIV in HIV+ women. Furthermore, HIV+ seroconcordant women, who had significantly higher plasma viral loads than HIV+ serodiscordant women, were also shedding more HIV in their genital secretions than serodiscordant women (with a median difference between groups of 1450 copies/ml of diluted secretion). This is the first report to my knowledge that HIV+ women with seroconcordant partners have higher genital tract viral loads than HIV+ women in serodiscordant relationships. Given the strong positive association between plasma viral loads and genital tract HIV loads, the finding that HIV+ seroconcordant women sustain higher plasma and genital HIV loads compared to serodiscordant women could indicate either that passive transudate from blood to genital tract, or active recruitment or homing of HIV-infected target cells to the genital mucosa under a chemokine gradient were driving HIV shedding. Local factors in the genital compartment, like inflammation, are likely to be important in determining the extent of HIV-infected target cell homing to the mucosa, possibly independently of plasma viral loads, thereby increasing local shedding of HIV (Nkwanyana *et al.*, 2009).

While systemic inflammatory cytokines were found to be significantly elevated in HIV seroconcordant compared to serodiscordant individuals (Chapter 3), no such difference was found in the genital inflammatory profile of seroconcordant and serodiscordant women in this Chapter. The finding that the difference in plasma viral load was 10-fold greater than the difference observed in genital secretions might explain why differences between groups in systemic inflammatory profiles were significant compared to inflammation in the genital tract. Alternatively, while plasma viral load and systemic inflammation were possibly directly related in Chapter 3, it is likely that little genital tract inflammation was directly linked to HIV replication at the mucosa as a result of competing and stronger drivers of local inflammation such as other STIs or BV.

Pro-inflammatory (TNF- α , IL-1 β , IL-6) and chemotactic cytokines (IL-8) are known to enhance HIV replication (Al-Harhi *et al.*, 1997) and attract neutrophils to sites of inflammation (Dinarello *et al.*, 2000). Increased concentrations of these cytokines within the female genital tract are likely to represent a hostile activated milieu for women, putting them at risk of infection by HIV. This finding of an association between genital tract cytokine concentrations and viral loads within the genital tract is similar to previous studies during chronic HIV infection (Lawn *et al.*, 2001; McGowan *et al.*, 2004). In this Chapter, a clear association was found between genital tract concentrations of two classical markers of inflammation, IL-1 α and IL-1 β , and genital tract HIV loads, independently of couple status and plasma viral load. In addition, IL-8 and IL-10 were also associated with genital HIV burden in serodiscordant women. HIV infection has previously been associated with an increase in IL-10 production by several cell subsets, but it remains unclear whether it has a role in HIV disease progression or protection (Ostrowski *et al.*, 2001; Biancotto *et al.*, 2007; Nilsson *et al.*, 2007; Brockman *et al.*, 2009). The observation of a lower genital HIV load in serodiscordant women compared to seroconcordant women, coupled with the association found between genital HIV burden and genital IL-10 concentrations, suggest that IL-10 in genital secretions may function synergistically with other immunoregulatory factors to balance the pro-inflammatory environment in this compartment, and thereby indirectly impact on HIV shedding in genital secretions. Further studies are however needed to determine causality.

Independently of plasma viral load, findings from this Chapter suggest that certain genital tract cytokines were associated with higher HIV genital loads, with certain cytokines that were elevated in women shedding HIV (such as MIP-1 β and IL-8) serving both pro-inflammatory and chemotactic functions. Upregulation of these cytokines could in turn lead to the recruitment and activation of more T-cells, which in turn would more likely result in increased production of cytokines with different functions in the genital tract (Abbas and Lichtman, 2007). Previous studies have shown that pro-inflammatory cytokines can stimulate production of growth factors as well as other secreted factors important in tissue repair, lymphangiogenesis and angiogenesis as well as inducing IL-10 to counterbalance the inflammatory process (Rafii *et al.*, 2003; Cursiefen *et al.*, 2004; Tammela *et al.*, 2005). Results in this study similarly show that upregulation of genital tract pro-inflammatory cytokines in women shedding HIV at their cervix was accompanied by increased concentrations of the hematopoietic cytokine G-CSF. Although this cytokine has generally

been associated with hematopoietic functions, secondary pro-inflammatory functions have also been associated with G-CSF, such as differentiation of granulocytes from stem cell progenitors, stimulating pro-inflammatory cytokine production by leukocytes, and chemotaxis of other immune cells (Gomez-Cambronero *et al.*, 2003; Wira *et al.*, 2005).

Plasma viral loads were shown to be completely independent of genital cytokine concentrations, confirming that systemic factors are unlikely to play a major role in the modulation genital inflammation. While chronic inflammation is an accepted hallmark of HIV pathogenesis, few prior studies have measured the inflammatory milieu systemically and in the genital tract in relation to plasma HIV load. A previous study from our group found a positive correlation between the frequency of activated T-cells in blood and from the lower genital tract of HIV-infected women, and that genital T-cell activation predicted HIV shedding (Jaspan *et al.*, 2012).

Several reports have shown that STIs predict both genital inflammation and HIV genital shedding (Rotchford *et al.*, 1999; LeGoff *et al.*, 2007; Kaul *et al.*, 2008; Shin and Kaul, 2008; Rebbapragada and Kaul, 2008; Kaul *et al.*, 2011). No laboratory based screening for STIs was conducted in this study, although signs and symptoms were recorded (genital ulcerative disease and vaginal discharge by clinical exam). Few women in this study did have clinical evidence of an STI (ulceration or discharge), and those women with symptoms of an STI were equally distributed between seroconcordant and serodiscordant women. While inflammation can persist long after STI symptom resolution (such as subclinical HSV infection or reactivation), the strong association of several inflammatory markers and HIV shedding in this study validate the multivariate model that identified IL-1 β and HIV plasma load as determinants modulating genital HIV burden.

In conclusion, this Chapter showed that genital tract inflammation was significantly associated with increased genital HIV shedding, a finding that was independent of plasma HIV loads or partner status. Elevated genital HIV shedding in HIV seroconcordant women compared to serodiscordant women together with the finding that genital tract inflammation further elevated shedding (irrespective of partner status) may suggest an increased risk for re-infection (or super-infection) of HIV seroconcordant partners or primary infection of HIV negative serodiscordant partners in women with genital tract inflammation. This also highlights one of the limitations of using plasma viral loads to predict HIV transmission risk. Although recent studies have reported that treating STIs would reduce HIV transmission risk

(Nagot *et al.*, 2007; Delaney *et al.*, 2009; Vanpouille *et al.*, 2009), findings from this Chapter additionally suggest that treatment of genital tract inflammation, irrespective of its causes, may also help to reduce genital HIV shedding, which in turn may also curb HIV risk.

CHAPTER 5

Impact of systemic immune activation and inflammation on the HIV susceptibility of HIV exposed uninfected individuals

5.1 Introduction

Studies of HIV transmission risk and resistance to infection in defined cohorts of HIV exposed-uninfected individuals has provided important insight into the mechanisms underlying resistance to HIV infection. Remaining seronegative in the face of HIV exposure has been documented in commercial sex workers (Jennes *et al.*, 2004), serodiscordant couples (Bernard *et al.*, 1998; Lo Caputo *et al.*, 2003; Suy *et al.*, 2007), injection drug users (Makedonas *et al.*, 2002), infants born to HIV infected mothers (Hygino *et al.*, 2008), occupational exposures in healthcare workers (Clerici *et al.*, 1994) and men having sex with men (Hladik *et al.*, 2003). In spite of considerable efforts to unravel the mechanisms of protection from HIV infection in these HIV exposed individuals, these remain largely undefined although they are likely to be multifactorial.

Numerous host factors have been associated with protection against HIV infection at the mucosa in these individuals; including co-receptor susceptibility (Paxton *et al.*, 1998), components of the innate and adaptive immune response (Furci *et al.*, 2002; Wichukchinda *et al.*, 2007); as well as several secreted cytokines including RANTES, SLP-1, MIP-1 α and MIP-1 β (Iqbal *et al.*, 2005; Hirbod *et al.*, 2008). In addition, elevated frequencies of HIV-specific IFN- γ and IL-2 secreting T-cells have been recognized in different cohorts of exposed-uninfected individuals (Kebba *et al.*, 2004; Pallikkuth *et al.*, 2007).

Recent studies have suggested that, in addition to protective innate and adaptive responses at the mucosa, lower levels of immune activation and “immunological quiescence” are strongly associated with resistance to HIV infection in these individuals (Koning *et al.*, 2005; Bégaud *et al.*, 2006; Jennes *et al.*, 2006). While it has been suggested by Card *et al.* (2008) that decrease in immune activation in resistance to HIV infection is associated with an elevated frequency of regulatory T-cells (Treg), an exact mechanism for the long term maintenance of the low level of immune activation has yet to be identified.

Multiple and sometimes conflicting immunological influences in the systemic and mucosal compartments have been proposed to confer protection against HIV infection in HIV exposed-uninfected individuals. The purpose of this Chapter was to investigate the interaction between systemic inflammation (measured by soluble cytokine concentrations) and systemic immune activation and explore their respective roles in resistance to HIV infection.

5.2 Materials and Methods

5.2.1 Cohort description

A total of 215 HIV negative (HIV-) individuals were included for this part of the study, of which 103/215 were in HIV- seroconcordant relationships and 113/215 were in serodiscordant relationships. In addition, a total of 144 HIV+ seroconcordant individuals were included as a comparison group. Chapter 2 (Section 2.2.1) provides a full description of these individuals. These participants were followed longitudinally, with immune activation and inflammatory cytokine production in blood and cervix being measured at 6, 18 and 30 months in the study. All aspects of the study were approved by the Research Ethics Committee of the University of Cape Town (UCT REC# 258/2006) and informed written consent was obtained from all individuals before initiation of the study.

5.2.2 PBMCs collection and processing

Blood (16ml) was collected from all study participants using standard venipuncture into sterile ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK). Blood was processed within 4 hours of collection; PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation as described in Chapter 2 (Section 2.2.2).

5.2.3 Cryopreservation of PBMCs

All PBMCs used in this study were cryo-preserved and subsequently stored in liquid nitrogen as described in Chapter 3 (Section 3.2.3).

5.2.4 Thawing of cryo-preserved PBMCs

Frozen PBMCs were thawed rapidly in a 37°C water bath as described in Chapter 3 (Section 3.2.4).

5.2.5 Counting of PBMCs using the automated Guava cell counter

Thawed PBMCs were diluted 20-fold in Guava Viacount reagent and counted using a Guava Automated cell counter as previously described in Chapter 2 (Section 2.2.3).

5.2.6 Staining for markers of T-cell activation by flow cytometry

PBMCs were stained in BD Falcon Round-Bottom tubes (BD Biosciences, San Jose, CA, USA) and 1 million PBMCs were used per staining reaction per participant as described in Chapter 3 (Section 3.2.6).

5.2.7 Measurement of cytokine concentrations in blood plasma

The concentrations of IL-1 β , IL-6, IL-12p70, TNF- α , IL-10, IL-2, IFN- γ , IL-7 and GM-CSF were measured in blood plasma from all individuals using High Sensitivity Human Cytokine LINCOp lex Premixed kits (LINCO Research, MO, USA). A description of the assay is according to the method described in Chapter 3 (Section 3.2.7).

5.2.8 Statistical analysis

Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Comparison of unpaired non-parametric data was done using the Mann-Whitney U test. Statistical inferences on binary sets of data were performed using the Fisher's exact test and odds ratios calculated. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons. Quantile regression analyses were used to estimate the median value of the independent (response) variable for given levels of the dependent (predictor) variables. Adjustment for multiple comparisons was performed using a false discovery rate step-down approach. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 11 (College Station, TX, USA). All tests were two-tailed and p-values of ≤ 0.05 were considered significant.

5.3 Results

5.3.1 Characteristics of HIV- unexposed and HIV- exposed individuals

Two hundred and fifteen HIV negative (HIV-) individuals, which included individuals who were from 52 unexposed couples and 112 HIV serodiscordant couples, were included to investigate the role of sexual partner HIV status on systemic immune activation and inflammation, as potential correlates of HIV risk (Table 5.1). Of 215 HIV- individuals, 103/215 (48%) had similarly HIV- partners, and 112/215 (52%) had HIV+ partners. The HIV- exposed participants were the partners of the HIV+ serodiscordant participants included in Chapter 3. While the majority of HIV+ serodiscordant individuals included in Chapter 3 were women, HIV- exposed individuals were predominantly male in this Chapter (73%). HIV- unexposed individuals were 4 years younger than HIV- exposed individuals (Table 5.1, $p=0.01$). This reflects the fact that HIV- exposed individuals were more likely to be men (73%) in this study and the Sub-Saharan African custom that men are on average older than women in many heterosexual couples (Eyawo *et al.*, 2010). Possibly also reflecting the age and gender distribution difference between HIV- exposed and unexposed individuals, it was not surprising to see that individuals in HIV unexposed relationship reported having had a longer sexual exposure than their exposed counterparts (Table 5.1).

HIV- exposed and unexposed individuals reported several other differences in sexual behavior; with HIV- unexposed individuals reporting higher frequencies of sex acts in the last month and lower frequencies of condom usage than the exposed ones. As discussed in Chapter 3 (Section 3.3.1), self reported condom use may be overreported in the HIV- exposed individuals compared to unexposed individuals because the exposed individuals perceive that this is the more socially desirable response to the question. Aside from these sexual behavior differences, CD4 percentages, proportion of individuals cohabiting, or having either ulceration or discharge were similar in exposed and unexposed individuals (Table 5.1). All men included in this study were circumcised. The HIV+ seroconcordant individuals studied in Chapter 3 were included as a control in this present Chapter.

Table 5. 1 Clinical and socio-behavioural characteristics of participants included in this study

Characteristics	HIV Positive		HIV Negative		P-value	
	n	Concordant	n	Concordant		Discordant
N		144		103	112	
Male [%] †		53		50	73	0.001
Age [yr; median (IQR)] *	141	37 (32-42)	102, 106	36 (29-43)	40 (33-48)	0.01
Living together with partner [%] †	142	70	103, 112	72	63	0.2
Age at first sex [median (IQR)] *	141	17 (16-18)	102, 106	18 (16-18)	17 (15-18)	0.2
Sexual exposure [median years of sex (IQR)] *	140	21 (15-25)	102, 106	19 (12-26)	21 (17-30)	0.01
Blood CD4% [median (IQR)] *	40	45 (24-65)	38, 30	71 (59-86)	74 (61-81)	0.9
Sex acts in the last month [median (IQR)] *	137	3 (1.5-6)	94, 112	4 (3-8)	3(2-5)	0.004
Condom usage [%] †	123	58	92, 102	29	75	P<0.0001
Genital ulceration in the last 6 months [%] †	61	8	93, 111	2	5	0.5
Vaginal discharge in the last 6 months [%] †	61	12	51, 30	6	10	0.5

†p-value calculated using Fischer's exact test

*p-value calculated using Mann-Whitney U-test comparing HIV- unexposed (seroconcordant) individuals to HIV- exposed (serodiscordant) individuals

IQR: Interquartile range

5.3.2 Impact of partner HIV status on T-cell activation in blood of HIV- individuals

The expression of activation markers CCR5, CD38 and HLA-DR and the nuclear proliferation marker Ki67 by T-cells in blood was measured in HIV- unexposed and exposed individuals in to investigate the impact of partner status on activation of T-cells in HIV- individuals. These markers allowed assessment of the frequency of (1) highly activated T-cells (defined by dual expression of HLA-DR and CD38), (2) activated proliferating T-cells (defined by dual expression of Ki67 and CD38); and (3) CCR5+ T-cells that represent the preferred target cells for HIV infection [activated CCR5+ cells (defined by dual expression of CCR5 and CD38) or proliferating CCR5+ cells (dual expression of CCR5 and Ki67)]. The overall level of activation (total activation) was defined as the cumulative expression of any permutation of the markers.

The global activation status of CD4⁺ T-cells from HIV- exposed and unexposed individuals was similar (p=0.51; Figure 5.1) and were significantly lower than global activation status of CD4⁺ T cells from HIV+ individuals (p<0.001 for both HIV- exposed and unexposed individuals). In contrast, HIV+ seroconcordant individuals had an overwhelmingly higher activation status for their CD8⁺ T-cell subset than HIV- exposed individuals (p<0.001; Figure 5.1). Interestingly, HIV- unexposed individuals had similar levels of total activation in their CD8⁺ T-cell subset as in HIV+ seroconcordant controls, both of which were significantly higher than frequencies observed in HIV- exposed individuals (Figure 5.1).

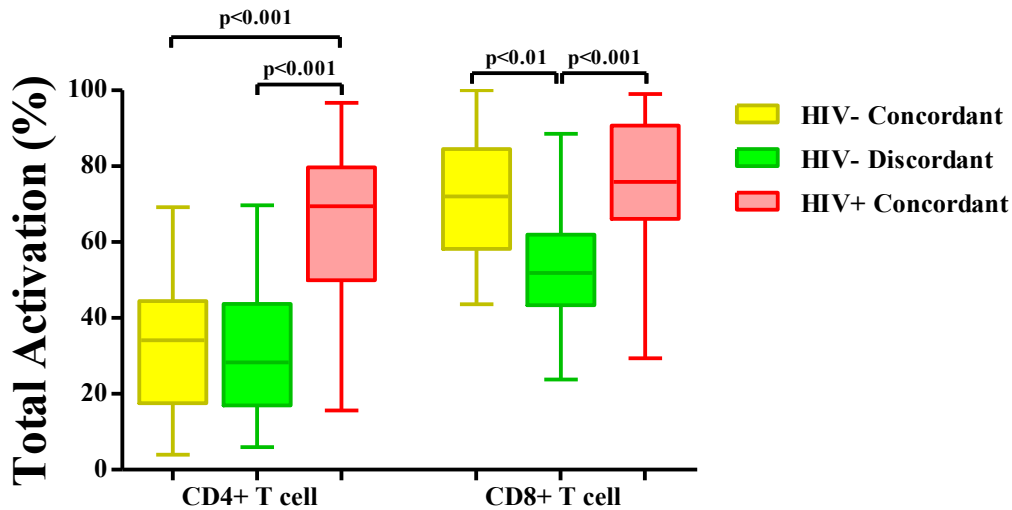


Figure 5. 1 Impact of partner HIV status on total activation of CD4⁺ and CD8⁺ T-cells in the blood from HIV- individuals (yellow for HIV- unexposed (seroconcordant) and green for HIV- exposed (serodiscordant)) compared to HIV+ individuals (red). Frequencies of immune activation markers on CD4⁺ and CD8⁺ T-cells derived from the blood of HIV- exposed and unexposed individuals were assessed for expression of CCR5, Ki67, HLA-DR, and CD38. Similar measurements assessed for HIV+ seroconcordant individuals (Chapter 3, Section 3.3.2) were included as comparisons. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons.

CCR5, CD38, HLA-DR and Ki67 expression by CD4⁺ and CD8⁺ T-cells were compared individually or in biologically important combinations [HLA-DR/CD38 (representing highly activated T-cells), Ki67⁺CCR5⁺ (representing proliferating T-cells which may be susceptible to HIV infection), CD38⁺CCR5⁺ (representing susceptible, activated T-cells) and CD38⁺Ki67⁺ (representing activated, proliferating T-cells)]. HIV- exposed individuals had significantly lower frequencies of CD4⁺ T-cells expressing the HIV co-receptor CCR5, alone or in combination with Ki67 or CD38, than HIV- unexposed individuals ($p=0.05$ for CCR5 alone, $p<0.05$ for CCR5/Ki67 and $p<0.05$ for CCR5/CD38; Figure 5.2A and C). These data suggest that CD4⁺ T-cells from HIV- exposed individuals were possibly less susceptible (more quiescent) to HIV infection compared to those in HIV- unexposed, on account of having lower frequencies of activated and proliferating CCR5⁺ cells to facilitate infection.

Similarly, HIV- exposed individuals had significantly lower frequencies of CCR5, alone or in combination with Ki67 and CD38, on the surface of their CD8⁺ T-cells than their HIV- unexposed counterparts (p=0.05 for CCR5 alone, p<0.01 for CCR5/Ki67, p<0.01 for CCR5/CD38; Figure 5.2B and D). In addition, HIV- exposed individuals also had lower frequencies of their CD8⁺ T-cells expressing HLA-DR than HIV- unexposed participants (p<0.01; Figure 5.2B).

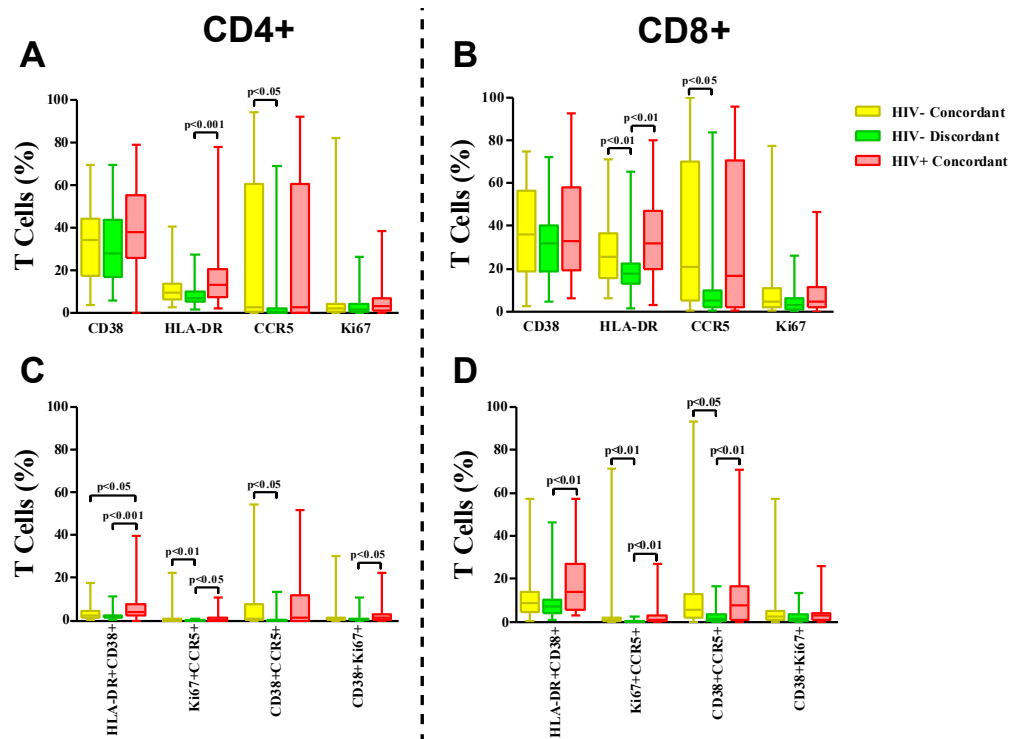


Figure 5. 2 Impact of partner HIV status on specific T-cell activation (defined by expression of CD38, HLA-DR, CCR5, or Ki67) in HIV- unexposed individuals (seroconcordant) (yellow) versus HIV- exposed (serodiscordant) (green) relationships. Frequency of specific activation marker expression (Ki67, HLA-DR, CD38) on CD4⁺ (panels A and C) and CD8⁺ (panels B and D) T-cells derived from the blood of HIV- exposed and unexposed was assayed. Similar measurements assessed for HIV+ seroconcordant individuals (Chapter 3, Section 3.3.2) were included in this comparison as positive controls. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons.

In addition to absolute frequencies of CCR5+ CD4+ and CD8+ T cells being lower in HIV-exposed participants compared to the unexposed individuals, a further analysis of the mean fluorescence intensity (MFI) of CCR5 on CD4+ and CD8+ T-cells showed that CCR5 MFIs were lower in HIV- exposed compared to unexposed individuals, significantly so for CD4+ T cells ($p=0.04$ for CD4+ and $p=0.07$ for CD8+ T cell subsets, Figure 5.3). This finding suggests that HIV- exposed individuals had significantly fewer CD4+ T cells expressing significantly lower amounts of CCR5 than unexposed individual. This important finding may explain why they remain HIV negative despite being in a stable, long term relationship with an HIV+ partner who is not taking HAART.

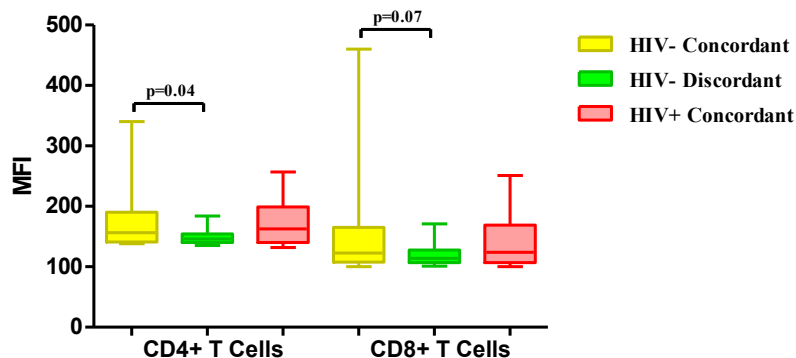


Figure 5. 3 Impact of partner HIV status on expression of CCR5 on CD4+ and CD8+ T-cells (mean fluorescence intensity). The cumulative MFI in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn’s post-test being applied to test for the effect of multiple comparisons.

5.3.3 Impact of gender on T-cell activation in blood

As more men than women were included in the HIV- exposed group, the levels of the individual markers for activation, proliferation and HIV co-receptor expression were investigated in men and women to determine if gender played a role in level of systemic activation. Generally, frequencies of all markers were similar in men and women, in either unexposed (concordant) (Top Panel) or exposed (discordant) (Bottom Panel) and for both CD4+ and CD8+ T-cells (Figure 5.4). While the CD4+HLA-DR+ subset of men in both unexposed and exposed relationship seemed to be higher than their respective female counterpart, this difference was not statistically significant. Similarly, no significant

differences were found when comparing the different combinations of activation markers when comparing men to women (data not shown).

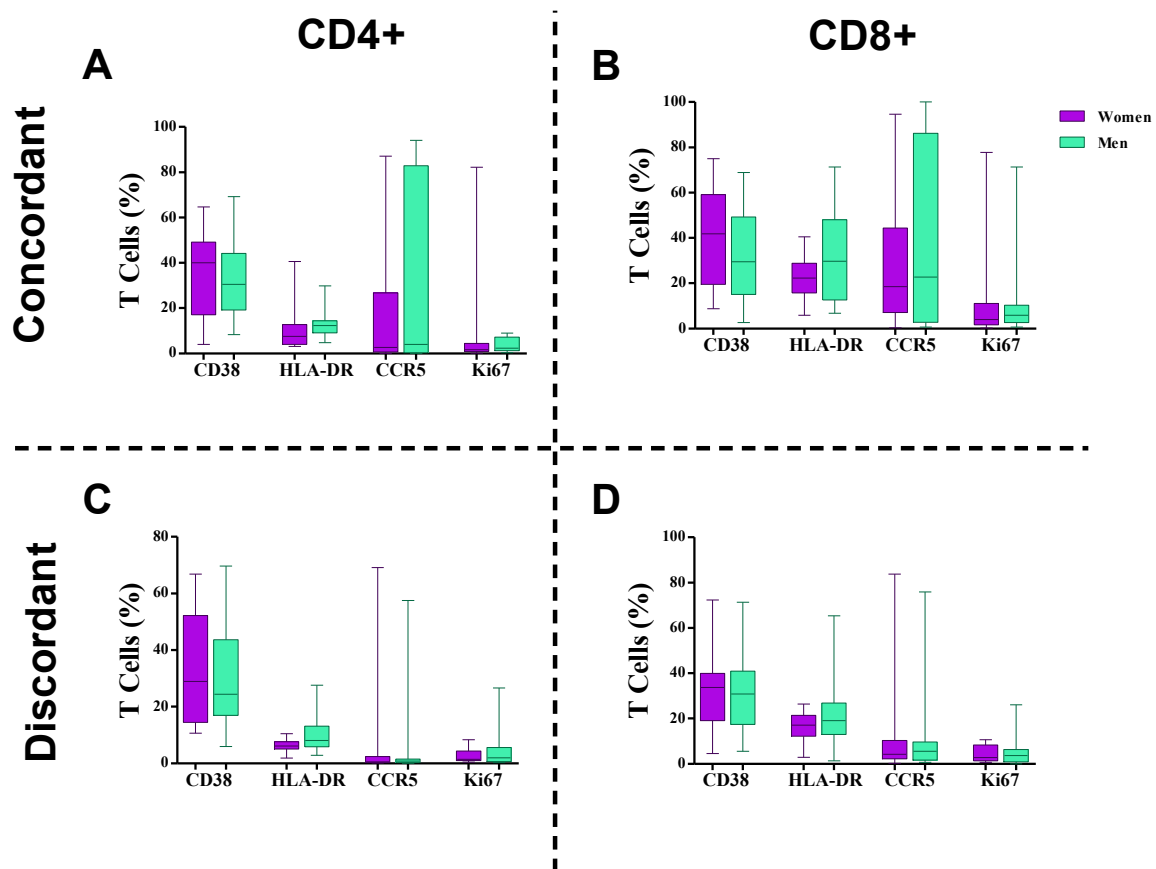


Figure 5.4 Impact of gender on systemic T-cell activation (defined by expression of CD38, HLA-DR, CCR5, or Ki67) in HIV- women (purple) versus men (green). Frequency of specific activation marker expression (CCR5, Ki67, HLA-DR, CD38) on CD4⁺ (Panel A, C) and CD8⁺ (Panel B, D) T-cells derived from the blood of HIV- women and men in unexposed (seroconcordant) (Top Panel) and exposed (serodiscordant) (Bottom Panel) was assayed. The cumulative percentage of activated CD4⁺ and CD8⁺ T-cells in each group of individuals is depicted by box-and-whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the frequencies of T-cells expressing the respective activation markers. Mann-Whitney U tests were applied to compare blood T-cell activation frequencies between men and women.

5.3.4 Impact of sexual partner HIV status on systemic inflammation in HIV- individuals

The concentrations of TNF- α , IL- β , IL-6, IL-10, IL-7, GM-CSF IL-12p70, IL-2 and IFN- γ were measured in plasma from HIV- unexposed and exposed individuals and compared to

concentrations measured in plasma from HIV+ individuals (Table 5.2). Plasma concentrations of IL-2 (p=0.02), IFN- γ (p=0.05) and GM-CSF (p=0.006) were significantly lower in HIV- exposed compared to unexposed individuals, with the difference between groups in GM-CSF concentrations remaining significant after adjustment for multiple comparisons. Compared to HIV+ individuals in seroconcordant relationships, HIV- exposed individuals had significantly lower plasma concentrations of IL-6 and TNF-a but significantly higher concentrations of GM-CSF. No significant differences were observed when comparing the level of circulating cytokines in men versus women in either exposed or unexposed relationships.

Table 5. 2 Impact of HIV status and couple HIV status on systemic cytokine levels

Function	Cytokine	Median cytokine conc (IQR; pg/ml)				
		HIV Negative		P-value [◊]	HIV Positive Concordant	P-value ^Δ
		Unexposed	Exposed			
Inflammatory	IL-β	0.45 (0.07-1.43)	0.35 (0.04-1.25)	0.5	0.72 (0.075-2.85)	0.07
	IL-6	4.66 (2.36-7.87)	3.90 (1.75-7.50)	0.2	5.26 (2.83-11.10)	<0.0001 [‡]
	IL-12p70	0.005 (0.005-0.83)	0.005 (0.005-0.58)	0.8	0.005 (0.005-0.42)	0.03
	TNF-α	6.00 (3.94-8.14)	5.85 (4.02-7.78)	0.7	9.45 (5.61-15.94)	0.04
Anti-inflammatory	IL-10	10.43 (5.60-22.26)	9.08 (5.88-17.95)	0.7	12.60 (7.75-23.44)	0.08
Adaptive	IL-2	0.28 (0.005-1.03)	0.0075 (0.005-0.69)	0.02	0.11 (0.005-0.70)	0.2
	IFN-γ	0.94 (0.14-2.91)	0.64 (0.02-1.93)	0.05	1.39 (0.27-6.33)	0.6
Hematopoietic	IL-7	1.86 (0.68-3.49)	1.51 (0.69-3.46)	0.7	1.58 (0.61-3.09)	0.3
	GM-CSF	0.64 (0.20-1.46)	0.32 (0.12-0.82)	0.006 [‡]	0.19 (0.005-0.64)	<0.0001 [‡]

Mann-Whitney U tests were applied to compare cytokine concentrations between the HIV- unexposed and HIV- exposed groups, and the HIV- unexposed and HIV+ seroconcordant groups. ‡ denotes p-value that remained significant after adjustment for multiple comparisons using the false discovery rate step-down approach (Columb and Sagadai, 2006). ◊ denotes p-value for comparisons between HIV- unexposed and HIV- exposed individuals. Δ denotes p-value for comparisons between HIV- unexposed and HIV+ seroconcordant individuals.

5.4 Discussion

Studies in HIV exposed-uninfected individuals provide a unique opportunity to determine correlates of protection associated with natural resistance to HIV infection. Serodiscordant couples, where an HIV uninfected individual is in stable relationship with HIV-infected partner, represent one of the best models in humans to study these correlates of protection. Central to productive HIV infection of cells is the binding of HIV both CD4 receptor as well as binding to the co-receptor CCR5. This Chapter showed that HIV- exposed individuals have a more quiescent immune profile compared to unexposed participants. The finding in this Chapter that significantly lower frequencies of CD4⁺ and CD8⁺ T cells from HIV-exposed individuals expressed CCR5 and had lower per cell amounts of CCR5 is therefore of great significance. The importance of this finding was further accentuated by the fact that these exposed individuals had a significantly smaller pool of activated susceptible T cells (CD38⁺CCR5⁺) and less of these susceptible cells were proliferating (Ki67⁺CCR5⁺) when compared to HIV- unexposed controls.

A recent study by Pandrea *et al.* (2007) showed that lower frequencies of CD4⁺CCR5⁺ T cells in the natural SIV hosts was associated with decreased level of SIV replication, and also a decreased homing of activated CD4⁺ T-cells to inflamed tissues. In this same study, they demonstrated that natural hosts for SIV infection (such as Sooty Mangabeys and African Green Monkeys, which also do not exhibit hallmark symptoms of SIV disease progression like macaques) have markedly reduced numbers of CD4⁺CCR5⁺ T cells in systemic blood and several lymphoid compartments like the bone marrow and lymph nodes. Previous studies in HIV exposed seronegative female sex workers in Kenya and Ethiopia also found that expression levels of CCR5 were reduced compared to unexposed control subjects (Fowke *et al.*, 1998; Messele *et al.*, 2001). These early studies did not take into account sexual partner HIV status so the extent of HIV exposure in exposed seronegative women as well as the extent of no exposure in unexposed controls was based on epidemiological and behavioural risk criteria. An early study from Paxton *et al.* (1998) linked the decreased infectibility of CD4⁺ T-cells from exposed-uninfected sex workers to lowered expression of CCR5 and concurrent elevated production of β -chemokines (the cognate ligand for CCR5). In addition, *in vitro* studies have revealed that CCR5 levels, their expression patterns, and β -chemokine production correlated with infectibility of PBMCs by HIV (Wu *et al.*, 1997; Blaak *et al.*, 2000). While resistance to HIV infection has been attributed in some cohorts to

homozygosity for the mutant alleles of the CCR5 receptor (CCR5 Δ 32; Huang *et al.*, 1996), this mutant allele is present at high frequencies (~10%) in Caucasian populations (Samson *et al.*, 1996; Novembre *et al.*, 2005) but at extremely low frequencies (0.1%) in black African populations (Williamson *et al.*, 2000).

Previous studies in persistently HIV exposed-uninfected individuals from Kenya, the Central African Republic, the Ivory Coast and the Netherlands have suggested that lower levels of T cell activation account for some of the in protection from HIV infection (Card *et al.*, 2008; Bégau *et al.*, 2006; Jennes *et al.*, 2006; Koning *et al.*, 2005). The study from Kenya focused on persistently HIV-uninfected commercial sex workers and evaluated the expression of three classical markers of cellular immune activation, namely HLA-DR, CD38 and CD69 on both CD4⁺ and CD8⁺ T-cells. The study from the Ivory Coast also focused on HIV exposed seronegative sex workers and measured activation on CD4⁺ and CD8⁺ T cells by CD69 expression (Jennes *et al.*, 2006). The study from the Netherlands focused on highly exposed but seronegative MSM and reported that protection was associated with lower CD38, HLA-DR and CD70 expression by CD4⁺ T cells (Koning *et al.*, 2005). Although several studies have suggested that HIV-exposed seronegative individuals have lower activation status than other low risk individuals, there have been other studies reporting elevated frequencies of activated CD8⁺CD38⁺ T-cells in HIV exposed seronegative sex workers when compared to low-risk blood donors (Jennes *et al.*, 2003). Findings from this Chapter [from stable, heterosexual black South African couples in which one partner is HIV- and the other is either HIV+ (serodiscordant) or HIV- (seroconcordant)] similarly suggest that activation of T cells was globally reduced in HIV- exposed individuals compared to those in unexposed relationships. While classic markers of activation were identified in other studies to be associated with this immune quiescence, this study showed that reduced activation by T cells was largely attributed to lower frequencies and lower relative expression of the HIV co-receptor CCR5 by both HIV target cells CD4 as well as CD8 T cells.

A study from Clerici *et al.* (2000) concluded that levels of systemic immune activation in individuals from Africa was influenced by environment rather than genetics when they compared individuals from Africa (HIV- individuals from Ugandan, living in Africa) to individuals from Europe (HIV- individuals of Italian decent and individuals of Ugandan decent living in Italy). Moreover, they observed that surface expression of CCR5 was enriched in African residents compared to Italian residents. They suggested that environmental factors, such as parasites, hygiene practices and nutrition are likely

contributing to differences in immune activation depending on geographic location. Recent studies by Cohen *et al.* (2010) confirmed that the findings by Clerici *et al.* (2000) were also prevalent in the genital tract of healthy young women from Sub-Saharan Africa. They reported an increased activated mucosal T-cell level in the genital tract of STI/HIV free Kenyan women, with a specifically elevated level of CD4⁺CD69⁺, CD4⁺CD69⁺CCR5⁺ and CD8⁺CD69⁺ T-cell subsets when compared to women from San Francisco. They proposed that this elevated level of genital immune activation could partly account for the higher HIV incidence in young women from Sub-Saharan Africa.

HIV- exposed individuals in this study had lower concentrations of the classical Th1 cytokines IFN- γ and IL-2 in blood plasma than HIV- unexposed controls. Other studies have similarly reported a significant reduction of IL-2 and IFN- γ expression in both CD4⁺ and CD8⁺ of highly exposed persistently seronegative individuals when compared to negative controls (Alimonti *et al.*, 2005; Nicastri *et al.*, 1999). These downregulated Th1 cytokines found in HIV- seronegative individuals might reflect less activated cells (anergic), which indirectly result in protection from HIV acquisition. The decreased concentrations of both IFN- γ and IL-2 could be involved in very low levels of activation of T cells. Moreover, a lowered expression of IFN- γ could affect the capability of macrophages to present antigens, thus resulting in a reduction of the cell-to-cell spread of infection (Gowda *et al.*, 1989). The finding in this Chapter that frequencies of HLA-DR expressing cells and concentrations of IFN- γ and IL-2 were significantly reduced in HIV- exposed compared to unexposed individuals are important, considering that IFN- γ as well as IL-2 are known to modulate HLA-DR expression. Reduced levels of these two cytokines may be directly involved in the downregulation of HLA-DR (Paxton *et al.*, 1996). In addition to regulating HLA-DR expression, IL-2 is involved in upregulation of CC chemokine receptors and chemotaxis of T cells, both functions necessary for recruitment of antigen-activated T-cells to sites of immune and inflammatory responses (Loetscher *et al.*, 1996).

GM-CSF in particular was significantly lower in plasma of HIV- exposed compared to unexposed individuals. Since GM-CSF promotes activation, maturation and differentiation of several immune cells (Kedzierska *et al.*, 1998; Shi *et al.*, 2006), this difference could impact on susceptibility to HIV infection. At high concentrations, GM-CSF might result in a rapid increase in numbers of leukocytes available in blood, which could then infiltrate and increase the frequency of HIV target cells in lymphoid tissues.

In conclusion, findings in this Chapter suggest a generalized lower level of systemic immune activation and inflammation in HIV- exposed individuals, who could potentially be exposed to HIV during sex with their HIV-infected partners, when compared to HIV- unexposed individuals. Moreover, a lower availability of susceptible cells for infection by the virus could partially explain why these individuals remain HIV- despite HIV exposure. Jaspan *et al.* (2011) showed that levels of activation in blood significantly and broadly predicted similar activation at the genital tract in women. It could be hypothesized from the findings in this Chapter that the reduction of susceptible target cells in blood would also result in the lower availability of these cells (targets for HIV infection) at the cervix. Elucidating the biological characteristics underlying protection against HIV can provide valuable insight on the protective mechanisms that may be harnessed for the development of new treatments and anti-HIV strategies.

CHAPTER 6

Impact of partner HIV status on genital tract inflammation in HIV negative women in exposed and unexposed relationships

6.1 Introduction

A number of studies worldwide have described a phenomenon of ‘resistance’ to HIV infection in women who remain HIV negative despite repeated exposure to the virus (Clerici *et al.*, 1994; Makedonas *et al.*, 2002; Hladik *et al.*, 2003; Jennes *et al.*, 2004; Suy *et al.*, 2007; Hygino *et al.*, 2008). The variability in risk for HIV infection has been associated with a multitude of factors, including viral, host genetic, immunological and sociobehavioural variables (Paxton *et al.*, 1998; Wichukchinda *et al.*, 2007; Hirbod *et al.*, 2008). Chapter 5 focused on systemic differences in levels of immune activation between HIV- individuals in HIV exposed and unexposed relationships, in which multiple markers of systemic activation (including CCR5, CD38, and HLA-DR expression by HIV target cells) were identified as being lower in exposed versus unexposed individuals. Studies of mucosal samples from HIV-exposed uninfected individuals offer an important opportunity to investigate biological events predicting HIV risk as well as those that predict protection.

Although several studies have described unique compartment specific drivers of inflammatory cytokines in blood and at the genital mucosa, relatively few have directly compared the relatedness or overlap between inflammatory or immune responses in the genital compartment and blood. Gumbi *et al.* (2008) reported that HIV-specific CD8⁺ T-cell IFN- γ responses in the female genital tract were of higher magnitude compared to responses in matching blood of HIV-infected women. Other studies have shown no association between level of inflammation in the female genital tract and in blood (Hedges *et al.*, 2006; Lajoie *et al.*, 2008; Blish *et al.*, 2012; Roberts *et al.*, 2012).

The purpose of this Chapter is to investigate the impact of sexual partner HIV status on female genital mucosal inflammatory cytokine markers, as predictors of risk for HIV infection. Furthermore, this Chapter describes the relatedness between important inflammatory markers in blood and in the female genital compartment.

6.2 Materials and Methods

6.2.1 Description of study participants

A total of 85 HIV- women were included for this part of the study, of which 69/85 were in HIV- unexposed relationships with HIV- stable male partners and 16/85 were in exposed relationships. Chapter 2 (Section 2.2.1) provides a full description of these individuals. These women were followed longitudinally, with inflammatory cytokine production in blood and cervix being measured at 6, 18 and 30 months in the study. For comparison, 78 HIV+ women in HIV+ concordant relationships were included. All HIV+ women were naïve to HAART.

6.2.2 PBMCs collection and processing

Blood (16ml) was collected from all women using standard venipuncture into sterile ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK). Blood was processed within 4 hours of collection; PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation as described in Chapter 2 (Section 2.2.2).

6.2.3 Genital supernatant collection and processing

Cervical mononuclear cells (CMCs) were collected using a Digene cervical sampler (Digene Corporation, Gaithersburg, MD, USA) as described in Chapter 4 (Section 4.2.2). The cervical supernatant collected after the centrifugation of the cervical specimen was aliquoted and kept at -80°C and thawed over ice, overnight prior to any assay.

6.2.4 Measurement of cytokine in blood and genital secretions

The concentrations of 9 cytokines (**IL-1 β** , **IL-6**, **IL-10**, IL-12p70, TNF- α , IL-2, IFN- γ , IL-7 and GM-CSF) were measured in blood plasma using High Sensitivity Human Cytokine LINCOp lex Premixed kits (LINCO Research, MO, USA). A description of the assay is available in Chapter 3 (Section 3.2.7).

The concentrations of 14 cytokines (**IL- β** , **IL-6**, **IL-10**, IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β , IL-1 α , IL-12p40, G-CSF, Eotaxin, Fractalkine, and IL-15) were measured in cervical supernatants using Human Cytokine LINCOp lex Premixed kits (LINCO Research, MO, USA). A description of the assay is available in Chapter 4 (Section 4.2.5). Cytokines listed in bold were those measured in both compartments although it should be noted that the sensitivity of the kits used to measure these were different depending on the sample analyzed.

6.2.5 Statistical analysis

Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Comparison of unpaired non-parametric data was done using the Mann-Whitney U test. Statistical inferences on binary sets of data were performed using the Fisher's exact test and odds ratios calculated. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons. Non-parametric association between groups was carried out through the Spearman rank test. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 11 (College Station, TX, USA). All tests were two-tailed and p-values of ≤ 0.05 were considered significant.

6.3 Results

Eighty five HIV- women were included in this part of the study to investigate the impact of partner HIV status on the comparative level of genital inflammation in HIV- unexposed women (69/85) and HIV- exposed women (16/85). In addition, 78 HIV+ women in HIV seroconcordant relationships were included for comparison. While HIV- women shared similar sociodemographic and reported a similar frequency of genital disease irrespective of partner status, HIV- exposed women reported a significantly higher condom usage than unexposed ones (70% versus 39%; $p=0.002$).

Table 6. 1 Clinical and socio-behavioural characteristics of women included in this study

Characteristics	n	Unexposed	n	Exposed	P-value
n		69		16	
Age [yr; median (IQR)] *	69	33 (27-41)	16	35(29-40)	0.5
Living together with partner [%] †	67	71	15	66	0.4
Sexual exposure [median years of sex (IQR)] *	66	18 (12-23)	15	19 (10-24)	0.9
Sex acts in the last month [median (IQR)] *	65	4 (2-8)	14	4 (3-9)	0.4
Condom usage [%] †	68	39	16	70	0.002
Genital ulceration in the last 6 months [%] †	69	5	16	5	0.8
Vaginal discharge in the last 6 months [%] †	69	6	16	7	0.6

†p-value calculated using Fischer's exact test

*p-value calculated using Mann-Whitney U-test

IQR: Interquartile range

6.3.1 Cytokine concentrations in genital secretions

The concentrations of 14 cytokines (IL- β , IL-6, IL-10, IL-8 IL-1 α , IL-12p40, IP-10, MCP-1, MIP-1 α , MIP-1 β , IL-15, Eotaxin, Fractalkine, and G-CSF) were measured in genital secretions from HIV- women in either exposed or unexposed relationships (Table 6.2). Concentrations of IL-1 α were significantly higher in HIV- exposed women when compared to HIV- unexposed women ($p=0.001$; Table 6.2). Overall, the level of most cytokines (except for G-CSF and IL-1 β) were elevated in the exposed women when compared to unexposed ones ($p=0.02$; Figure 6.1). Genital cytokine concentrations available from 78 HIV+ seroconcordant women were included for comparison. HIV+ seroconcordant women

generally had higher cytokine concentrations in their genital compartments than either HIV-exposed or unexposed women, and this was significant for IL-1 α , IL-6, IL-8, G-CSF and IP-10 (Table 6.2). It was interesting to note that HIV-exposed women had profiles closer to HIV+ concordant women than HIV-unexposed women, suggesting a signature of inflammation-associated HIV risk was detectable in these women despite them not being HIV infected.

Table 6. 2 Impact of HIV status and couple HIV status on cervical cytokine levels

Cytokine	Median cytokine conc (IQR; pg/ml)				P-value ^Δ
	HIV Negative		P-value [◊]	HIV Positive	
	Unexposed	Exposed		Concordant	
Eotaxin	12.91 (4.36-19.02)	16.37 (10.49-20.43)	0.3	15.5 (3.5-21.22)	0.4
Fractalkine	70.63 (30.26-129.4)	76.27 (39.72-224.3)	0.3	77.41 (29.66-144.8)	0.7
IL-8	75.36 (28.48-219.8)	149.3 (40.91-303.9)	0.5	153.6 (56.67-344.5)	0.01 [‡]
IP-10	61.44 (22.77-159.1)	102.5 (21.63-208.5)	0.7	441.1 (172.7-1002)	p<0.0001 [‡]
MCP-1	29 (8.89-102.8)	37.82 (23.7-71.58)	0.6	42.83 (16.41-122.6)	0.1
MIP-1α	5.62 (1.36-28.50)	20.71 (1.36-43.47)	0.1	13.64 (1.36-38.16)	0.2
MIP-1β	20.25 (1.96-40.39)	28.93 (8-66.93)	0.1	29.26 (10.10-43.71)	0.06
IL-1α	14.53 (4.53-32.95)	45.31 (23-128.5)	0.001 [‡]	34.96 (15.52-99.38)	p<0.0001 [‡]
IL-1β	3.11 (0.26-7.87)	2.61 (0.5-8.62)	0.9	4.07 (0.69-7.14)	0.4
IL-6	9.42 (2.13-21.62)	9.9 (3.43-31.36)	0.7	18.39 (4.9-55.6)	0.008 [‡]
IL-12p40	25.82 (0.26-61.92)	36.09 (6.07-60.13)	0.6	34.51 (5.64-67.33)	0.4
IL-10	2.35 (0.77-6.15)	5.37 (0.56-10.26)	0.3	2.6 (1.26-5.65)	0.5
G-CSF	388.6 (51.53-952.8)	349.3 (122.6-857.7)	1	759.8 (200.7-1847)	0.01 [‡]
IL-15	2.22 (1.06-3.81)	2.63 (0.75-5.39)	0.8	2.07 (1.24-4.1)	0.9

IQR: Interquartile range

Mann-Whitney U tests were applied to compare cytokine concentrations between the HIV-unexposed and HIV-exposed groups, and the HIV-unexposed and HIV+ seroconcordant groups. ‡ denotes p-value that remained significant after adjustment for multiple comparisons using the false discovery rate step-down approach (Columb and Sagadai, 2006). ◊ denotes p-value for comparisons between HIV-unexposed and HIV-exposed individuals. Δ denotes p-value for comparisons between HIV-unexposed and HIV+ seroconcordant individuals.

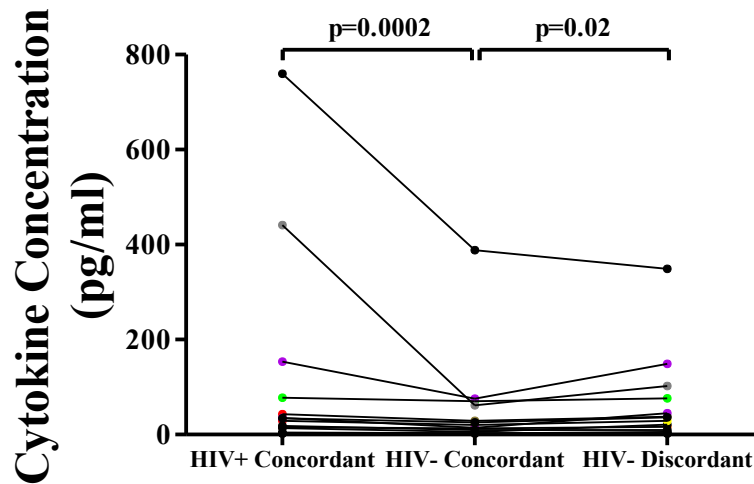


Figure 6. 1 Comparison of genital cytokine levels in HIV+ seroconcordant, HIV- unexposed (seroconcordant) and exposed (serodiscordant) individuals. The median value of each of the 14 cytokines measured in genital secretions was compared between groups using Wilcoxon signed rank test.

6.3.2 Compartmental relatedness between cytokines

To evaluate the relatedness between compartments in inflammatory markers, the association between concentrations of IL-1 β , IL-6 and IL-10 (the cytokines which were measured in both compartments) was investigated in HIV- (Figure 6.2A) and HIV+ women (Figure 6.2B). Irrespective of HIV or partner status, no association was observed between matching plasma and genital tract concentrations of IL-1 β , IL-6 and IL-10, suggesting that these cytokines are likely to be independently produced in the female genital tract compared to blood.

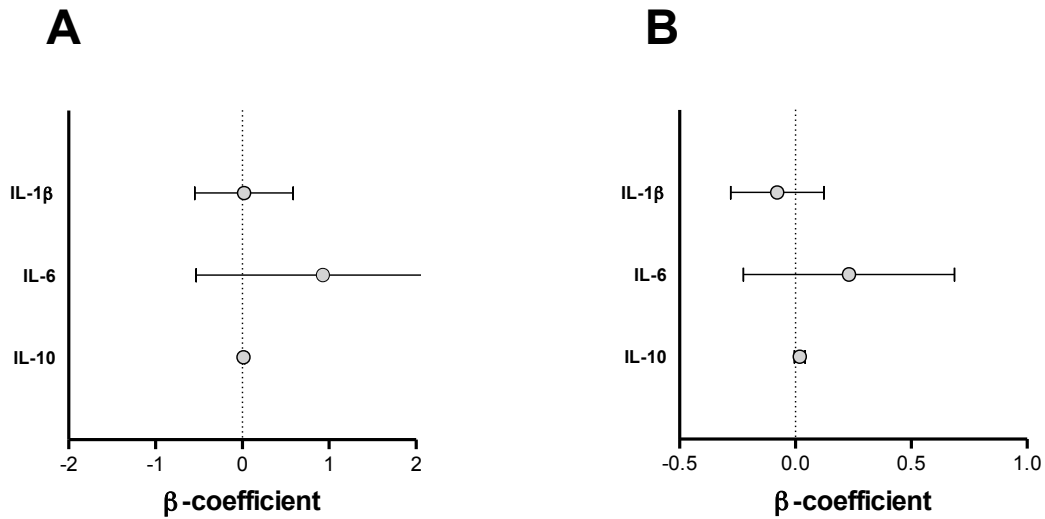


Figure 6. 2 Relationship between cervical level of IL-1 β , IL-6 and IL-10 and their matching blood plasma level of HIV- (A) and HIV+ (B) women. Matching levels of IL-1 β (A), IL-6 (B) and IL-10 (C) in the cervical compartment and blood plasma were investigated using simple linear regression. $p < 0.05$ were considered significant.

Figure 6.3 depicts the compartmentalization of cytokines by paired sample ratios of their cervical to blood plasma concentration. Ratios >1 or <1 indicate enrichment of a cytokine in cervix and blood plasma, respectively. This analysis suggests that the genital tract is dominated by a more inflammatory profile (irrespective of HIV status), whereas blood is characterized by a more anti-inflammatory/regulatory profile.

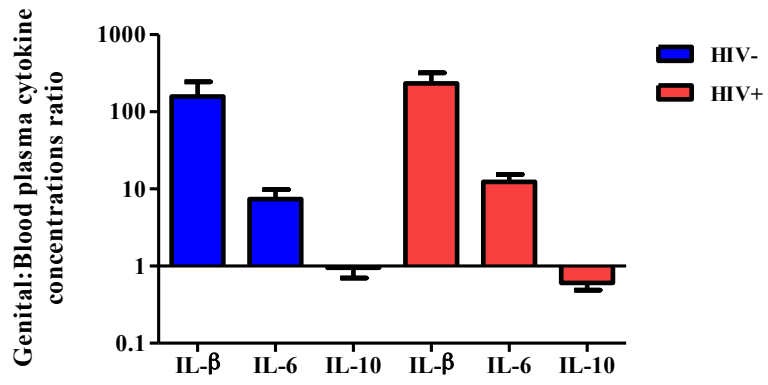


Figure 6. 3 Comparison of the genital: blood plasma cytokine ratios from HIV- (blue) and HIV+ women (red). Mean and standard error of cytokine concentration ratios in cervical and blood plasma are shown for HIV- and HIV+ women.

6.4 Discussion

Sexual transmission remains the predominant mechanism for HIV acquisition and the efficiency of transmission reflects the biology of this specific mucosal compartment (Broliden, 2010). The presence, activation status and phenotype of immune cells and receptors at the genital tract therefore represent an influential combination of factors influencing the outcome on exposure to HIV. The female genital tract provides a highly alterable environment with constant physiological changes in the form of induced inflammation, hormonal cycle and seminal deposition.

While no significant differences were found in the blood plasma inflammatory profile of HIV- unexposed and exposed individuals, inflammatory markers were generally higher at the cervix of exposed women compared to unexposed one, with IL-1 α concentrations being significantly different between the two groups. While inflammation in the female genital tract could lead to recruitment of HIV target cells and other immune cells to this compartment, the elevated inflammatory profile measured in HIV- women in HIV exposed relationships could represent signature of exposure to HIV rather than elevated risk. This interpretation is supported by the finding in Chapter 5 that HIV- exposed individuals had significantly lower frequencies of activated and proliferating CD4+ HIV target cells compared to unexposed individuals, and the previous observation from our group that the extent of immune activation in blood significantly and broadly predicted similar T cell activation in the genital tract of women (Jaspan *et al.*, 2011).

It was interesting to observe that the inflammatory profile in genital secretions from HIV- exposed women were more similar to HIV+ individuals than HIV- unexposed women, which could be reflective of HIV exposure rather than HIV risk. In contrast to the overall higher genital inflammatory cytokines in HIV- exposed compared to unexposed, Chege *et al.* (2012) reported contrastingly blunted pro-inflammatory cytokine responses, measured by mRNA expression rather than protein, in both the genital tract and blood of HIV-exposed seronegative female sex workers in Kenya. In addition to relative immune quiescence, however, HIV-exposed uninfected status has also been linked with HIV-specific T-cells with a pro-inflammatory function profile (Piacentini *et al.*, 2008). It could be argued that cellular immune quiescence in the female genital mucosa establishes a reduction in the pool of susceptible target cells for HIV to infect after initial sexual exposure, decreasing HIV

acquisition risk, and such repeated exposure to the virus may in turn generate the mucosal HIV-specific responses that have been observed. Whether these responses contribute to protection on exposure has not been established, but their low frequency suggests that they may contribute to protection, while at the same time maintaining a low enough density of activated and susceptible target cells at the genital mucosa.

Few studies have investigated the relatedness between innate immune responses in blood and at the genital mucosa, and results from this Chapter suggest that the female genital tract and blood are immunologically separate compartments, with cytokine responses being likely driven by local stimuli within the mucosa compared to blood. In support of this finding, Hedges *et al.* (2006) previously reported that serum concentration of IL-1 β , IL-6 and IL-8 were not influenced by any changes in genital levels of these cytokines, which they suggested were associated rather with change in vaginal flora. A study to compare expression of immunoregulatory cytokines in the cervicovaginal lavages with matching serum of commercial sex workers (HIV+ and HIV-) and HIV- low risk individuals in Benin similarly suggested differences in immunoregulatory cytokine expression patterns between the two compartments (Lajoie *et al.*, 2008). Lisco *et al.* (2012) showed that semen and blood are distinct immunological compartments in which concentrations of cytokines are profoundly different. Blish *et al.* (2012) similarly found no association between genital and systemic cytokine concentrations. Strategies to decrease HIV transmission should take into account local genital tract inflammation, as this is not reflected in plasma measurements.

CONCLUSION

In spite of the considerable scientific advancements in understanding the interactions between HIV, the individual and the community, the biological correlates of protection against HIV infection remain elusive. The observation that a select group of individuals appear to resist HIV infection has led to a host of epidemiological studies aimed at defining protective biological and behavioural attributes that confer protection against infection of these individuals. Data gathered from studying such cohorts of individuals could inform on the design of appropriate strategies that would mimic the effective responses of these resistant individuals. In this dissertation, HIV serodiscordance among black stable heterosexual couples in Sub-Saharan Africa was investigated to determine potential correlates of protection against HIV infection.

Chapter 2 focused on HIV+ individuals who were either in HIV serodiscordant or seroconcordant relationships. It explored the potential virological and immunological risks associated with being an HIV+ individual in a relationship with a stable partner who is either HIV seroconcordant or serodiscordant, using objective biomarkers of HIV disease progression (including plasma HIV load and absolute blood CD4 counts). The most compelling finding from this Chapter was that HIV+ individuals in HIV seroconcordant relationships with similarly HIV+ stable sexual partners had 0.5 log₁₀ higher plasma HIV loads than HIV+ individuals in HIV serodiscordant relationships (with HIV- partners). Even though HIV loads in plasma were negatively associated with absolute CD4 counts overall in this cohort, this 0.5 log₁₀ higher viral load in HIV+ seroconcordant compared to serodiscordant individuals did not predict a worse clinical outcome in seroconcordant individuals (as indicated by lower absolute CD4 counts at future visits over a 2 year period of follow-up). Although this appears counterintuitive, there are several published studies in discordant couples similarly reporting differences in viral loads depending on partner status although some studies report differences in CD4 counts over time while others do not (Malamba *et al.*, 2005; Chatterjee Rogers *et al.*, 2005; Kumarasamy *et al.*, 2010). In this study, it is possible that the difference in plasma viral loads between seroconcordant and serodiscordant individuals (0.5 Log = 14700 copies RNA/ml), although being significantly different between groups, was not high enough in magnitude to result in a worse clinical outcome as measured by blood CD4 count.

In HIV+ individuals, plasma RNA load is generally accepted as a good predictor of the rate of loss of CD4 cells at a population level (Lima *et al.*, 2009). However, there remains a wide variability in the rate of CD4 depletion among infected individuals (Grabar *et al.*, 2000; Florence *et al.*, 2003; Jevtovic *et al.*, 2005; Badri *et al.*, 2008). In Chapter 2, it was shown that initial CD4 count at baseline was a strong predictor of the magnitude of the eventual loss of CD4 cells over the following 12 months, accounting for 77% of CD4 depletion in blood from baseline to month 12. While a change in plasma HIV load was predictive of a change in blood CD4 count over the same period, it accounted for only 35% of the variability in CD4 counts. While this association was stronger than that reported by Rodriguez *et al.* (2006), it remains of paramount importance to define other factors influencing CD4 counts, using more in depth techniques in treatment decisions and for understanding HIV pathogenesis. Since CD4 cell counts and viral loads can vary widely among individuals, due to a wide range of genetic and biological factors, the inclusion of any of these factors that could account for any additional variation, over and above that explained by the model could prove determinant in the improvement of the timing of therapy initiation.

While the molecular basis for the difference in HIV plasma load between seroconcordant and serodiscordant individuals remains unclear, viral load setpoint represents an accepted predictor of clinical disease course during HIV infection. This therefore suggests that HIV+ seroconcordant individuals in this study may be at greater risk of a more rapid disease progression (Lavreys *et al.*, 2006; Saathoff *et al.*, 2010). It is likely that HIV seroconcordant couples are infected with viruses with similar phenotype or characteristics, which may result in higher viral load setpoints. Future studies from this cohort will aim to use 454 sequencing to identify whether seroconcordant individuals are sharing viruses with their partners and whether there is any evidence for dual-, co- and superinfection. This will determine if any continuous viral movement between partners exists and will allow us to determine which of the partners was the likely donor versus recipient. Moreover, to investigate whether HLA matched or mismatched couples display differences in frequencies/rates of superinfection and immunopathogenesis, future studies will evaluate HLA types of couples. Data suggest that HLA incompatibility between sexual partners confers protection against HIV transmission (Jennes *et al.*, 2013).

From the host perspective, a number of immune cell subsets are also key in HIV pathogenesis, in particular HIV-specific effector cells endowed with effective antiviral functions. The role of cytotoxic CD4⁺ T-cells in the control of HIV has been supported by

recent findings reported by Zheung *et al.* (2009), where the ability of Nef-specific CD4⁺ T-cells to suppress HIV replication in HIV-infected CD4⁺ T-cells and macrophages was demonstrated. Intracellular cytokine staining assays following Gag-specific stimulation in Chapter 2 revealed a higher magnitude of IFN- γ and MIP-1 β production by CD4 T-cells from HIV⁺ seroconcordant individuals when compared to serodiscordant individuals. This, however, was not associated with better control of HIV as has previously been reported in HIV⁺ individuals by Soghoian *et al.* (2012). CD8⁺ T-cells have the capacity to kill infected cells through the ability to produce and secrete an array of cytokines with antiviral properties, such as IFN- γ , TNF- α , MIP-1 α and β , RANTES, perforin and granzyme. Chapter 2 assessed Gag-specific CD8⁺ T-cell functional properties and revealed higher frequencies of IFN- γ and MIP-1 β production by HIV⁺ individuals in seroconcordant relationships compared to those in serodiscordant relationships. Moreover, a significant positive association was found between HIV-specific IFN- γ responses by CD8⁺ T-cell and HIV plasma loads, confirming that frequencies of CD8⁺ T cell responses were tracking antigenic load (Ogg *et al.*, 1998; Betts *et al.*, 2001; Edwards *et al.*, 2002).

The direct depletion of CD4⁺ T-cells by HIV infection was shown to only partially account for the decrease of the CD4⁺ T-cell pool in systemic circulation in Chapter 2, and a range of bystander mechanisms have been postulated as contributors to CD4⁺ T-cell death (Grossman and Paul, 2000; Sousa *et al.*, 2002; Holm and Gabuzda, 2005). Recent views consider HIV/AIDS as a dynamic and progressive disease stemming from a mixture of hyperimmune activation and chronic inflammation. The broader comprehension gathered in the field of HIV immunology over the past 3 decades, made possible by technological advancement such as multiparametric flow cytometry analysis, offers the opportunity of monitoring a constellation of cellular immune markers relevant for immune activation, inflammation, control of replication or the exhaustion and modification of the immune functions. Following productive HIV infection, the host's immune system on the one hand plays a predominant role in keeping this persistent virus in check, while on the other hand, this long-term control is accompanied by chronic hyper activation and inflammation, such that a progressive exhaustion of the host immune competence ensues and this leads to the onset of non-AIDS defining co-morbidities. Chapter 3 focused primarily on the monitoring of cellular activation and soluble inflammatory markers in the blood of HIV⁺ participants.

Assessment of systemic immune activation levels has become a routine indicator in the immunomonitoring of HIV⁺ patients, as these levels are significantly associated and

predictive of HIV disease progression, even in viral suppressed participants on therapy (Hunt *et al.*, 2003; Vrisekoop *et al.*, 2008; d’Ettorre *et al.*, 2011). In Chapter 3, the expression of receptors CD38, HLA-DR and Ki67 on T-cells, which generally reflect the activity of the immune system against viral antigens, were assimilated to the degree of systemic immune activation. In addition to these receptors, the expression of the coreceptor CCR5 was also assessed. While the total activation status of CD4⁺ and CD8⁺ T-cells in systemic circulation of HIV⁺ seroconcordant individuals were higher than that of HIV⁺ serodiscordant individuals, it came as a surprise that serodiscordant individuals displayed a significantly higher level of CD8⁺ T-cell activation as measured by the individual expression of CD38 and HLA-DR. This could be explained by the fact that CD8⁺ T-cell activation occurs at a lower viral threshold because of their cytotoxic potential, but progressively, it is expected that hyperactivation will lead to CD8 cell exhaustion and anergy (Karim *et al.*, 2012). One of the limitations of this study is the lack of an accurate timescale for when the participants became infected with HIV. If the seroconcordant participants had been infected for a longer period compared to the serodiscordant ones, they may have reached this stage of exhaustion and anergy for their CD8⁺ T-cell subset.

CD38 expression remains a well documented marker of early T-cell activation and is moreover associated with increased cytokine production and increased T-cell differentiation (Ausiello *et al.*, 1996; Orendi *et al.*, 1998; Savarino *et al.*, 2000). While most studies assessed immune activation through the measurement of frequency of T-cell expressing CD38, the prognostic value of T-cell activation is improved when assessed by the coexpression of CD38 with HLA-DR (Benito *et al.*, 2004), suggestive of a hyperactivated profile. Findings from Chapter 3 following the comparison of the systemic activation profile of participants with low (<1500 cps/ml) and higher (>1500 cps/ml) HIV viremia revealed that individuals with higher plasma HIV load had significantly higher level of CD8⁺ T-cells expressing CD38 individually or dually with HLA-DR. The further association observed between CD38 and HLA-DR coexpression on T-cells and plasma HIV load and blood CD4% lend support to earlier observation that T-cells dually activated with CD38 and HLA-DR are significantly predictive of a worse disease outcome as observed in separate studies (Kovac *et al.*, 2010; Karim *et al.*, 2012).

In agreement with other reports, cross sectional cytokine data from Chapter 3 showed that a key driving force in the establishment of a continued HIV cycling, and probably progression to worse HIV disease outcomes, is the ongoing and aberrant systemic inflammation. The

constant priming of the immune system for action results in the ongoing release of pro-inflammatory cytokines, such as TNF- α and IL-1 β . Consistent with earlier observations of higher HIV viremia in seroconcordant individuals compared to serodiscordant ones, seroconcordant participants manifested a significantly higher plasma concentration of proinflammatory cytokines leading to the setting up of a vicious cycle involving HIV replication, systemic immune activation and chronic plasma inflammation, whereby the increase in any one of these three factors would be analogous to ‘adding fuel to the fire’. The lack of difference seen when comparing absolute blood CD4 count in seroconcordant and serodiscordant individuals could be partly attributed to a greater ability of the seroconcordant individuals to switch into anti-inflammatory mode, as supported by a higher circulating level of IL-10, potentially resulting in a slower erosion of immune protection. Multivariate analysis revealed that the dual expression of CD38 and HLA-DR on CD4⁺ T-cells and circulating level of TNF- α were the best predictors of HIV plasma viral load.

As understanding of the attributes of the immunological response to HIV infection grows and the role of chronic systemic inflammation shows more relevance, it is necessary that this acquired knowledge becomes integrated in the clinical management of the infection. Chapters 2 and 3 highlighted the fact that decisions concerning the implementation of therapy can no more be purely based on HIV viremia and absolute blood CD4 count. It is imperative that the current systemic immune activation and inflammation profile be taken into consideration when treatment programs are set up. There can be significant clinical benefits in the inclusion of affordable and accessible laboratory markers of immune activation and inflammation in the treatment or monitoring of patients with chronic HIV infection.

The central finding of this dissertation centres on the CCR5 coreceptor expression on T-cells of participants with different HIV and couple status, and its implication in disease progression in HIV⁺ participants and HIV transmission risk in HIV⁻ participants. CCR5 is the major coreceptor for infection by macrophage tropic strains of HIV-1 and therefore has a vital role in the transmission and pathogenesis linked to the virus (Deng *et al.*, 1996; Dragic *et al.*, 1996). Memory CCR5⁺CD4⁺ activated T-cells represent the preferred targets for HIV and it has been confirmed that up to 80% of the GALT CD4⁺ T-cells, consisting mainly of CCR5⁺ activated cells, can be depleted within the first month of primary HIV infection (Brenchley *et al.*, 2004). The most compelling piece of evidence outlining the importance of CCR5 in HIV infection has been demonstrated by a 32 bp deletion in the CCR5 ORF, the CCR5 Δ 32 mutation, which translates to a phenotype of reduced CCR5 surface expression in

individuals heterozygous for the gene, and conferring full resistance to HIV in individuals homozygous for the gene, as they fail to express CCR5 on cell surfaces (Liu *et al.*, 1996; Wu *et al.*, 1997).

Using data from Chapter 3 showing a significantly higher level of expression of CCR5 on the T-cells (both CD4⁺ and CD8⁺) of seroconcordant participants compared to serodiscordant participants, it can be proposed that plasma HIV load difference observed between the two groups could be partly attributable to this differential expression of CCR5. Increased CCR5 density was determined to correlate with high HIV viral load in a study by Reynes *et al.* (2000). Moreover, the observation that seroconcordant individuals have a higher frequency of CD4⁺CD38⁺CCR5⁺ T-cells in their systemic circulation lends support to the argument that these individuals manifest a higher HIV viremia compared to serodiscordant individuals on account of a greater HIV infection and replicative capabilities. This is further confirmed by the fact that the CD4⁺CCR5⁺ subset in circulation is even greater in individuals with a higher plasma HIV load (>1500 cps/ml) when compared to those with lower viral burden (<1500 cps/ml). The expression of chemokine receptor CCR5, and potentially its ligands MIP-1 β , are widely regarded as central to the pathogenesis of HIV infection as their regulation is thought to influence leukocyte migration, as well as HIV infection. CCR5 density has been identified as being one of the determinants of the efficiency of CCR5 in the chemotactic response to its ligands (Desmetz *et al.*, 2006). In Chapter 2, a significantly greater frequency of MIP-1 β was observed when intracellular cytokine staining assays were performed, in both CD4⁺ and CD8⁺ T-cell subsets of seroconcordant individuals when compared to serodiscordant individuals. Finally, from an immunopathological perspective, elevated CCR5 density, as measured by mean number of molecules/cell in HIV+ patients has been linked to faster disease progression (Reynes *et al.*, 2001; Gervaix *et al.*, 2002) as well as poorer response to therapy (Ketas *et al.*, 2007; Heredia *et al.*, 2008).

Individuals in various cohorts globally have been documented to have what appears to be some form of resistance to HIV infection in spite of several exposures (Clerici *et al.*, 1994; Bernard *et al.*, 1998; Makedonas *et al.*, 2002; Lo Caputo *et al.*, 2003; Hladik *et al.*, 2003; Jennes *et al.*, 2004; Suy *et al.*, 2007; Hygino *et al.*, 2008). This variability in susceptibility to infection by HIV and its immunopathogenic consequences has been widely investigated and was found to correlate with a plethora of immunological, genetic, viral and sociobehavioural factors. Nevertheless, the exact mechanisms of protection in these individuals are yet to be elucidated. In order to evaluate the immunological factors that may contribute to the relative

resistance in the exposed uninfected HIV- partners in HIV serodiscordant couples, the ‘immune quiescence’ hypothesis described in previous studies (Card *et al.*, 2009; McLaren *et al.*, 2010; Chege *et al.*, 2012) was investigated. Immune quiescence refers to the low baseline level of immune activation and inflammation. Vatakis *et al.* (2010) showed that quiescent T-cells can be infected but viral replication would be rendered inefficient, as HIV preferentially infects activated T-cells offering all the necessary host factors for efficient HIV infection (McLaren *et al.*, 2010).

In line with findings from previous studies documenting an increased HIV susceptibility with increased activation (Shapira-Nahor *et al.*, 1998; Naranbhai *et al.*, 2012) and decreased susceptibility with lower activation (Card *et al.*, 2009, 2012; Songok *et al.*, 2012), it was reasonable to hypothesise that the exposed seronegative individuals included in Chapter 5 would have a lower level of immune activation on account of their lower susceptibility to HIV infection. When compared to HIV- controls in HIV unexposed relationship, HIV-exposed individuals manifested a phenotype of reduced systemic activation. Firstly, HIV-exposed participants expressed a lower frequency of CD8⁺HLA-DR⁺ T-cells, consistent with previous findings of reduced systemic activation described in a HESN MSM cohort (Koning *et al.*, 2005 - reduced expression of HLA-DR, CD38, CD70 and Ki67) and serodiscordant couple cohorts (Camara *et al.*, 2010 - reduced expression of CD38; Begaud *et al.*, 2006 - reduced expression of HLA-DR). As observed with HIV+ serodiscordant individuals in Chapter 3, HIV- exposed individuals expressed significantly lower frequencies of CCR5 coreceptors on both CD4⁺ and CD8⁺ T-cells when compared to HIV- unexposed controls. Moreover, the CCR5⁺ T-cells in HIV- exposed individuals had a reduced proliferative capacity (Ki67⁺CCR5⁺) and lower activation status (CD38⁺CCR5⁺) when compared to HIV-unexposed controls. Keeping in mind that HIV preferentially replicates in activated T-cells, these findings altogether translates to an immune quiescent state where protection against HIV infection is conferred through the limitation of target cell availability.

There exist several lines of evidence showing a relation between the lowered frequency of CD4⁺CCR5⁺ T-cells and decreased level of replication/infection by firstly, SIV in non-human primate models (Pandrea *et al.*, 2007) and secondly, HIV in exposed but uninfected FSW (Fowke *et al.*, 1998; Messele *et al.*, 2001). In addition to a lowered expression of CCR5 on CD4⁺ T-cells in the FSW enrolled in their study, Paxton *et al.*, 1998 also reported on a concurrently elevated level of β -chemokines, the cognate ligand for CCR5. *In vitro* studies by Platt and collaborators (1998) have revealed the existence of a specific range for CCR5

density threshold on cells required for efficient replication of R5 viruses. Moreover, the density of CCR5 molecules on CD4⁺ T-cells has been shown to correlate positively with replication of R5 HIV-1 (Lin *et al.*, 2002; Heredia *et al.*, 2007). The present study confirmed CCR5 as a critical and central factor in HIV infection. It is however known that this coreceptor has interpopulation, racial and gender variability in its expression. Picton *et al.*, (2012) in two separate studies, firstly documented the differential expression patterns of CCR5 in two racially distinct South African populations, showing notably that the CCR5 expression, both in term of density (number of receptors per cell) and frequency was higher within all cell subsets measured in African individuals compared to Caucasian individuals. They also evaluated the influence of CCR5 haplotypes on CCR5 expression in healthy HIV uninfected individuals, highlighting the protective effect of the HHC haplotype in South African Caucasians and the HHA haplotype in South African Africans, both linked to a lowered density of CCR5 expression on leukocytes. The individuals who were homozygous for the CCR5Δ32 mutation conferring resistance to HIV infection were excluded from their analysis. The influence of this mutation is not thought to be a major confounder within the cohort described in this dissertation. While population studies have revealed that the mutation is present at a frequency of 10% in Europe (Samson *et al.*, 1996; Novembre *et al.*, 2005), it is however very rare in Africans (Williamson *et al.*, 2000).

Soluble inflammatory factors in plasma were compared in HIV- unexposed and exposed participants, and together with the differential expression pattern of CCR5 on T-cell surfaces of these two groups of individual is the observation that HIV- exposed individuals have a lower concentration of IL-2 in systemic circulation. There are previous reports of IL-2 upregulating the CCR5 expression on T-cell surfaces (Zou *et al.*, 1999; Weissman *et al.*, 2000; Yang *et al.*, 2001). The reduced level of systemic IL-2 and IFN- γ concentrations in HIV- exposed individuals compared to their unexposed counterparts is generally suggestive of a lowered systemic activation status and hence lowered HIV susceptibility. GM-CSF was another cytokine that was significantly reduced in the systemic circulation of HIV- exposed individuals, and again, as this hematopoietic factor promotes activation, maturation and differentiation of several immune cells (Kedzierskae *et al.*, 1998; Shi *et al.*, 2006), this difference could impact on the susceptibility to HIV infection by increasing the frequency of HIV target cells available in blood that could infiltrate and increase risk of HIV acquisition in lymphoid tissues.

The majority of HIV transmission worldwide occurs at the genital mucosa and therefore knowledge of mucosal immunology is essential in HIV susceptibility studies. As such, it is of primary importance to design strategies that can confer protection to the human mucosal port of entry to HIV. Study of the female genital compartment in this dissertation was aimed at improving our understanding of genital mucosal immunology and its potential linked to the systemic immune system. HIV+ and HIV- women in exposed and unexposed relationship were respectively compared to their counterpart. It has been established in previous studies that there exists a positive association between HIV plasma load and genital HIV load ((Iversen *et al.*, 1997; Hart *et al.*, 1999; Shepard *et al.*, 2000; Kovacs *et al.*, 2001; Vettore *et al.*, 2006; Taton *et al.*, 2011). A similar observation was made in this study and in line with the significantly higher plasma HIV burden observed in seroconcordant women compared to serodiscordant women in Chapter 2, seroconcordant women were shown to shed more virus at the genital tract when compared to serodiscordant ones. This represents a greater risk of transmission to their partners. To determine if this difference in genital viral load observed between the two groups was indicative of passive transudate from blood to the genital tract, or the active recruitment and homing of HIV-infected target cells to the genital mucosa under a chemokine gradient, the concentration of local inflammatory and regulatory factors were measured in genital secretion. While systemic inflammatory cytokines were found to be significantly elevated in HIV seroconcordant compared to serodiscordant ones, no such difference was found at the genital mucosal level. The lack of difference in genital inflammatory profile between seroconcordant and serodiscordant women could be down to the 10-fold greater difference observed in plasma HIV load (14 700 cps/ml) compared to genital HIV load (1450 cps/ml) between seroconcordant and serodiscordant individuals.

The association between specific genital cytokine levels and genital HIV load was confirmed when stratifying women by genital HIV load. Women who were shedding HIV at their cervix had significantly higher level of inflammatory, chemotactic and regulatory cytokines compared to women who were not shedding. After adjusting for plasma viral load, genital concentrations of IL-1 α and IL-1 β remained strong predictors of genital HIV shedding. IL-10 and IL-8 also remained significant after adjustment for plasma HIV load confirming that both and inflammatory and chemotactic gradients are in play to determine genital shedding. Sexually transmitted infections may also have an impact on genital mucosal immunity and have been associated with an elevated risk of HIV transmission through physical disruption or the modulation of immune factors associated with HIV acquisition (Rotchford *et al.*, 1999;

LeGoff *et al.*, 2007; Kaul *et al.*, 2008; Shin and Kaul, 2008; Rebbapragada and Kaul, 2008; Kaul *et al.*, 2011). One of the shortcomings in this study was that no laboratory based screening for STIs was conducted, although signs and symptoms (genital ulceration and vaginal discharge) were documented through clinical exam. Syndromic management guidelines for the treatment of STIs and other reproductive tract infections has been introduced by the WHO in the early 1990s to circumvent problems that may be experienced with limited laboratory facilities, high cost of laboratory tests and coordination of patients receipt of test results (Johnson *et al.*, 2011). Few women in this study did have clinical evidence of an STI (ulceration or discharge), and those women with symptoms of an STI were equally distributed between seroconcordant and serodiscordant women.

Comparison of the inflammatory profile of the genital compartment of women in HIV-exposed and unexposed relationship revealed that the inflammatory markers in exposed women were generally but not significantly higher than the HIV- unexposed women. IL-1 α was the only significantly elevated cytokine and this could potentially represent a signature of HIV exposure rather than elevated risk of HIV acquisition. This interpretation is supported by the finding in Chapter 5 that HIV- exposed individuals had significantly lower frequencies of activated and proliferating CD4+ HIV target cells compared to unexposed individuals, and the previous observation from our group that the extent of immune activation in blood significantly and broadly predicted similar T cell activation in the genital tract of women (Jaspan *et al.*, 2011). Finally, Chapter 6 confirmed that there exist clear differences in terms of immune parameters when comparing the female genital tract and blood. When matching levels of IL-1 β , IL-6 and IL-10 in genital secretion and blood plasma were measured, no significant association between the genital compartment and systemic circulation was observed. Cytokine responses within the genital compartment could be locally driven by local stimuli rather than transudation of immune factors from blood. Several studies have confirmed that the female genital tract and blood are immunologically separate compartments (Hedges *et al.* 2006; Lajoie *et al.*, 2008; Blish *et al.*, 2012).

Most studies conducted in exposed seronegative individuals have looked at heterogeneous populations, given the difficulties to recruit such individuals and have moreover used cross-sectional designs. As such, the interpretation of results in term of the protective role of immune responses on resistance to HIV infection remains difficult. While it was confirmed that the difference observed in plasma HIV load between seroconcordant and serodiscordant individuals was maintained over 3 consecutive time points, it will be interesting to see if the

difference seen in inflammation and immune activation profile are sustained over time. Another area of potential research is the genotyping of HLA genes. These play a role in activating the immune system to respond to foreign substances. There is an increasing body of evidence emerging with regard to the role of HLA in HIV transmission. The differences in correlates of protection among HIV-exposed uninfected individuals have been the focus of several investigations and the MHC has emerged as a vital factor determining susceptibility or resistance to infection. Concordance and discordance in HLA class I alleles between HIV transmission pairs has been shown to impact on the likelihood of HIV transmission with the sharing of HLA-B allele resulting in a two-fold greater risk of HIV transmission (Dorak *et al.*, 2004). Possession of other specific HLA alleles has also been implicated with a reduced risk of HIV seroconversion, such as A2/6802 (MacDonald *et al.*, 2000). As for the role of specific HLA alleles in the immunopathogenesis of HIV infected individuals, studies have suggested a protective effect for HLA-B27 and HLA-B57, whereby these individuals tend to progress more slowly than usual to AIDS (Migueles *et al.*, 2000; Carrington and O'Brien, 2003). HLA typing will be performed for a subset of the participants included in this dissertation in a future study to see if this factor plays any role in protection to HIV infection or disease progression. Moreover, deep-sequencing will be carried for the HIV+ seroconcordant couples included in this study to establish if there exists any intra-couple transmission of different viral variants.

In conclusion, the studies described in this dissertation provides valuable insight into the understanding of HIV susceptibility and disease progression profile through the characterisation of different immune parameters in HIV positive and negative seroconcordant and serodiscordant couples. Firstly, it was noted that HIV+ seroconcordant individuals had a significantly higher plasma HIV load compared to their serodiscordant counterparts and that this difference however did not translate into a worse disease outcome in seroconcordant participants, as measured by absolute blood CD4 count. While it was observed that the level of viral replication only partially predicted the rate of CD4 decline, this could be explained by a process wherein the level of HIV replication either determines or reflects the existing level of T-cell activation, and as such only indirectly impact on the CD4 T-cell count. Therefore, the observations that CD4⁺ T-cells are infected by HIV and depleted during the course of infection may merely be an unfortunate coincidence (Roederer, 1998), while the actual basis for the HIV associated CD4 decline may be that these cells are more depleted than CD8⁺ T-cells to the chronic damage induced by immune activation. The different associations

observed between systemic and genital markers of immune activation and inflammation and HIV viral load, both systemically and genitally, leads us to suggest that currently, the standard guideline used to monitor HIV infection, namely clinical assessment, flow cytometric determination of absolute blood CD4 count and molecular assays to quantify plasma HIV burden lacks sensitivity in determining disease stage, progression and therapy initiation and responses. We suggest the inclusion of these extra parameters in conjunction with current laboratory measures, to provide a more 'tailor-made' therapeutic assistance to infected patients. This will however represent a huge challenge on the account of the already high burden of HIV/AIDS in certain regions, such as Sub-Saharan Africa. Finally, CCR5 expression on HIV target cells represents a potential avenue for the development of an anti-HIV strategy to reduce the number of successful viral transmissions in the event of an exposure to HIV.

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