Studies on immunity in the male genital tract

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DEDICATION

This thesis is dedicated to my mother and friend Julia Olivier. You have been the foundation that supported me throughout my path up to this point in my life. Your unconditional love and friendship have been my salvation. Without you there would be no sanity and no thesis.

I love you, always!

Baie dankie vir al die ondersteuning en liefde!

"I've got a good mother and her voice is what keeps me here,

feet on ground, heart in hand, facing forward, be yourself"

- Jann Arden

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LIST OF ABBREVIATIONS

^{0}C	Degree Celsius
ACD	Acid citrate dextrose
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
ARV	Anti-retroviral
BAL	Bronchoalveolar lavage
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCL11	Chemokine ligand 11
CCL2	Chemokine ligand 2
CCL3	Chemokine ligand 3
CCL4	Chemokine ligand 4
CCL5	Chemokine ligand 5
CCR2	Chemokine receptor 2
CCR5	Chemokine receptor 5
CD	Cluster designation
CEF	Cytomegalovirus/Epstein Barr virus/Influenza virus
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor type 4
DC-SIGN	Dendritic cell-specific-Inter-cellular adhesion molecule 3- grabbing non integrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ESN	Exposed sero-negative
FCS	Foetal calf serum
FEM-PrEP	Female pre-exposure prohylaxis
FITC	Fluorescein isothyocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
Gag	Group associated antigen
GALT	Gut-associated lymphoid tissue
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
gp120	Glycoprotein 120
h	Hour
HAART	Highly active antiretroviral therapy
hAP	Human acrosomal protein
HBV	Hepatitis B virus

HCV	Hepatitis C virus
HIV-1	Human Immunodefiency type 1
HLA-DR	Human leukocyte antigen
HSV-2	Herpes Simplex Virus type 2
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IL-12p40	Interleukin 12
IL-12p70	Interleukin 12
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
IP-10	Interferon gamma-induced protein 10 kDa
IQR	Interquartile range
LC	Langerhans cell
LDL	Lower than detection level
LPS	Lipopolysaccharide
LTRA	Leukotriene receptor antagonist
µg	Microgram
µM	Micro molar
MALT	Mucosa-associated lymphoid tissue
MCP-1	Monocyte chemotactic protein 1
mg	Milligram
MGT	Male genital tract
MHC	Major Histocompatibility Complex
MIG	Monokine induced by IFNγ
min	Minutes
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
MIP-3α	Macrophage inflammatory protein 3 alpha
ml	Milliliter
mM	Milli molar
mm^3	Cubic millimeter
MSM	Men who have sex with men
Nef	Negative factor

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
nNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
PAP	Prostatic acid phosphatase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCA	Principal cluster analysis
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
pg	Picogram
pH	Potential of hydrogen
PIs	Protease inhibitors
PMA	Phorbol myristate acetate
Pol	Polymerase
PrEP	pre-exposure prophylaxis
rAd5	Recombinant adenovirus serotype 5
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
RNA	Ribonucleic acid
RPMI	Rosewell Park Memoral Institute Medium
SDF-1α	Stromal cell-derived factor 1 alpha
SEVI	Semen-derived Enhancer of Virus Infection
SIV	Simian Immunodeficiency Virus
SLPI	Secretory leukocyte protease inhibitor
SMCs	Seminal mononuclear cells
SSC	Side scatter
STI	Sexually transmitted infection
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
U	Unit
VL	Viral load

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Abstract

Background. The male genital tract is a major site of HIV acquisition and transmission. It is an obvious site for inducing immune responses to candidate HIV vaccines, to prevent infection or halt the spread of the virus. There are relatively few published studies characterising T cells in the male genital tract. A challenge that hampers studies at this mucosal surface is obtaining samples with sufficient immune cells. Therefore, the first aim of this study was to establish an optimised method to isolate immune cells from the male genital tract. Cellular activation and inflammation in the genital tract have important implications for both transmission and acquisition of HIV, since they provide target cells for viral replication. Thus, the second and third aim of this study was to investigate mucosal T cell activation and inflammatory cytokine profiles in semen in HIV-infected and uninfected men, and compare the immune milieu of the genital tract with the systemic compartment.

Methods. For the optimisation of immune cell isolation from the male genital tract, semen samples from 199 men were used to enumerate T cell yields after the use of combinations of pronase digestion, cell strainers, ficoll isolation and magnetic bead capture. Using the optimised method, the viability and function of T cells were evaluated by flow cytometry. Additional leukocyte subsets, namely B cells, monocytes, neutrophils, Langerhans cells and NK cells were enumerated in semen using flow cytometry. For measuring T cell activation, frequencies of CCR5-expressing and activated (CD38+) T cells in semen and blood of 42 HIV-uninfected men were compared with those in 26 HIV-infected, ARV-naïve and 12 HIV-infected, ARV-treated men. Seminal and plasma viral loads were quantified and correlated with frequencies of activated T cell subsets. Cytokine multiplex assays were used to quantify cytokine and chemokine concentrations in the seminal and blood plasma of 28 HIV-uninfected, 26 HIV-infected, ARV-naïve and 12 HIV-infected, treated men. Univariate and multivariate analyses were performed on associations between cytokine concentrations and viral loads, as well as activated T cell subsets in both the genital and systemic compartments.

Results. This study found that pronase digestion and the use of cell strainers resulted in improved $CD3^+$ T cell yields compared to gradient separation, whilst selection of $CD3^+$ cells using magnetic beads resulted in improving seminal lymphocyte differentiation by flow cytometry. This method allowed for the subsequent functional analysis of $CD4^+$ and $CD8^+$ T cells, and found abundant NK and DC-SIGN-expressing cell populations in semen. In the second part of the study, HIV replication, activation and inflammation were investigated. There was a positive correlation between viral loads in semen and blood. ARVs successfully suppressed

viral replication in the male genital tract in the majority of infected men on treatment. There was substantially greater immune activation in semen compared to blood, regardless of HIV infection or suppressive ARV treatment. The highest levels of immune activation in semen were found in untreated HIV-infected men, and this was mirrored in blood. Notably, although ARV-treatment reduced the numbers of activated T cells in semen, frequencies were not restored to those observed in HIV-uninfected men. Although frequencies of activated $CD8^+$ T cells correlated between compartments in HIV-infected men, there were no associations between immune activation and viral loads. HIV-uninfected men in partnerships with HIV-infected women had elevated frequencies of $CD8^+CCR5^+$ T cells in their blood, but no differences were seen in the genital tract. Semen and blood differed substantially with respect to the cytokine milieu, with the majority of cytokines and chemokines found at higher concentrations in semen than blood, both in HIV-infected and uninfected men. HIV-infection caused a dysregulation of the network of cytokine/chemokine associations in semen. While there were no significant associations found between levels of cytokines and seminal viral load, higher levels of G-CSF in semen of HIV-infected men were associated with higher levels of $CCR5^+CD8^+$ T cells in semen.

Conclusions. This study described an optimised method for the isolation of $CD3^+$ T cells from semen, that can be used in immune assays to investigate the phenotype and function of these cells. The methods developed in this study could be applied to assess vaccine-induced immunity in HIV vaccine trials. In addition, this study characterised a range of immune cell types found in semen, suggesting further avenues of study in terms of characterising humoral and innate responses in the male genital tract. Furthermore, this study showed a high degree of immune activation in semen, regardless of HIV infection. HIV target cells were abundant in HIV-uninfected men, while 75% of HIV-infected men were shedding virus, which has implications for both HIV acquisition and transmission. No direct relationship between T cell activation in the genital tract of HIV-infected men and viral replication was observed, and together with the high degree of T cell activation in the semen of HIV-uninfected men, implies that there may be ongoing inflammation due to other causes, resulting in immune activation. Although there were substantial differences in the immune milieu of blood compared to the genital tract, there were no differences in the levels of cytokines and chemokines in the semen of HIV-infected and uninfected men, or HIV-infected men on suppressive ARV treatment. Thus, even in the absence of HIV infection, the male genital tract appears to maintain a state of inflammation, which may be as a result of undetected and untreated co-infections that may also mask the effect of HIV replication. Lastly, this study showed no obvious link between inflammation and viral load, emphasizing that the relationship between inflammation, cellular activation and viral replication is likely to be complex.

CHAPTER 1
LITERATURE REVIEW

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This thesis focuses on immunity in the male genital tract in the context of HIV infection. The male genital tract plays a central role in sexual transmission of HIV. HIV transmission occurs predominantly through sexual contact with semen as the carrier (Krieger *et al.,* 1995, Vernazza 2005). The importance of the male genital tract in HIV transmission has been highlighted by several key discoveries in the last few years. A considerable reduction in female-to-male HIV transmission was shown in three randomised controlled trials of circumcision. In South Africa, Kenya and Uganda, female-to-male HIV transmission was reduced by 60%, 53% and 48% respectively, following male circumcision (Auvert *et al.,* 2005, Bailey *et al.,* 2007, Gray *et al.,* 2007). Recently, sexual transmission of HIV from males to females was shown to be enhanced by amyloid fibrils in semen (Munch *et al.,* 2007). These fibrils, termed semen-derived enhancer of virus infection (SEVI), were shown *in vitro* to trap HIV virions in a network of fibrils, thereby potentially extending vaginal exposure to HIV acquisition. HSV-2 is the most prevalent co-infection with HIV, and can cause genital ulcers (Weiss 2004). HSV-2 not only elevates levels of genital pro-inflammatory cytokines (Rebbapragada *et al.,* 2007), but even asymptomatic reactivation of HSV-2 elevates HIV levels in the genital tract (Mbopi-Keou *et al.,* 2000). Although antiviral treatment of HSV-2 lowered HIV levels at the rectum and in the genital tract (Zuckerman *et al.,* 2007, Baeten *et al.,* 2008, Dunne *et al.,* 2008), a recent randomised trial showed no reduction in HIV-1 transmission upon treatment for HSV-2 with acyclovir, despite a decrease in plasma HIV viral load (Celum *et al.,* 2010).

This introduction will review the anatomy of the male genital tract, potential target cells for HIV infection within the genital tract, factors that may enhance HIV replication and transmission, including immune activation and inflammation, the male genital tract as a potential HIV reservoir and HIV-specific immunity in the genital tract.

1.1 The HIV pandemic

In an attempt to combat the HIV pandemic, there have been successes and failures. By the end of 2009 the number of people receiving antiretroviral (ARV) treatment increased 13-fold, however an estimated 10 million people still need therapy globally. In the 10 years between 1999 and 2009, there has been a 27% increase (26.2 million – 33.3 million) in the estimated number of people living with HIV (UNAIDS, 2010). Overall there has been a global drop in the number of new infections in the past few years, with 22 countries in sub-Saharan Africa showing a decrease in the occurrence of HIV infection by more than 25%. Despite this decrease, there were still 1.8 million new infections in sub-Saharan Africa in 2009 (Avert, 2011) [Table 1.1]. World-wide in 2009, a third of all AIDS-related deaths, new infections and people living with HIV lived in 10 southern African countries (UNAIDS, 2010). Furthermore, approximately 40% of all HIV-infected adult women live in southern Africa (UNAIDS, 2010).

Table 1.1 Regional statistics for HIV and AIDS, end of 2009

Region	People[a] living with HIV/AIDS	People[a] newly infected	Adult prevalence[b]	AIDS-related deaths
Sub-Saharan Africa	22.5 million	1.8 million	5.0%	1.3 million
North Africa & Middle East	460,000	75,000	0.2%	24,000
South and South-East Asia	4.1 million	270,000	0.3%	260,000
East Asia	770,000	82,000	<0.1%	36,000
Oceania	57,000	4,500	0.3%	1,400
Central & South America	1.4 million	92,000	0.5%	58,000
Caribbean	240,000	17,000	1.0%	12,000
Eastern Europe & Central Asia	1.4 million	130,000	0.8%	76,000
North America	1.5 million	70,000	0.5%	26,000
Western & Central Europe	820,000	31,000	0.2%	8,500
Global Total	33.3 million	2.6 million	0.8%	1.8 million

[a] Adults and children

[b] Proportion of adults aged 15-49 who are living with HIV/AIDS

1.1.1 The HIV burden in sub-Saharan Africa

Despite a decrease in the number of new HIV infections in sub-Saharan Africa, the total number of people living with HIV have steadily increased, reaching 22.5 million, which constituted 68% of the global total in 2009 (UNAIDS, 2010). Southern Africa is still the most severely affected region, with an estimated 11.3 million people living with HIV, which constituted an increase of nearly one third from estimates a decade earlier (UNAIDS, 2010). In sub-Saharan Africa, the majority of newly infected HIV cases occurred during unprotected heterosexual intercourse, transmission to newborns and breastfeeding. The greatest risk factor for HIV infection in this region continues to be unprotected sex with multiple partners (UNAIDS, 2010). South Africa remains one of the only countries in the world where, since the 1990s, child and maternal mortality has increased (UNAIDS, 2010) [Figure 1.1].

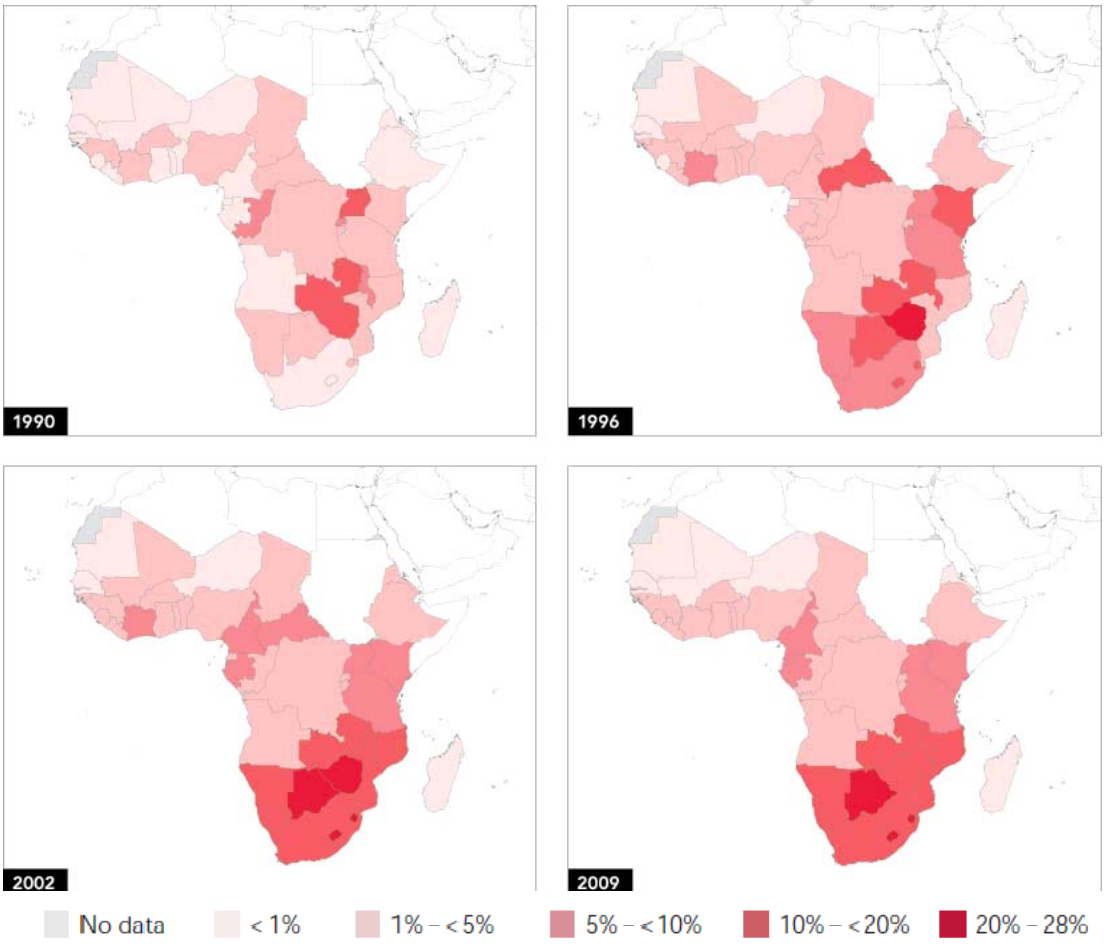


Figure 1.1 HIV prevalence in sub-Saharan Africa. Sub-Saharan Africa's disease burden has increased progressively since 1990 (taken from UNAIDS, 2010).

In fact, in South Africa, 35% of deaths in children under five as well as the largest cause of maternal mortality is due to AIDS (UNAIDS, 2010). The availability of ART has increased dramatically in sub-Saharan Africa, from 2 – 37%, while AIDS-related deaths have decreased by 18% in southern Africa alone (UNAIDS, 2010).

1.1.2 Preventing new HIV infections: HIV vaccines and other biomedical prevention tools

Despite the success of highly active antiretroviral therapies (HAART) in suppressing HIV replication, the development of an HIV vaccine to avert new infections remains a priority. There are several major challenges in developing a prophylactic HIV vaccine. These include the degree of genetic diversity of the virus, which results in a variety of subtypes and recombinant forms circulating in different geographical areas. This presents the challenge of whether a vaccine based on one subtype would potentially protect against all subtypes equally well (McBurney *et al.,* 2008). Due to its high rate of mutation, HIV has the ability to evade the host immune response (Korber *et al.,* 2009), which may imply that vaccines might have to target regions of the virus that cannot mutate without a fitness cost. Furthermore, the correlates of immune protection against HIV infection are largely unknown, and although the macaque SIV model of infection has given us many answers, it is limited in predicting the efficacy of candidate HIV vaccines (Kim *et al.,* 2010). The focus of vaccine design has been on inducing cell-mediated immune responses capable of reducing viral load (Kim *et al.,* 2010), or neutralising antibodies that would be capable of providing sterilising immunity (Walker & Burton, 2008). Variability in the frequency, magnitude, breadth and functional nature of cellular immune responses to viral vectors carrying HIV genes has emphasized the need for further research (Harari *et al.,* 2008; Priddy *et al.,* 2008; Catanzaro *et al.,* 2007). New adenovirus vectors show promise as vaccine vectors (as reviewed in Barouch 2010), and recent data on persistent replicating CMV vectors show that sterilising immunity may be possible with T cell-generating vaccines (Hansen *et al.,* 2011). The recent discovery of more broadly cross neutralising antibodies against HIV in infected individuals also raises hopes of developing a vaccine capable of generating such antibodies (as reviewed in Stamatatos *et al.,* 2009).

Two recent clinical trials of candidate HIV vaccines have illustrated the challenges in HIV vaccine development. In the Phase IIb Step trial, designed to test the 'T cell

vaccine' concept, a recombinant adenovirus vector (rAd5) containing HIV-1 *gag*, *pol* and *nef* genes from HIV-1 subtype B did not protect participants from HIV acquisition or achieve lower viral load in those who became infected (Buchbinder *et al.,* 2008; Sekaly 2008). A parallel trial, Phambili, conducted in South Africa was as a result halted early and also did not show any efficacy (Gray *et al.,* 2011). In the Step trial, those volunteers that received the vaccine exhibited higher rates of HIV infection compared to the placebo group. Researchers have hypothesized that pre-existing antibodies to Ad5 in participants elevated the risk of HIV infection after vaccination with the rAd5 vector (Perreau *et al.,* 2008), and a recent study found a link between Ad5-specific $CD4^+$ T cells and an elevated risk of HIV-1 acquisition during the Step trial (Hutnick *et al.,* 2009). However, vaccine recipients in the Phambili trial did not show enhanced acquisition (Gray *et al.,* 2011). The RV144 Phase III efficacy trial in Thailand tested a modified canarypox vaccine carrying gp120, *gag* and *protease* (ALVAC) as a prime, boosted with a bivalent gp120 subunit vaccine (AIDSVAX B/E) (Rerks-Ngarm *et al.,* 2009). Although the vaccine showed 31.2% efficacy in modified intent-to-treat analysis of participants, there were no decreases in viral load in those that became infected. Although this represents a moderate degree of protection, this is the first HIV vaccine trial to show any effect in reducing HIV infections; follow up trials are planned, as well as in-depth studies to identify the correlates of protection from HIV infection.

Although a prophylactic vaccine for HIV has not yet been developed, additional biomedical measures to prevent HIV transmissions that have been developed in parallel have recently shown a great deal of promise. These involve using antiretroviral therapy to prevent new infections. In the recent CAPRISA 004 trial, the antiretroviral tenofovir formulated in a vaginal gel decreased HIV acquisition by 54% in women using the gel > 80% of the time (Abdool Karim *et al.,* 2010). The use of oral pre-exposure prophylaxis (PrEP), in the form of emtricitabine combined with tenofovir (Truvada), in men who have sex with men in the iPrEx trial, showed a 44% reduction in incidence of HIV infection among participants receiving PrEP compared to those not receiving PrEP (Grant *et al.,* 2010). A similar study (FEM-PrEP) investigated the same protection in women, but was stopped due to the ineffectiveness of Truvada in preventing HIV infection (http://www.fhi.org). Extremely promising findings from the HPTN052 study showed a 96% reduction in the risk of HIV-1 transmission from HIV-infected individuals to their partners through oral prophylaxis

at high CD4 counts (http://www.hptn.org). These trials herald a new era in HIV prevention using antiretrovirals.

1.2 HIV is a disease of the mucosal immune system

Transmission of HIV occurs mainly across mucosal surfaces and the ability of the virus to cross this normally effective barrier takes place by means of disruption of epithelial integrity, transcytosis, infection through M-cells and infection and/or transference by mucosal dendritic cells (Shattock & Moore 2003, Mestecky 2006). However, regardless of the site of viral entry into the body, HIV (and SIV), particularly during acute and early productive infection, replicates in the mucosal tissues of the genital tract and gastrointestinal tract where it is responsible for the destruction of a majority of the $CD4^+$ T cells in the body (Veazey *et al.,* 1998, Brenchley *et al.,* 2004, Li *et al.,* 2005, Mattapallil *et al.,* 2005). CD4 and the co-receptor CCR5 are used by HIV for cell entry, and in mucosal effector sites, the majority of effector memory T cells express CCR5 (Poles *et al.,* 2001, Mehandru *et al.,* 2004, Veazey *et al,* 2003), leading to a massive depletion in $CD4^+CCR5^+$ T cells from mucosal sites (Brenchley *et al.,* 2004, Mehandru *et al.,* 2004, Shacklett *et al.,* 2003).

1.2.1 Structure and function of mucosal immune system

The mucosa-associated lymphoid tissue (MALT) is the term that describes lymphoid tissue found in association with mucosal surfaces (Lydyard & Grossi 2006). MALT consists of organised and non-organised secondary lymphoid tissues, like the tonsils, the bronchus-associated lymphoid tissue (BALT) in the lungs, the gut-associated lymphoid tissue (GALT) and the urogenital lymphoid tissues (Lydyard & Grossi 2006) [Figure 1.2]. The MALT exhibits varying degrees of organised lymphoid tissue. For example, the main organised lymphoid tissue in the GALT are found in structures called Peyer's patches that contain M cells, rich in T and B cells as well as dendritic cells and macrophages (MacDonald & Monteleone 2005), all ideal targets for HIV infection. In contrast, the genital tract has no organised lymphoid structures, and in the female genital tract of macaques and humans potential HIV target cells, like CD4 T cells, are evenly distributed below the epithelium (Ma *et al.,* 2001, Pudney *et al,* 2005) and sometimes cluster in small founder foci (Zhang *et al.,* 2004). From studying SIV infection in macaques, subsequent to a productive infection in the genital tract, HIV reaches the draining iliac lymph nodes where concentrated populations of T cells are infected and may then spread throughout the body (Miller *et al.,* 2005).

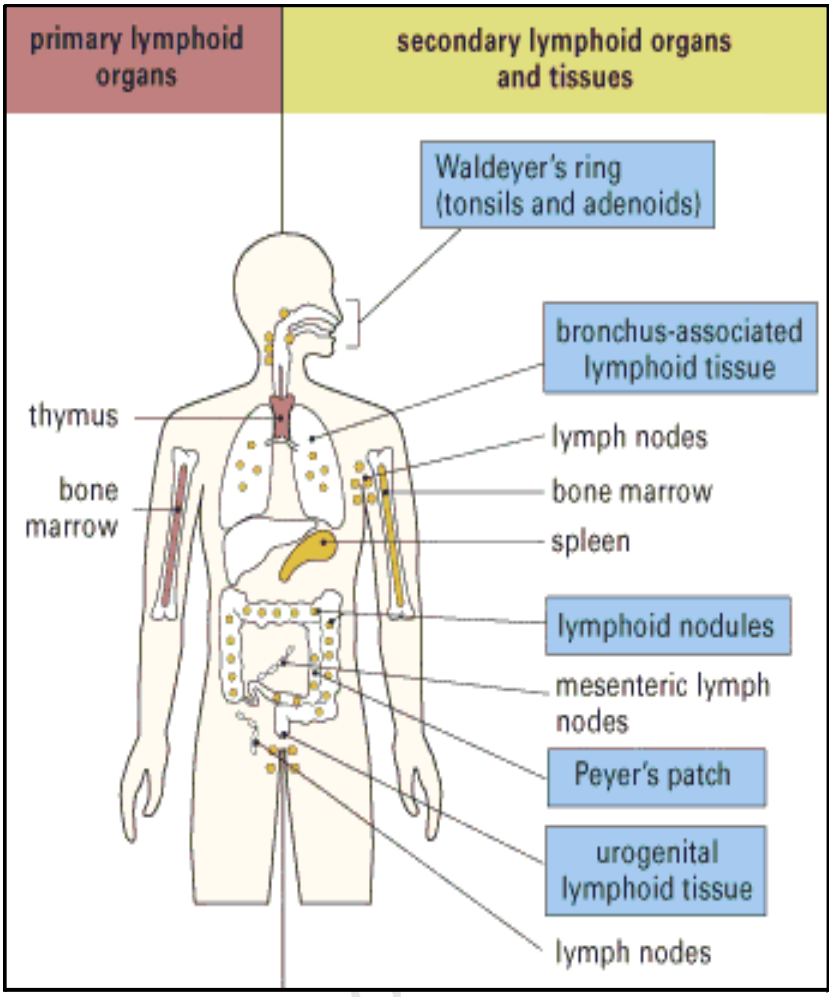


Figure 1.2 Major lymphoid organs and tissues. Tonsils, Peyer's patches and other mucosa-associated lymphoid tissues (MALT) [blue boxes] react to antigens entering mucosal barriers (taken from Lydyard & Grossi, 2006).

1.2.2 Organisation of the male genital tract

The male genital tract (MGT) consists of the testes, the excurrent duct system (rete testis, efferent ducts, epididymis and vas deferens), accessory glands (seminal vesicles, prostate and bulbourethral glands) and the penis (Cao & Hendrix 2008) [Figure 1.3]. The epithelia of the male genital tract consist mainly of two types, simple and stratified epithelia. A single layer of simple columnar, cuboidal or pseudostratified epithelia are found in accessory organs, rete testis, epididymis, vas deferens and penile urethra, while stratified squamous epithelia can be found at the meatus (distal end of penile urethra), fossa navicularis (opening) and foreskin (Anderson & Pudney 2005). Both the foreskin and meatus exhibit differential levels of keratinisation (McCoombe & Short 2006). The outer foreskin is highly keratinized while the inner foreskin has a lower degree of keratinisation (Patterson *et al.,* 2002). Mucus glands found in most simple epithelia in the MGT protect against pathogens through secretion of large amounts of mucus, for example the mucin, MUC6, secreted by the seminal vesicles. Conversely, stratified epithelium is glandless with numerous layers physically hindering pathogen invasion (Anderson & Pudney 2005).

Testis and excurrent duct system

The tissue within the testis consists of a germinal compartment connected by interstitial tissue. Sertoli cells, the "nurse-maid" cells that assist in spermatogenesis, immature germ cells and spermatozoa are the main cells inside the seminiferous tubules (germinal compartment), whereas the interstitial tissue contain blood vessels, lymphatics, mast cells, macrophages and Leydig cells (Anderson & Pudney 2005). The testis provides an immunologically advantaged location protecting germ and sperm cells against the body's autoimmune reaction (Tung 1995). Protection of these germ cells from the immune immune system is achieved by tight junctions between neighbouring Sertoli cells forming the blood-testis barrier (Dym & Fawcett 1970, Johnson & Setchell 1968, Shehu-Xhilaga *et al.,* 2005). Entry of macromolecules such as immunoglobulins and complement into the seminiferous tubules is also restricted (Anderson & Pudney 2005).

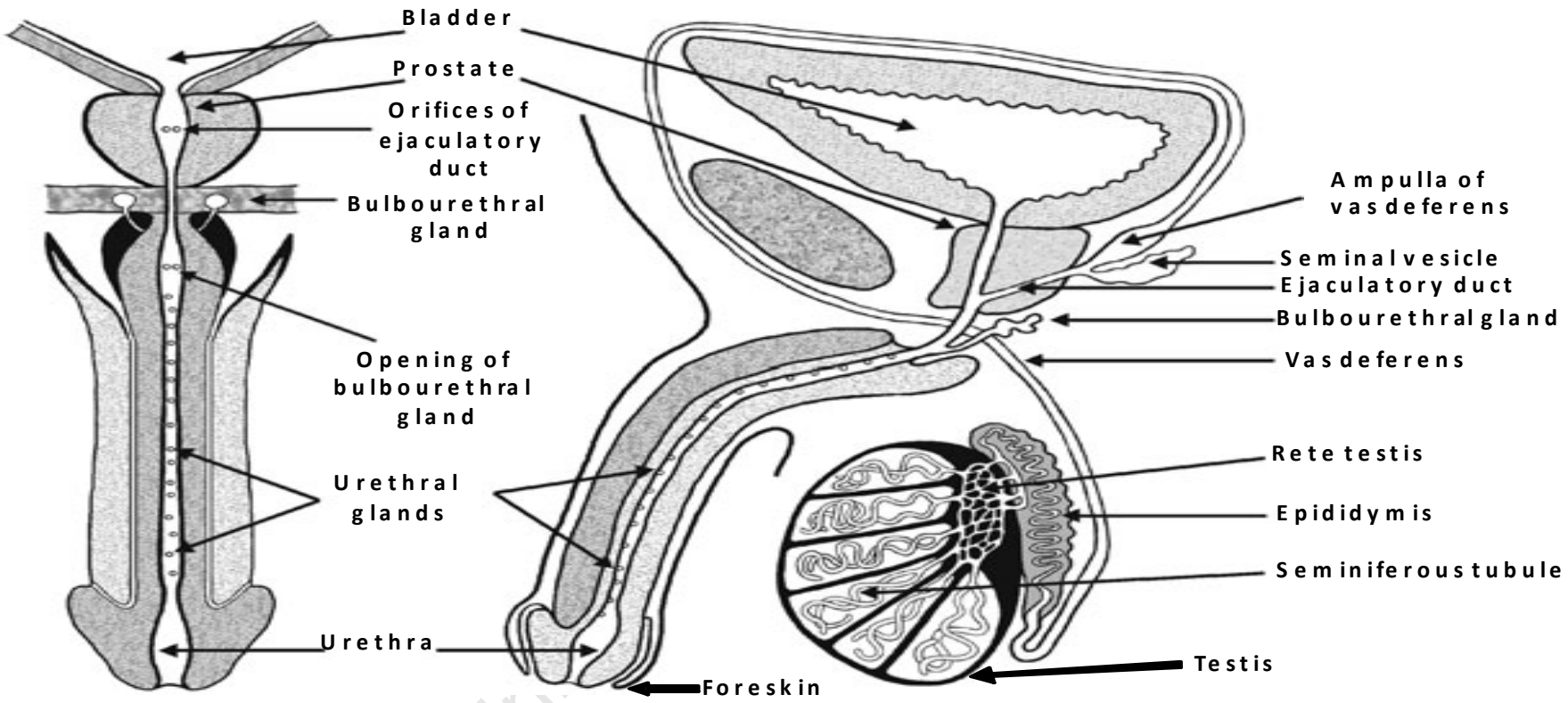


Figure 1.3 Anatomy of the male genital tract. (Taken from Cao & Hendrix, 2008)

The rete testis, efferent ducts, epididymis and vas deferens all form part of the excurrent duct system (Shehu-Xhilaga *et al.,* 2005). From the seminiferous tubules immature sperm cells move through the rete testis which connects the mediastinum to the tightly wound up epididymis through a series of efferent ducts (Anderson & Pudney 2005). Within the epididimys, sperm cells attain maturity by forming of the sperm tails and achieving fertilising capacity, while the muscular duct (vas deferens) is responsible for expelling the mature spermatozoa into the urethra (Anderson & Pudney 2005).

Accessory glands

The seminal vesicles, prostate and bulbourethral glands make up the accessory glands of the male genital tract. The seminal vesicles are a pair of large glands on either side of the vas deferens, extending to the base of the prostate (Anderson & Pudney 2005, Shehu-Xhilaga *et al.,* 2005). Nearly 30% of seminal fluid, consisting of mucus and nutrients, is secreted by the seminal vesicles. The urethra originates from the urinary bladder and it is here where the prostate surrounds the urethra (Anderson & Pudney 2005). Regulated by male hormones, the remaining 60% of seminal fluid, mainly proteins and other nutrients essential for sperm function, is contributed by the largest male accessory organ, the prostate (Shehu-Xhilaga *et al.,* 2005). The bulbourethral glands or Cowper's glands, are small glands located on either side of the urethral bulb. The function of the bulbourethral glands is to secrete lubricant (mostly glycoproteins) to facilitate the ejaculation of sperm (Chughtai *et al.,* 2005).

The penis, foreskin and penile urethra

The penis consists of three cylindrical hollow bodies, the corpora cavernosa, the corpus spongiosum surrounding the urethra, and the urethra that broadens into the glans penis. The loose connective tissue that moves freely over the glans penis is called the foreskin (Shehu-Xhilaga *et al.,* 2005). As part of the ejaculatory duct system of the genital tract, the penile urethra transports spermatozoa at the time of ejaculation. Directly before ejaculation, numerous glands in the penile urethra produce a highly viscous secretion that is released, called pre-ejaculatory fluid (Anderson & Pudney 2005).

1.2.3 Semen

Semen consists of seminal plasma and seminal cells. The testis, epididymis, seminal vesicles, bulbourethral glands, prostate and periurethral glands contribute secretions to make up seminal plasma (Coombs *et al,* 2003). Seminal cells in semen consist of spermatozoa, immature germ cells, macrophages, T cells and polymorphonuclear cells (Anderson *et al.,* 1991, Quayle *et al.,* 1998). In the semen of HIV-infected men, the virus is found as free viral particles and present in infected T cells or macrophages (Le Tortorec & Dejucq-Rainsford 2010). Leukocytes in semen are derived from organ epithelia and different ducts of the male genital tract (Aitken, & Baker 1995).

1.2.4 Immune cell targets for HIV infection

Although the male genital tract contains immune cells that can support HIV replication in the epithelium and interstitial tissue of the semen-producing organs (el-Demiry *et al.,* 1985), it is not known precisely where in the MGT HIV replicates. Immune cells are found in all tissues comprising the male genital tract (Anderson & Pudney 2005)[Figure 1.4]. Langerhans cells and $CD4^+$ $CCR5^+$ cells were found in the foreskin and other epithelial layers of the penis (McCoombe & Short 2006). Although $CD8^+$ T cells dominate in the penile urethra, the presence of $CD4^+$ T cells has also been shown (Anderson & Pudney 2005). Within the testis, the most likely targets for HIV-1 infection are tissue-specific macrophages and $CD4^+$ T cells (Hedger 2002). Of all the organs of the male genital tract, the testis has been implicated as being the most important as a possible viral sanctuary, which is defined as any part of the body (organ or tissue) where ARVs are less effective in penetrating allowing HIV-1 to persist.

Macrophages are found in the mucosa of the rete testis, the efferent ducts, the epididymis and the vas deferens, while T cells are compartmentalised between the epithelium and lamina propria (Anderson & Pudney 2005). The lamina propria is a thin layer of connective tissue directly below the epithelium that together with the epithelium forms the mucosal layer. It is rich in capillaries and mucus-producing glands (Young *et al.,* 2006). $CD8^+$ T cells are restricted to primarily the epithelium lining, whereas $CD4^+$ T cells are found mainly in the lamina propria (Ritchie *et al.,* 1984, el-Demiry *et al.,* 1984) As with the epididymis, the accessory glands consist of an epithelial layer and lamina propria. In the seminal vesicles, the epithelia have a secretory function, whereas the lamina propria is made up of connective tissue

consisting of mast cells and macrophages (Shehu-Xhilaga *et al.,* 2005). T cells have also been shown to reside in both the epithelium and the lamina propria of the mucosa of the seminal vesicles (Anderson & Pudney 2005). In both the epithelium and lamina propria of the prostate, macrophages and lymphocytes may be found (Anderson & Pudney 2005). When the prostate becomes inflamed, due to, for example, bacterial infection, a significant number of $CD4^+$ T cells traffic there (McClinton *et al,* 1990; Moser *et al.,* 2002). Prostatitis is common, occuring with a prevalence of 40% in healthy, HIV-uninfected men (Blumenfeld *et al,* 1992). Macrophages along with both $CD4^+$ and $CD8^+$ T cells are present in the urethra (Anderson & Pudney 2005), although the majority of cells have been reported as $CD8^+$ T cells. Antigen-presenting cells are abundant along the whole penile urethra, while Langerhans cells are in the majority in the meatus, fossa novicularis and the foreskin (Anderson & Pudney 2005). The foreskin also contains activated memory $CD4^+$ T cells expressing both CCR5 and CXCR4 receptors ideal for HIV infection (Patterson *et al.*, 2002, Hussain & Lehner 1995). DC-SIGN is expressed on both dendritic cells and macrophages in the foreskin (Soilleux & Coleman 2004). The concentration of target cells for HIV infection in the foreskin illustrates why circumcision may be so effective at reducing HIV infection.

To investigate which organs of the male genital tract may be involved in recurrent viral shedding into semen, SIV RNA was quantified in a range of genital tract organs of SIV-infected macaques (Le Tortorec *et al.,* 2008). The seminal vesicles and prostate had the highest levels of SIV RNA and since these organs together contribute nearly 90% of seminal fluid (Wolff & Anderson 1988), it is likely that these organs are the source of most viral particles (Le Tortorec *et al.,* 2008). Contrasting results from a study quantifying HIV RNA in several genital fluids sampled in different sites within the human male genital tract, found that the prostate was not the major source of seminal HIV, but rather the urethra, seminal vesicles, periurethral and bulbourethral glands (Coombs *et al.,* 2006). Regardless of what the major sources of HIV replication are, these studies emphasise that almost all parts of the male genital tract may shed either SIV/HIV at differential levels in semen (Le Tortorec *et al.,* 2008, Coombs *et al.,* 2006).

Whether or not spermatozoa can transmit HIV remains a controversy. Despite reports from several groups that provide evidence that sperm cells may bind HIV virions through mannose, glycolipid receptors or even CCR5 receptors (Dussaix *et al.,* 1993,

Bandivdekar *et al,* 2003, Brogi *et al.,* 1998, Fanibunda *et al.,* 2008, Gadella *et al.,* 1998), other groups have not found evidence of HIV infection of viable spermatozoa (Quayle *et al.,* 1998, Pudney *et al.,* 1998). So whether HIV can enter sperm cells or merely "hitch" a ride still has to be conclusively proven.

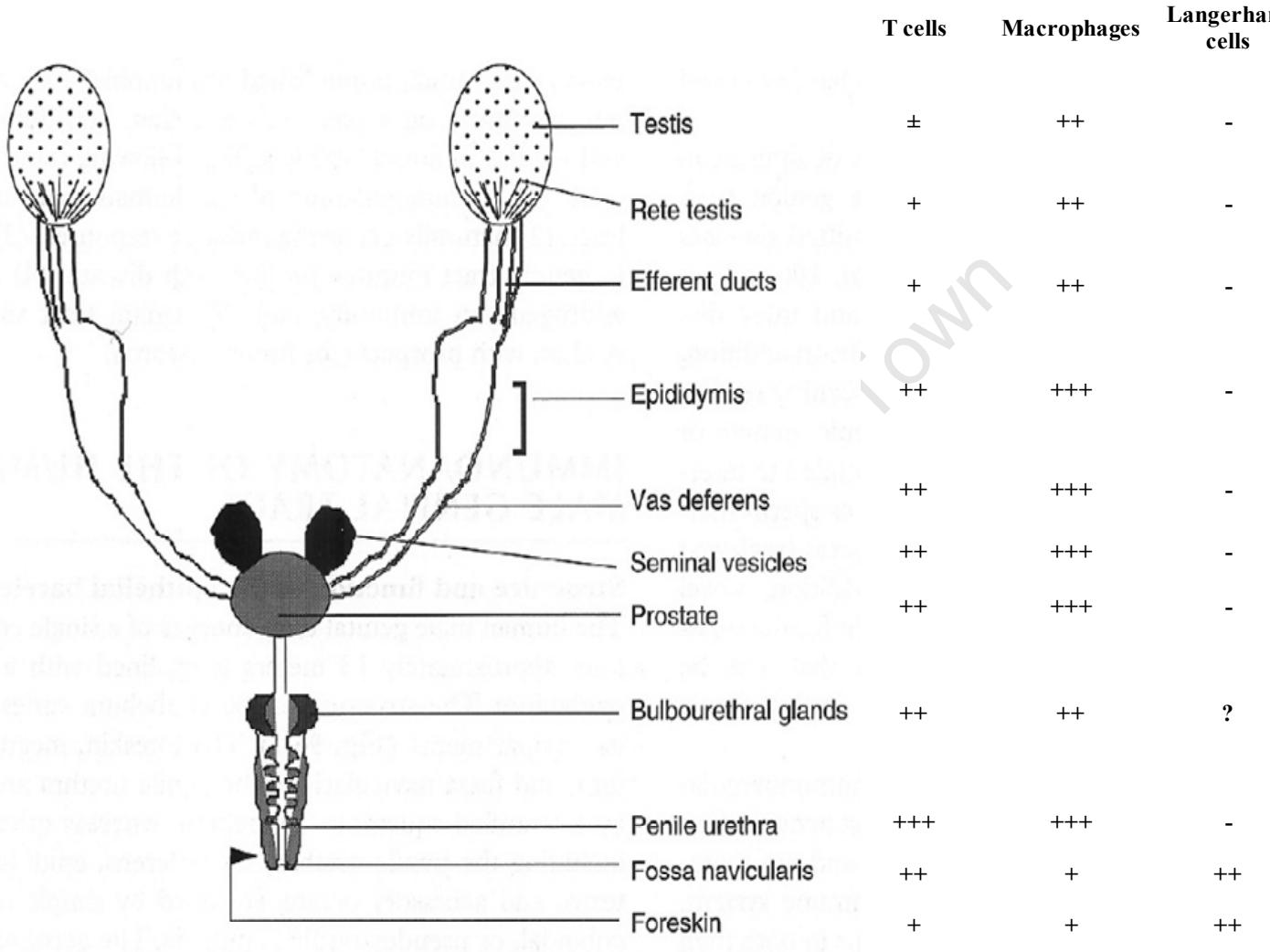


Figure 1.4 Schematic representation of the male genital tract. Distribution and abundance of T lymphocytes, macrophages and Langerhans cells (Taken from Mestecky *et al.*,, 2006).

1.3 HIV acquisition and transmission in the male genital tract

The majority of HIV infection occurs through heterosexual penile-vaginal or vaginal-penile transmission. The rate of sexual transmission in serodiscordant, heterosexual couples was found to be 0.0012 per coital act; strikingly, during the first 3 months after seroconversion, this risk increased 12-fold (Wawer *et al.,* 2005), emphasizing that the risk of HIV transmission is at its highest shortly after seroconversion, when the infected person may not be aware of their status and viral loads are high (Baeten & Overbaugh 2003, Brenner *et al.,* 2007). The risk of acquisition for uncircumsised men compared to circumcised men was increased two-fold per sexual encounter (Baeten *et al.,* 2005). Several studies estimate that male-to-female transmission within heterosexual encounters is more efficient than female-to-male transmission (Nicolosi *et al.,* 1994, Padian *et al.,* 1997, Carpenter *et al.,* 1999). However, this is not evident in all studies (Quinn *et al.,* 2000, Guthrie *et al,* 2007) and in fact, it has been suggested that at the level of a single sex act, there is no difference in the direction of transmission favouring either male or female infection (Powers *et al.,* 2008).

Powers *et al.,* provide evidence that the ubiquitous estimation of 0.001 (1 in 1000 contacts) for the rate of heterosexual HIV transmission (Royce *et al.,* 1997) may be an underestimation. In a meta-analysis, Powers *et al.,* suggest that factors such as STIs, genital viral load, additional sex partners, age of participants, stage of infection, design of studies and circumcision of male partners may all play a role as confounders of HIV infectivity (Powers *et al.,* 2008). Transmission of HIV from an infected male to an uninfected female partner may be affected by seminal viral load. In semen, viral loads may vary from exceedingly high to disproportionately low, when compared to plasma viral loads (Sheth *et al.,* 2006). An empiric study by Chakraborty *et al.,* proposed that at 100 000 copies of HIV RNA per ejaculate, the probability of HIV transmission rises to 1 in 100 acts of sexual intercourse compared to when there are 1000 HIV RNA copies per ejaculate, where the probability of transmission is reduced to 3 in 10 000 acts of intercourse (Chakraborty *et al.,* 2001).

It is known that circumcision protects men from HIV-1 acquisition (Auvert *et al.,* 2005, Bailey *et al.,* 2007, Gray *et al.,* 2007). Many studies have investigated a proposed mechanism for HIV acquisition and transmission in the female genital tract, however very few have investigated the same in the male genital tract. A recent study

compared the transmissibility of CCR5 and CXCR4-utilising viruses in different explant tissues of the the male genital tract (Fischetti *et al.,* 2009). They found that due to a preponderance of HIV target cells in the foreskin, glans, meatus and urethra of the penis, CCR5 HIV infection was successful, whereas CXCR4-tropic strains could not be productively disseminated (Fischetti *et al.,* 2009).

1.3.1 How does HIV cross the genital mucosal barrier?

The first studies investigating a productive HIV infection in the genital tract were performed in the female genital tract. The inherent properties of the mucosal barrier can be a natural deterrent to HIV transmission. The genital tract produces a host of mucins that are responsible for protection against pathogens and lubrication of this surface (Russo *et al.,* 2006). Further protection is provided by an intact genital mucosal barrier, and the presence of genital innate immune factors and the low numbers of HIV-infected $CD4^+$ T cells may explain lower HIV levels in genital secretions compared to blood (Kaul *et al.,* 2008). HIV binds to DC-SIGN on dendritic cells with subsequent infection of $CD4^+$ T cells locally or in lymph nodes, or direct infection of $CD4^+CCR5^+$ lymphocytes, to establish a productive infection in the female genital tract (Belyakov & Berzofsky 2004). Transcytosis involves the fusion of an HIV-infected cell and an epithelial cell to form a viral synapse, where viral budding at the synapse leads to internalisation of the virions by epithelial cells and release at the basal side of the epithelial layer (Bomsel 1997, Bobardt *et al.,* 2007). In stratified epithelia, such as the vagina, exocervix and anus, HIV-infection via Langerhans cells (LCs) is proposed to be the means by which HIV crosses the mucosal barrier. Sampling or capturing of HIV virions by Langerhans cells across the mucosal epithelia leads to transfer to T cells, either locally or in the lymph nodes (Hladik & McElrath 2008, de Witte *et al.,* 2008).

There are few studies investigating HIV transmission across the epithelia of the male genital tract. The presence of HIV target cells in the penile urethra (Pudney & Anderson 1995), the epithelia of the remainder of the penis (McCoombe & Short 2006), and especially the preponderance of target cells in the foreskin enhances the risk of HIV acquisition and productive infection (Figure 1.5). As in the female genital tract, both Langerhans and $DC\text{-}SIGN^+$ cells are present in the foreskin, and other non-keratinized epithelia of the human penis may play an important role in binding, internalisation and spread of HIV to draining genital lymph nodes (McCoombe &

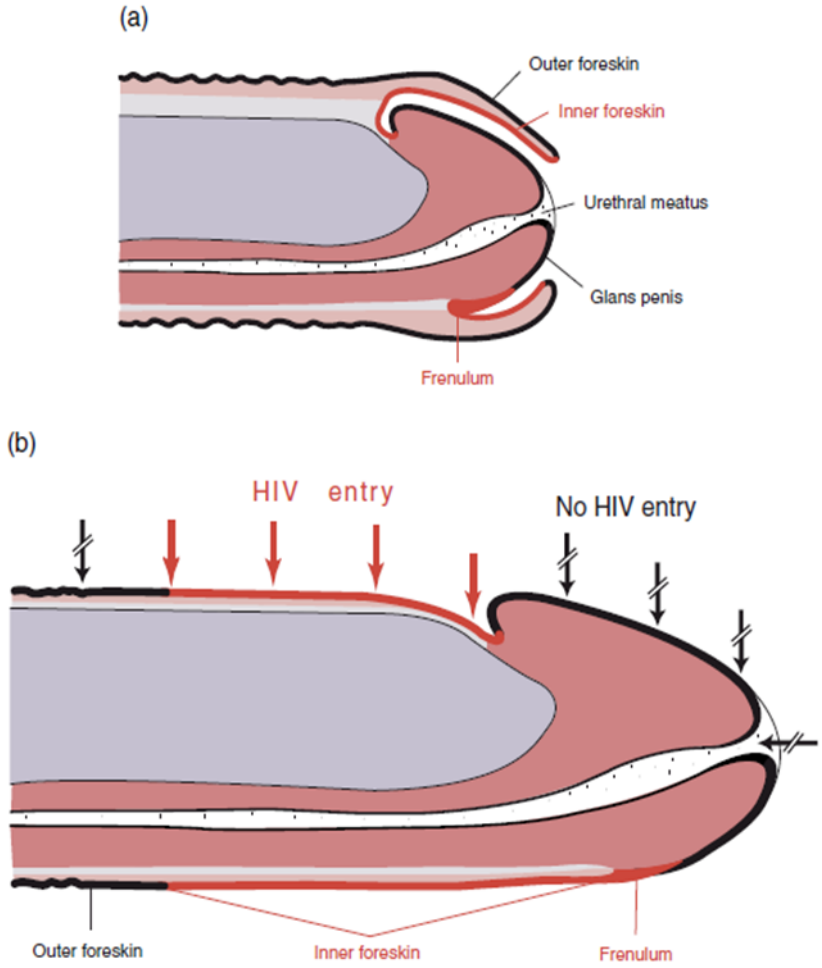


Figure 1.5 Potential areas of HIV-1 entrance. (a) Flaccid uncircumcised penis. (b) Erect uncircumcised penis with foreskin retracted showing likely sites of HIV-1 entry. (Taken from McCoombe & Short, 2006)

Short 2006). A recent study described the development of an *ex vivo* foreskin explant model to investigate the early events after HIV exposure of foreskin (Ganor *et al.*, 2010). This study found abundant LCs and T cells in the inner foreskin. HIV entry through the inner foreskin occurred more effectively than the outer, suggesting that the keratinized outer foreskin provides protection that may only enable HIV entry and infection of LCs subsequent to skin abrasions, supporting earlier findings (Kawamura *et al.*, 2008). Importantly, Ganor *et al.*, showed that HIV infection in this model was more effective with HIV-infected cells, rather than cell-free virus. They observed the formation of LC-T cell conjugates in the inner foreskin, as well as viral synapses between HIV-infected cells and apical foreskin keratinocytes. The LC-T cell conjugates occurred at high concentrations of virus, confirming a previous study that showed that at low viral concentrations, LCs protected from HIV infection (de Witte *et al.*, 2007) [Figure 1.6].

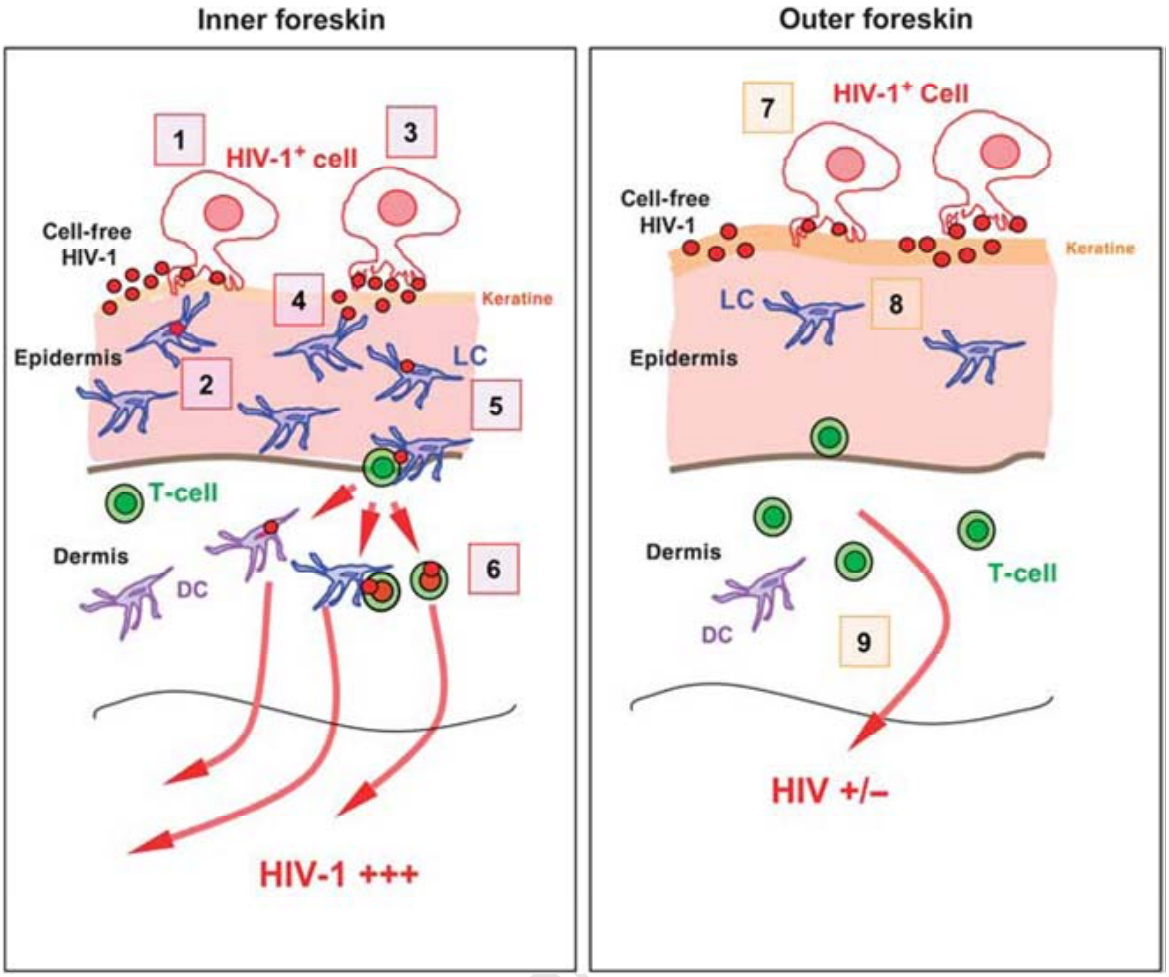


Figure 1.6 Initial events during HIV-1 transmission across the foreskin. (Left) Inner foreskin, synapses form, leading to particle budding (1). Low virion concentrations: Langerhans cells (LCs) remain within the epidermis; virions are destroyed by LCs (2). In contrast, high concentrations of virions: (3) LCs move to mucosal surface; virions are captured (4) LCs migrate back to the epidermal – dermal interface; conjugates with T cells allow virion transfer to T cells (5). Conjugates or single infected T cells disseminate the infection (6). (Right) Outer foreskin, synapse formation also occurs (7). However, newly budded virions may remain trapped within the thick layer of keratin (8). Thus, virus entry into the epidermis is limited, and infection cannot spread (9) [Taken from Ganor *et al.*, 2010].

1.3.2 Factors that enhance local replication and transmission of HIV

STIs can lead to enhanced HIV shedding in the genital tract and increased transmission of HIV, as well as increased suseptibility in uninfected persons (Corbett *et al.,* 2002, Kaul *et al,* 2000). Herpes simplex virus (HSV)-2 is one such STI. People with both infections may transmit HIV more efficiently than people infected with HIV alone, and people that are infected with HSV-2 can acquire HIV more readily (Schacker 2001). HSV-2 causes genital ulcer disease, but HSV-2 shedding in the genital tract can also be asymptomatic (Corey *et al.,* 2004, Wald & Corey 2003, Chen *et al.,* 2000). A three-fold risk of HIV acquisition in both men and women has been associated in several studies with pre-existing HSV-2 infection (Freeman *et al.,* 2006), whereas a six-fold increase in HIV acquisition was shown in highly exposed sex workers infected with HSV-2 (Kaul *et al.,* 2004). Even in the absence of HSV-2 shedding or ulceration, HSV-2 was associated with elevated levels of $CD4^{+}CCR5^{+}$ T cells and DC-$SIGN^{+}$ dendritic cells (Rebbapragada *et al.,* 2007) and in fact, HIV genital shedding has been associated with local HSV-2 replication (Kaul *et al.,* 2008, Baeten *et al.,* 2004, McClelland *et al.,* 2002) [Figure1.7]. Since symptomatic HSV-2 may cause lesions in the genital tract, HIV-1 transmission is more likely, although in asymptomatic patients, re-activation is sub-clinical and shows similar microscopic damage as well as elevated levels of lymphocytes as clinical disease (Corey *et al.,* 2004).

Non-ulcerative STIs such as gonorrhoea and chlamydia have been found to increase HIV shedding in the genital tract by recruiting target cells for possible infection (Fleming & Wasserheit 1999). Gonococcal urethritis has been shown to elevate seminal viral loads in HIV-infected men (Winter *et al.,* 1999, Sadiq *et al.,* 2005b, Taylor *et al.,* 2003). Chlamydia infections, that are often asymptomatic, have been associated with increased inflammation in the male genital tract (Kokab *et al.,* 2010) and *in vitro*, this has been shown to increase HIV replication (Ho *et al.,* 1995). In HIV-infected men, non-specific urethritis causes up to eight times higher shedding of HIV-1 than in healthy men (Dyer *et al.,* 1998), and even non-STI conditions such as prostatitis can elevate inflammation in the MGT (Motrich *et al.,* 2005), potentially increasing the risk of HIV acquisition by recruiting target cells for HIV.

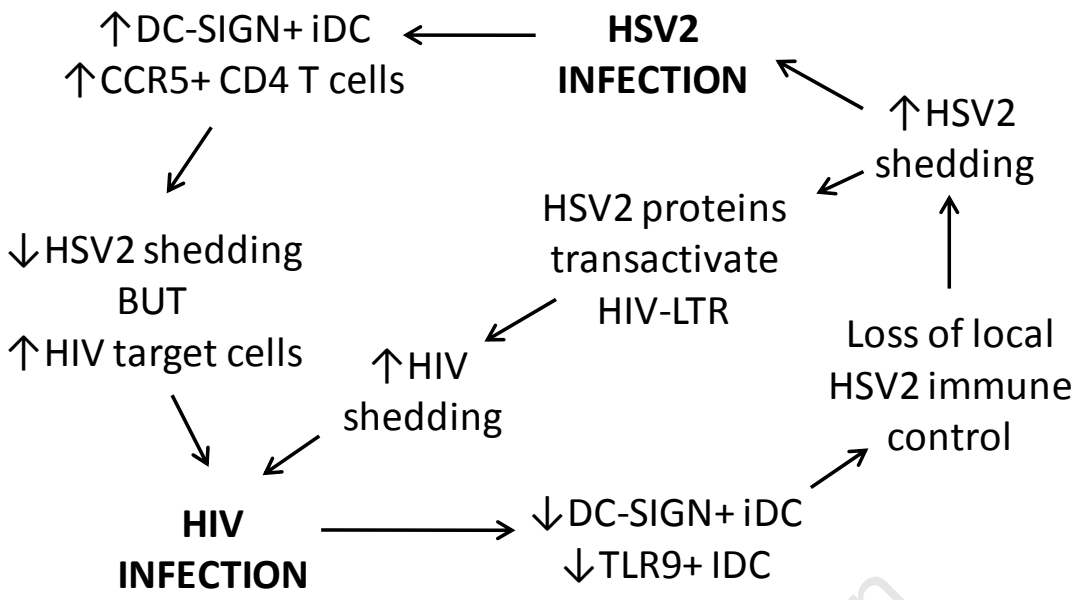


Figure 1.7 Model showing synergy between HIV-1 and HSV-2. (taken from Kaul *et al.,* 2007)

Other STIs that may cause genital ulcers are the bacterial infections syphilis and chancroid. During primary and secondary syphilis, mucosal lesions may allow viral entry in co-infected people, and HIV may also be transmitted from these lesions (Fleming & Wasserheit 1999). One study reported a significant increase in plasma HIV viral load and a reduction in $CD4^+$ T cells in men with primary and secondary syphilis (Buchacz *et al.*, 2004), whereas no association was found between syphilis and either HIV seminal or plasma viral loads in a subsequent study (Sadiq *et al.,* 2005a). Chancroid infections in men are associated with an influx of $CD4^+$ T cells into the genital tract (Magro *et al.,* 1996, Van Laer *et al.,* 1995). Symptomatic chancroid-infected men are up to five times more likely to acquire HIV from female partners than asymptomatic men (Cameron *et al.,* 1989).

1.3.3 Factors associated with protection from HIV infection in the genital tract

There are several innate immune factors in the genital tract that have demonstrated anti-HIV activity *in vitro*. These include MIP-3α, RANTES, lactoferrin, SLPI and α/β-defensins (Iqbal *et al.,* 2005). A recent study showed that the genital innate immune factor, elafin/trappin-2 is associated with HIV-1 protection in women (Iqbal *et al.,* 2009). Although these molecules may contribute to antiviral defence, some, such as MIP-3α, RANTES and α-defensin, may also facilitate transmission of HIV through increased recruitment of target cells to the sites of infection (Kaul *et al.,* 2008); (Vendrame *et al.,* 2009, Levinson *et al.,* 2009, Li *et al.,* 2009). Most studies have investigated the presence of these innate immune factors in the female genital tract. In semen and urethral lavages of men, SLPI and lactoferrin were detected (Sheth *et al.,* 2005, Anderson *et al,* 2011), and lactoferrin was shown to be significantly positively associated with HIV-1 RNA levels (Sheth *et al.,* 2005). RANTES levels have also been quantified in the semen of both healthy and HIV-infected men (Politch *et al.,* 2007, Anderson *et al.,* 2010). In the semen of healthy men, RANTES levels were detected at moderate levels, whereas in HIV-infected men, low levels were detected. It remains to be seen whether these factors in the male genital tract protect or enhance HIV-1 transmission.

Circumcision has been shown to be highly effective at protecting men against acquisition of HIV-1 (Auvert *et al.,* 2005, Bailey *et al.,* 2007, Gray *et al.,* 2007). The outer surface of the foreskin is more keratinized than the inner part (McCoombe & Short 2006, Patterson *et al.,* 2002, Hussain & Lehner 1995), and studies have linked keratinization to protection against HIV-1 acquisition (Patterson *et al.,* 2002; Pask *et al.,* 2008). In contrast, the inner foreskin is less keratinized and also has a higher density of target cells for HIV infection (McCoombe & Short 2006, Donoval *et al.,* 2006). As mentioned previously, a recent study has proposed that HIV-1 uses dendritic cells and keratinocytes in the foreskin to increase transmission (Ganor *et al.,* 2010). Thus, excision of the foreskin results in removal of potential HIV-1 target cells and can aid in protection from HIV infection.

As discussed, co-infection with STIs can lead to increased shedding of HIV (Galvin & Cohen 2004). Previous studies have shown that treatment of urethritis with antibiotics can reduce HIV shedding in semen by as much as two-thirds (Moss *et al.,* 1995, Cohen *et al.,* 1997). Four randomized trials in women showed that HIV levels can be reduced in plasma and the genital tract by treating HSV-2 (Baeten *et al.,* 2008, Dunne *et al.,* 2008, Nagot *et al.,* 2007, Delany *et al.,* 2009). In men, a randomised trial showed the same result in plasma and rectum (Zuckerman *et al.,* 2007), while a recent study showed a 44% reduction in levels of seminal HIV after treatment of HSV-2 (Zuckerman *et al.,* 2009). Despite successes in reducing the genital HIV burden through treating STIs, clinical trials aimed at decreasing HIV acquisition and transmission have largely failed. Thus far only one trial in Tanzania showed a significant decrease of 38% in HIV incidence after treatment of non-viral STIs such as chancroid, syphilis, gonorrhoea, chlamydia and trichomoniasis (Grosskurth *et al.,* 1995, Grosskurth *et al.,* 2000), whereas HSV-2 suppressive treatment trials have all failed to show a decrease in HIV infection (Celum *et al.,* 2010, Watson-Jones *et al.,* 2008).

1.3.4 Semen as an inhibitor or enhancer in HIV transmission

In male to female transmission of HIV, there are a number of seminal factors that have been implicated in providing either an inhibitory or enhancing effect to transmission of HIV (Figure 1.8). Seminal plasma contains 52 cationic polypeptides that have been shown to enhance endogenous anti-HIV activity *in vitro* in two cell lines (Martellini *et al.,* 2009). Semen can also act as an enhancer of HIV transmission within the female recipient by more non-specific means. The pH of semen varies from 7.0 – 8.4 (WHO, 2010). The natural antiviral acidic environment of the vagina is temporarily neutralised by semen (Wolters-Everhardt *et al.,* 1986), which may facilitate HIV survival and subsequent transmission.

Amyloid aggregates in semen have been shown to dramatically enhance HIV infection *in vitro* through the trapping of HIV virions, which promotes target cell attachment in vitro (Munch *et al.,* 2007). These amyloid fibrils have been termed 'semen-derived enhancer of virus infection' (SEVI), and are formed through the action of the enzyme prostatic acidic phosphatase (PAP). The same group found that SEVI fibrils are highly

cationic, enhancing HIV attachment and subsequent infection of target cells (Roan *et al.,* 2009), and that a compound, aminoquinoline surfen, inhibits SEVI-mediated enhancement of HIV infection (Roan *et al.,* 2010). Another mechanism of semen enhancement that has been highlighted recently involves interactions between spermatozoa and HIV virions. Heparin sulphate expressed on the surface of spermatozoa can bind to HIV, and may transmit virus directly to DC-SIGN on dendritic cells in the female genital tract (Ceballos *et al.,* 2009).

Semen can also enhance infection by leading to localised vaginal inflammation. Up-regulation of expression of inflammatory genes in vaginal epithelial cells has been demonstrated in response to semen exposure (Sharkey *et al.,* 2007). Semen contains high concentrations of transforming growth factor (TGF)-β and prostaglandins, both of which have been shown to have pro-inflammatory effects and attract immune cells in the vagina (Robertson *et al.,* 2002, Templeton *et al.,* 1978). Paradoxically, TGF-β also suppresses macrophage activation serving as the main constituent in seminal plasma responsible for immune tolerance within the female genital tract (Robertson *et al.,* 2002, Ashcroft 1999). Seminal plasma can also induce the expression of MIP-3α, a soluble factor responsible for the recruitment and maturation of Langerhans and dendritic cells (Berlier *et al.,* 2006b). MIP-3α recently been shown to attract these cells to the endocervix in SIV infected macaques, in turn recruiting $CD4^+$ T cells, thereby facilitating early establishment of foci of infection and dissemination systemically (Li *et al.,* 2009, Haase 2010).

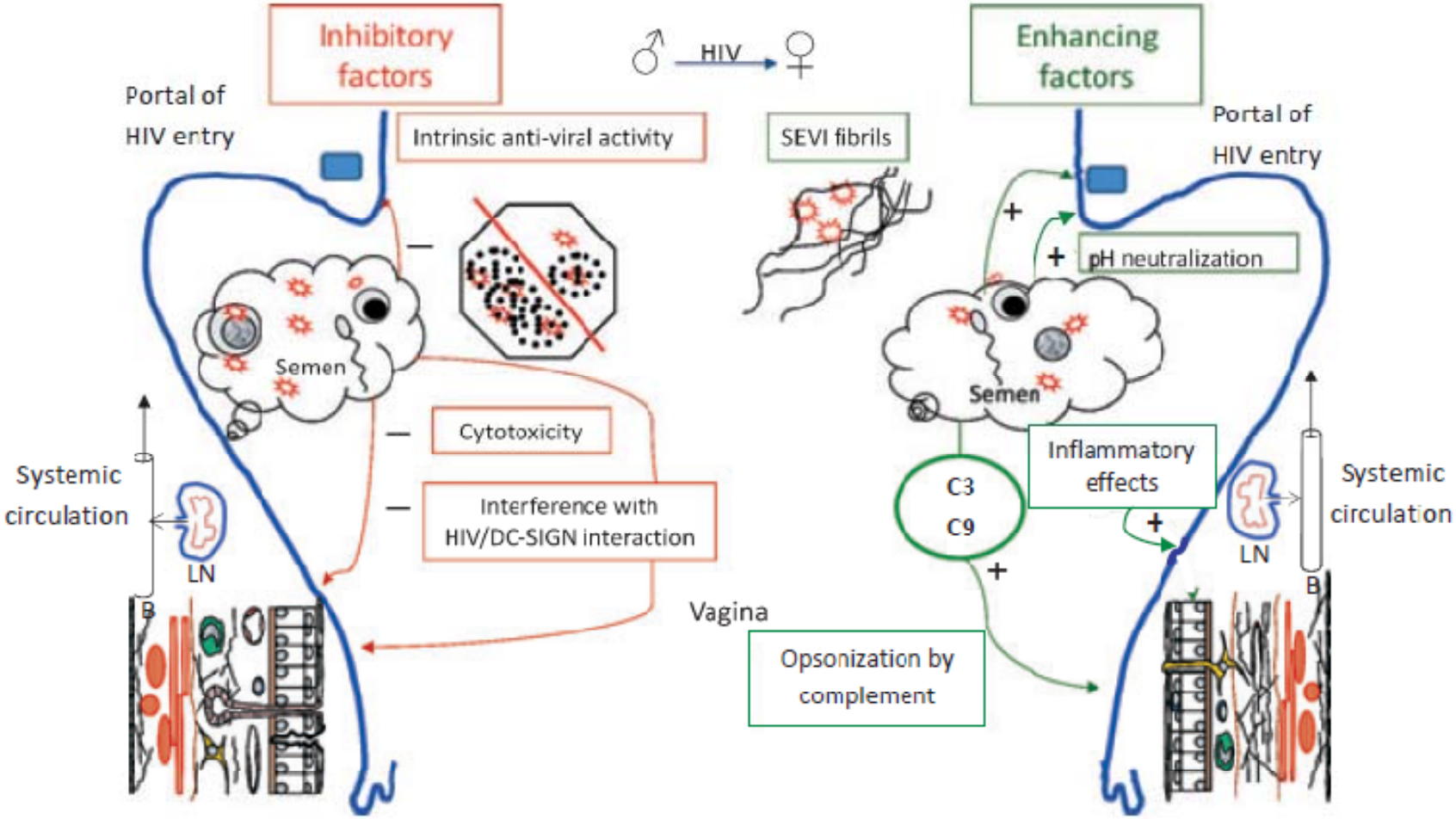


Figure 1.8 Factors in semen that may enhance or inhibit male-to-female transmission. (Taken from Doncel *et al.*, 2010)

1.4 Immune activation and inflammation in HIV transmission and pathogenesis

Disease progression in HIV infection occurs as the result of destruction and loss of $CD4^+$ T cells, and dysfunction of a range of immune cells. Massive depletion of activated $CD4^+$ T cells that express CCR5 takes place in mucosal tissues at all stages of HIV infection (Brenchley *et al.,* 2004). Not only are the majority of $CD4^+$ T cells lost by direct infection, but also as bystanders, and there is substantial evidence pointing to hyperactivation of the immune system and activation-induced cell death as a major mechanism driving HIV disease progression (Hunt 2007, Douek *et al,* 2009, Appay & Sauce 2008) [Figure 1.9].

1.4.1 HIV elevates immune activation and immune exhaustion

HIV infection is characterised by widespread T cell activation that is not always antigen-specific (Papagno *et al.,* 2004, Doisne *et al.,* 2004). Besides increased levels of non-specifically activated T cells during HIV infection, B cells are also polyclonally activated, and increased levels of pro-inflammatory cytokines and chemokines are evident (Douek *et al,* 2009).

Chronic immune activation has been found to be detrimental in HIV infection. The levels of activated $CD8^+$ T cells in chronic infection are more predictive of clinical progression to AIDS and death than plasma $CD4^+$ T cell counts or HIV RNA concentration (Giorgi *et al.,* 1993, Liu *et al.,* 1998, Giorgi *et al.,* 1999). Furthermore, in people at risk of HIV acquisition, higher levels of T cell activation prior to infection may be associated with faster HIV progression subsequent to HIV infection (Hazenberg *et al.,* 2003). Further evidence that immune activation may be involved in HIV disease progression is provided by studies of non-pathogenic SIV infection of primates. In SIV-infected sooty mangabeys and African green monkeys, despite high viral loads, T cell hyperactivation is not found and these animals do not develop AIDS (Silvestri *et al.,* 2003). In contrast, in SIV-infected macaques and HIV-infected humans, elevated T cell activation accompanies $CD4^+$ T cell loss and progression to AIDS (Silvestri *et al.,* 2003).

Immune activation increases the potential for T cells and macrophages to be infected,

by up-regulation of surface expression of CCR5 (Wu *et al.,* 1997, Bleul *et al.,* 1997). Furthermore, trafficking of T cells to lymphoid tissues is increased by activation-induced up-regulation of adhesion molecules, further enhancing HIV replication and potential systemic spread of the virus (Shattock *et al.,* 1996). Transcription of integrated virus is noticeably sped up in activated T cells, promoting expression of HIV from latently-infected cells (Calman *et al.,* 1988, Kawakami *et al,* 1988). In addition, T cell homeostasis may be disrupted by the high turnover of CD4 and CD8 T cells (Kovacs *et al.,* 2001). Continued immune activation causes damage to lymphoid tissues, such as the thymus and lymph nodes, impairing the regenerative capacity of the immune system. The ability of the thymus to generate mature, functionally competent naive T cells is reduced in HIV-infected individuals (Douek *et al.,* 1998). Persistent immune activation has also been linked to progressive collagen deposition and destruction of lymph node architecture (Schacker *et al.,* 2002b). This damage may further decrease the half-life of resting naïve and memory T cells, restricting cells from entering or leaving lymph nodes, as well as reducing the ability of APCs to effectively prime naïve T cells, thereby affecting the HIV-specific T cell immunity (Hellerstein & McCune 1997).

HIV infection can lead to immune activation by direct or indirect means. HIV antigenic stimulation directly activates T cells, inducing HIV-specific $CD4^+$ and $CD8^+$ T cells (Betts *et al.,* 2001). Up-regulation of pro-inflammatory mediators along with increased activation of both lymphocytes and macrophages have been suggested to be the directly induced by HIV gene products. For example, gp120 and Nef can activate macrophages and lymphocytes (Merrill, Koyanagi & Chen 1989, Rieckmann *et al.,* 1991, Lee *et al.,* 2003, Swingler *et al.,* 1999, Wang *et al.,* 2000, Simmons *et al,* 2001). Immune activation may be exacerbated by re-activation of co-infections, such as CMV and EBV (Doisne *et al.,* 2004). In addition, HIV depletes $CD4^+$ T cells in the gastro-intestinal tract mucosa, leading to enteropathy, a condition characterized by damage to the epithelium of the gut, increased intestinal permeability as well as malabsorption (Sharpstone *et al.,* 1999, Smale *et al,* 2000).

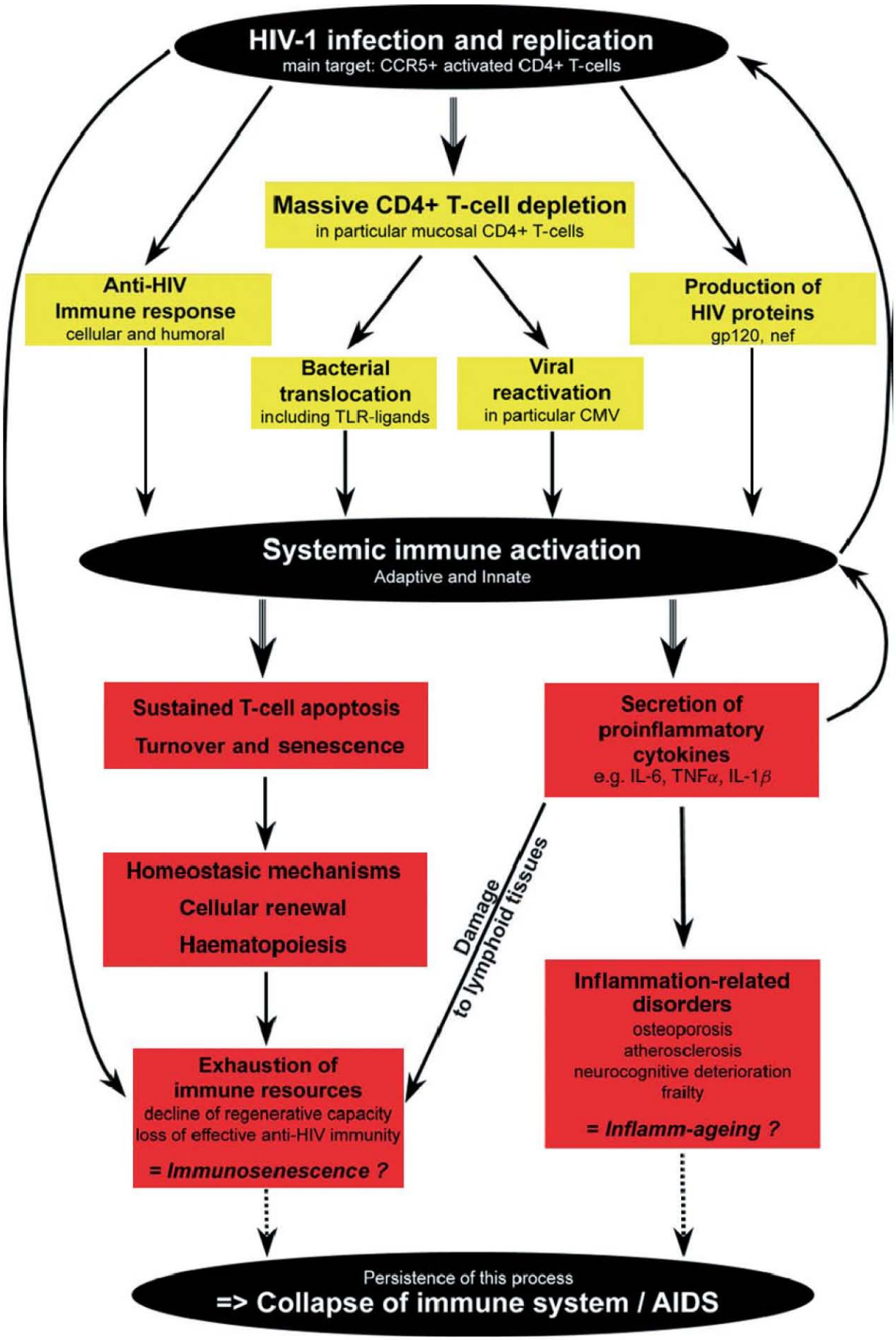


Figure 1.9 A proposed model for HIV pathogenesis. Possible causes for immune activation are in yellow, while the effects are in red. (Taken from Appay & Sauce, 2008)

Because of this increased permeability in the gut, significantly increased levels of bacterial products, such as bacterial DNA, lipopolysaccharides (LPS) and other bacterial components from gut microbial flora can 'leak' into plasma, and this 'microbial translocation' has been linked to systemic immune activation (Brenchley *et al.,* 2006, Jiang *et al.,* 2009).

1.4.2 Inflammation in HIV transmission and pathogenesis

During acute HIV infection, an HIV-induced "cytokine storm" has been described, where high levels of pro-inflammatory cytokines are found in plasma, which peak in conjunction with viral load (Stacey *et al.,* 2009). It appears that cytokines that are normally produced to control HIV replication by orchestrating innate and adaptive immunity may well contribute to disease progression, due to their sustained and high level production. Pro-inflammatory cytokines can promote the activation of $CD4^+$ T cells (Decrion *et al.,* 2005), continually providing new targets for viral infection.

A recent study by Li *et al.,* investigated the earliest steps in of SIV infection after cervicovaginal inoculation of macaques. They found an aggregation of plasmacytoid dendritic cells (pDCs) producing IFNα and β as well as MIP-1α and MIP-1β. These chemokines were found to attract $CD4^+CCR5^+$ T cells that may serve as SIV target cells (Li *et al.,* 2009). In the female genital tract pro-inflammatory cytokines are associated with ulceration and increased HIV shedding (Lawn *et al.,* 2000). Persistent shedding of HIV in semen has been reported (Coombs *et al.,* 1998, Gupta *et al.,* 2000, Bujan *et al.,* 2004) which may in turn elevate inflammation in the male genital tract sustaining viral replication. Microbial translocation together with concomitant systemic activation can be worsened by reduction of transepithelial resistance (monolayer integrity) in gut mucosal tissues through increased levels of IL-1β and TNFα (Stockmann *et al.,* 2000, Nazli *et al.,* 2010); in the same way, pre-existing inflammation may enhance transmission of HIV by thinning and disruption of the vaginal epithelium (Haase 2010).

Sustained local inflammation in the genital tract results in the influx of target cells for HIV, which has implications for susceptibility, or HIV-infected cells and thus local replication of HIV, which has implications for transmission. In the female genital tract, increased levels of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6 and IL-8, were significantly associated with HIV shedding (Gumbi *et al.,* 2008). In the

semen of HIV-infected men, several pro-inflammatory cytokines (IL-6, IL-8, IL-12 and IFNγ) were associated with increased HIV shedding (Sheth *et al.,* 2006). Seminal plasma levels of RANTES and IL-1β have also been shown to positively correlate with seminal viral load (Storey *et al.,* 1999, Berlier *et al.,* 2006a). Pro-inflammatory mediators identified to be up-regulated in HIV-infection differ from study to study, depending on the panel of cytokines and chemokines investigated. A recent study investigating cytokine levels in HIV-infected male genital tissue explants of the foreskin, glans and urethra showed high levels of IL-6, IL-8, MIP-1α, MIB-1β, G-CSF, MCP-1 and IP-10 (Fischetti *et al.,* 2009). A comprehensive investigation of cytokines and chemokines present in seminal plasma of HIV-infected men revealed significant up-regulation of IL-1β, IL-4, IL-6, IL-7, IL-8, GM-CSF and MCP-1 (Anderson *et al.,* 2010) compared to uninfected men, although adjustment for multiple comparisons was not performed. Since the genital tract is an effector site, exposed to multiple insults up-regulation of levels of cellular activation and inflammation is to be expected. From these studies above, IL-1β, IL-6 and IL-8 were the inflammatory mediators that were most often identified in the genital tract. The majority of the studies above controlled for the presence of other STIs, and despite the variation of pro-inflammatory mediators in different studies, there appears to be evidence that inflammation in the genital tract is linked to HIV replication.

1.5 HIV replication in the genital tract: distinct viral reservoirs

The genital tract poses a challenge for effective viral suppression. Despite ART reducing seminal viral loads to undetectable levels in most patients (Bujan *et al.,* 2004, Ghosn *et al.,* 2008, Chan *et al.,* 2008a), certain antiretroviral drugs have been measured at sub-optimal concentrations in the genital tract (Kashuba *et al.,* 1999, Lafeuillade *et al.,* 2002, Ghosn *et al.,* 2004a, Chan *et al.,* 2008b). Differential penetration of antiretroviral drugs into genital organs and tissues can lead to sustained viral replication there, as well as the emergence of drug-resistant strains (Taylor *et al.,* 2003, Ghosn *et al.,* 2004a, Eyre *et al,* 2000, Ghosn *et al.,* 2004b, Taylor, Pereira 2001, Eron *et al.,* 1998), which may be spread by means of sexual transmission (Grant *et al.,* 2002, Little *et al.,* 2002, Markowitz *et al.,* 2005).

1.5.1 Antiviral drug resistance in the male genital tract

Antiretroviral therapy may adequately suppress viral loads in blood, without achieving suppression in the genital tract (Lorello *et al.,* 2009; Haggerty *et al,* 2006) [Figure 1.10]. In fact, several studies have shown genetically divergent strains between blood and semen (Eyre *et al,* 2000, Ghosn *et al.,* 2004b, Smith *et al.,* 2007, Kroodsma *et al.,* 1994) for men on antiretroviral therapy. The lack of successful suppression of seminal viral replication may be due to a disparity in penetration of different antiretrovirals in semen compared to blood (Chan 2005, Pereira *et al.,* 2002, Taylor & Davies 2010, Lowe *et al.,* 2006, Lafeuillade *et al.,* 2003). Lorello *et al.,* found that 2/13 men on efavirenz had detectable seminal viral loads (Lorello *et al.,* 2009), whereas Sheth *et al.,* found no association between seminal HIV shedding and any specific antiretroviral agents or classes (Sheth *et al.,* 2009). A recent meta-analysis investigating antiviral drug concentrations in the genital tract showed a 0 – 48% seminal viral shedding depending on the ARV regime and the length of time on treatment (Taylor & Davies 2010).

1.5.2 The male genital tract as a potential viral reservoir

Several studies have shown that the male genital tract gives rise to distinct viral populations from blood (Coombs *et al.,* 1998, Gupta *et al.,* 2000, Eyre, Zheng & Kiessling 2000, Ghosn *et al.,* 2004b, Zhu *et al.,* 1996, Vernazza *et al.,* 1994, Byrn *et al.,* 1997, Kiessling *et al.,* 1998, Ping *et al.,* 2000, Pillai *et al.,* 2005, Diem *et al.,* 2008), suggesting that the unique tissue-specific environment in the male genital tract results in the evolution of distinct HIV strains.

A recent study investigating genetic differences between viral strains in semen and blood of acutely SIV-infected macaques showed that at the time of peak viral replication, there were no significant differences between compartments, with distinct seminal viral differences only developing after 16 weeks of infection in 4 out of 7 animals (Whitney *et al.,* 2011). The authors suggest that during peak viremia there is likely more trafficking of virus between the male genital tract and blood and that compartmentalization only occurs after a decrease in viral load (Whitney *et al.,* 2011). In humans, a recent study proposed a model that demonstrated that not only does compartmentalization result in viral shedding in semen, but that there may be direct transfer of virus from blood to the genital tract. Furthermore, the authors postulate that uninfected target cells may be infected by infected $CD4^+$ T cells or virions that

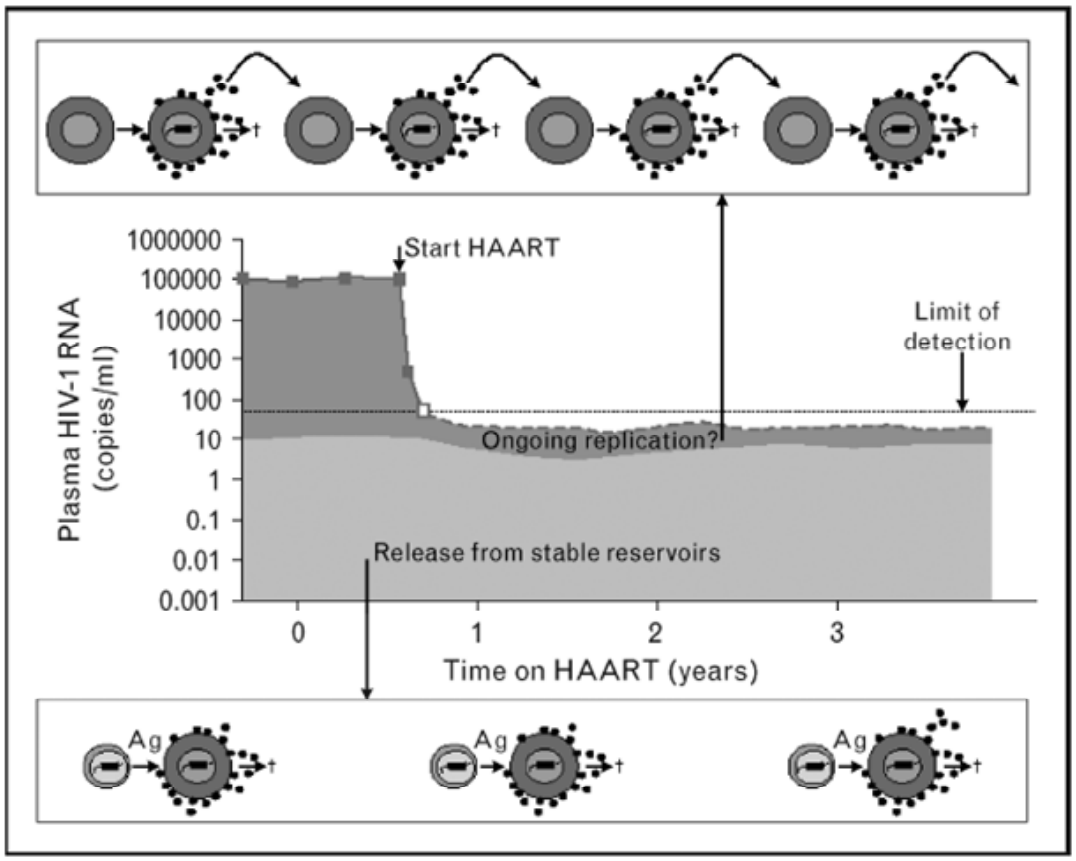


Figure 1.10 HAART does not guarantee low-level viraemia. Anti-retroviral therapy may reduce HIV-1 RNA below the detection level of most clinical assays (50 copies/ml). More sensitive methods detecting HIV-1 RNA < 50 copies/ml may represent continuing rounds of replication (top) or release of HIV-1 from stable reservoirs (bottom). (Taken from Haggerty *et al.*, 2006)

infiltrate the seminal tract, establishing local areas of infection within the male genital tract (Anderson *et al.,* 2010). In the case of compartmentalization, the question remains, what part of the male genital tract is responsible for contributing to shedding of virus in semen? Due to the blood-testis barrier, antiretroviral entrance into the testes are constrained (Choo *et al.,* 2000, Livni *et al.,* 2004), and therefore it has been postulated that the testis is the most probable site of a viral sanctuary in the male genital tract (Shehu-Xhilaga *et al.,* 2005).

1.6 HIV-specific immunity in the genital tract

A range of studies have identified HIV-specific T cell responses in the cervix and semen of HIV-infected individuals (Quayle *et al.,* 1998, Sheth *et al.,* 2005, Musey *et al.,* 1997, Musey *et al.,* 2003b, Shacklett *et al.,* 2000, Kaul *et al.,* 2000, Kaul *et al.,* 2003). HIV-specific responses have been shown to be shared between the blood and mucosa of HIV-infected individuals (Musey *et al.,* 2003a, Ibarrondo *et al.,* 2005). An important question is whether these responses play a role in the control of local HIV replication, either in the context of natural infection or if induced prior to exposure in the context of vaccination.

1.6.1 The female and male genital tracts

Important insights into adaptive immunity to HIV in the genital have been gained from studying vaginal transmission in the SIV-macaque model, demonstrating that even robust SIV-specific T cell responses in the female genital tract and gut did not prevent or contain SIV infection (Reynolds *et al.,* 2005). Reynolds *et al.,* suggested that a contributing factor for the massive depletion of $CD4^+$ T cells early in infection is the delay in the development of SIV-specific $CD8^+$ T cell responses, which takes up to two weeks. This results in the establishment of a productive SIV infection that cannot be controlled by subsequent T cell responses (Reynolds *et al.,* 2005), what these authors termed "too little, too late". Similar studies of early HIV infection in the male genital tract have not been undertaken. In humans, clonality of HIV-specific $CD8^+$ T cells was compared between the blood, semen and cervix, and it was shown that the majority of clones were shared between blood and the mucosal sites investigated (Musey *et al.,* 2003a), implying a trafficking of responses from blood to the genital tract. Similar

HIV-specific responses by $CD8^+$ T cells between blood and the GALT have also been demonstrated (Ibarrondo *et al.,* 2005).

An important question is whether HIV-specific T cells at these sites have any effect on controlling local viral replication. A study by Gumbi *et al.,* found that there was no association between the magnitude of HIV Gag-specific $CD8^+$ T cell responses and viral shedding in the female genital tract (Gumbi *et al.,* 2008), suggesting that T cell responses in the genital tract may not be as a result of local HIV viral replication alone; and that these cells may not be involved in effective viral control. A similar study of semen of HIV-infected men found that HIV-specific $CD8^+$ T cells correlated positively with the levels of HIV in semen (Sheth *et al.,* 2005), mirroring the situation often described in blood, where it appears that antigen load drives the magnitude of T cell responses, and there is no obvious relationship between T cell frequency and viral control. This may be due to non-optimal functioning, as has been shown for mucosal HIV-specific responses (Ferre *et al.,* 2009), where individuals controlling HIV had $CD8^+$ responses in the rectal mucosa with greater functionality than non-controllers.

1.6.2 Does an HIV vaccine need to induce mucosal immunity?

As discussed, the majority of HIV transmission occurs via mucosal surfaces, and the lymphoid tissue of the gut is an early target for HIV replication. Therefore, immunity at mucosal surfaces, especially the genital tract and the gut, is paramount for vaccine-mediated protection. Thus, vaccination that can induce effective mucosal responses, whether they are T cell or antibody responses, is needed.

Several studies in macaques using different vectors and vaccination regimes showed that SIV-specific T cells and antibodies can be induced at various mucosal surfaces (Bertley *et al.,* 2004, Fuller *et al.,* 2002, Nilsson *et al.,* 1998, Tenner-Racz *et al.,* 2004). A study in mice and macaques showed that effective mucosal T cell responses elicited by an intramuscular recombinant adenovirus (rAd) vaccine were found in the spleen, lymph nodes, gut and vaginal surfaces of mice, whereas in macaques robust, longlasting responses were shown in lymph nodes, the gut bronchoalveolar lavages (BAL) and the vaginal tract (Kaufman *et al.,* 2008).

In humans, Musey *et al.,* demonstrated HIV-1-specific $CD8^+$ T cell responses detected in rectal mucosa following intramuscular vaccination with a live recombinant

canarypox ALVAC-HIV vaccine containing HIV-1 genes encoding for *env*, *gag* and parts of the *pol* gene (Musey *et al.*, 2003b). This demonstrated that even systemic vaccination may induce mucosal immunity, likely due to the trafficking of these responses from blood. Also in a macaque model of SIV infection, a recent study showed a reduction of SIV levels in the semen of macaques after vaccination with a DNA-rAd5 candidate vaccine expressing Gag and Pol (Whitney *et al.,* 2009). Sixteen weeks after vaccination, macaques were infected intravenously with SIV. A comparison between vaccinated animals and the unvaccinated control group showed a significant reduction of more than one log in seminal viral load at peak viremia between vaccinated and unvaccinated animals. Furthermore, seminal viral loads in the vaccinated animals decreased to undetectable levels by day 42 after challenge, and remained so for the remaining 10 weeks of the study. The data in this study implies that vaccination can not only lower seminal viral loads, but significantly impact on lowering the risk of sexual transmission.

A vastly understudied area is humoral immunity to HIV in the male genital tract, with a single study to date characterising HIV-specific antibody responses in semen (Mestecky *et al.,* 2009). Further studies on the presence of neutralising antibodies in both natural infection and in vaccine trial volunteers are warranted. It seems clear that an effective HIV vaccine will need to elicit not only effective systemic immunity, but most importantly provide protection where infection occurs, namely the genital mucosa.

1.7 Aims and objectives

Overall aim of the thesis

The aim of this thesis was to develop methodologies for the isolation and characterization of T cells from semen, as a representation of local immunity in the male genital tract. These methodologies were then applied to characterizing cellular activation and inflammation in the male genital tract in a cohort of HIV-infected and uninfected men, in order to gain insights into the acquisition and transmission of HIV.

Rationale for developing methodologies for the isolation of T cells from semen

Studies of mucosal immunity in the genital tract are significantly hampered by inherent difficulties associated with mucosal sampling and the resultant low yields of mucosal cells obtainable from clinical specimens (Gumbi *et al.,* 2008, Nkwanyana *et al.,* 2009). There is a need for the development of optimized protocols for isolating and evaluating sufficient numbers of viable lymphocytes from the male genital tract, as no standardized protocols currently exist. Such methods would aid in improving our understanding of both local immunity and the assessment of responses to candidate HIV vaccines.

Rationale for investigating immune activation and inflammation in the male genital tract

Semen is the main vector for HIV transmission. Inflammation in the male genital tract may therefore have important implications for onward transmission of HIV. Local inflammation may increase local viral replication (Mitchell *et al.,* 2011), and higher viral loads in semen would likely increase the probability of successful transmission (Pilcher *et al.,* 2004, Wilson *et al.,* 2008). Inflammation may also influence HIV acquisition, leading to target cell activation and infiltration into the genital tract (Nkwanyana *et al.,* 2009), increasing the susceptibility of uninfected individuals to productive HIV infection. No studies to date have specifically investigated the impact of local T cell activation and virus levels in semen, and a few studies have evaluated a limited range of inflammatory cytokines in semen and their relationship with viral shedding. Extending these studies may improve our understanding of the local microenvironment of the genital tract and how genital tract

T cell activation, inflammation and local viral replication relate to each other and influence HIV transmission and acquisition.

Specific objective 1

To develop methods for the optimal isolation of $CD3^+$ T cells from semen, as well as to characterize the types of immune cells present in semen (Chapter 2).

Specific objective 2

Using methods developed in Chapter 2, the objective was to characterize and compare levels of T cell activation in the semen and blood of HIV-infected and HIV-uninfected men, and to evaluate the impact of local viral replication on immune activation in the genital tract (Chapter 3).

Hypothesis

Levels of T cell activation will be higher in semen from HIV-infected men compared to HIV-uninfected men, and there is a direct correlation between T cell activation in semen and seminal HIV viral load.

Specific objective 3

The objective of this part of the thesis was to investigate inflammation in the male genital tract during HIV infection, by characterizing the presence and levels of a range of cytokines and chemokines in seminal plasma. The association between the levels of inflammatory cytokines and T cell activation, as well as local viral replication, was investigated (Chapter 4).

Hypothesis

HIV-infected men maintain higher levels of inflammation in the genital tract than uninfected men, and that this is associated with higher levels of T cell activation and HIV shedding in semen.

CHAPTER 2

Isolation and characterisation of leukocytes from semen

Table of Contents

2.1 Introduction

HIV-1 is predominantly a disease of the mucosa. Infection, transmission and much of the pathogenesis of the disease occurs at the mucosal surfaces of the genital tract and the gastrointestinal tract (Haase 2010). In the context of developing strategies for preventing HIV infection by vaccination, inducing immune responses in the genital tract is paramount. However, few HIV vaccine studies in humans to date have measured the ability of candidate vaccines to induce mucosal immunity (Musey *et al.,* 2003b). This is likely due to the difficulty in sampling genital tract sites, and the fact that there are few well-established methods for processing the cells obtained. A good understanding of the immune milieu of genital tract sites, including the types of cells and their abundance, as well as soluble factors present, is lacking. This chapter focuses on methodologies for obtaining and studying T cells from the male genital tract, as well as the characterisation of immune cells present in this compartment.

T cells play a central role in immune control of HIV-1 infection (Koup *et al.,* 1994, Schmitz *et al.,* 1999, Allen *et al.,* 2005). Many current HIV vaccine strategies are aimed at eliciting cellular responses against HIV (as reviewed in Gamble & Matthews 2010). In natural HIV infection, the presence of HIV-specific $CD8^+$ T cells has been demonstrated both in the female genital tract (in the cervix), as well as in the male genital tract, using semen (Quayle *et al.,* 1998, Shacklett *et al.,* 2000). Recently it was demonstrated that a significant decrease in viral load in the semen of SIV-infected macaques is possible through vaccine-induced cellular immune responses, which, although only measured in blood, may have been present in the genital tract (Whitney *et al.,* 2009). In addition to cellular immune responses, an effective HIV-1 vaccine would very likely need to elicit humoral responses at mucosal surfaces, capable of combating HIV-1 by means of neutralising antibodies (Shacklett *et al.,* 2009).

Isolation of functional T cells from the genital tract, and especially semen, can be challenging, as the genital tract harbours fewer immune cells than blood or other mucosal surfaces such as the gastrointestinal tract where an abundance of T cells can be found in organised lymphoid tissue (Anderson *et al.,* 1998). Low numbers of immune cells from the genital tract limits the ability to characterise the cells and

perform functional assays to detect immune responses, which is an important aspect for example of assessing candidate HIV vaccines for further development. Further challenges include the effective treatment of viscous samples and removal of highly autofluorescent sperm and germ cells from semen samples.

A need exists for the establishment of standardised methodologies for the isolation and characterisation of T cells from the male genital tract. Standardised methods are important for provision of consistent and reliable data during clinical trials that are likely to be conducted at multiple sites in areas of high HIV prevalence. The aim of this study was to investigate immune cells in semen, as a representative sample of the male genital tract. Different factors influencing T cell yields from semen, including the optimal storage temperature and time to processing of ejaculates, as well as various methods for isolation of T cells from semen, were investigated. In addition, characterisation of the immune composition of semen was performed. A simple, optimised method of leukocyte isolation from semen that maximizes recovery of functional T cells while minimising interfering fluorescence from residual sperm and germ cells for flow cytometric analysis is described. This standardised method may be applied to clinical trials of candidate HIV vaccines, where it may be important to establish whether cellular responses are generated not only in the peripheral blood compartment, but also in the genital tract.

2.2 Materials and Methods

2.2.1 Participant recruitment and sample collection

One hundred and ninety nine men of unknown HIV status were recruited from the Embryology and Andrology Laboratory at Groote Schuur Hospital, Cape Town. The study was approved by the Faculty of Health Sciences Human Research Ethics Committee (HREC REF 045/2007), University of Cape Town, and written informed consent was obtained from all volunteers. Ejaculates were collected in sterile specimen jars following voluntary self masturbation. Days of sexual abstinence prior to ejaculation were self-reported. Sample volume, sperm count, pH and viscosity were noted.

2.2.2 T cell isolation from semen

At the clinic, semen samples were immediately diluted with 6 ml of transport medium [RPMI-1640 medium supplemented with 5 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (GIBCO Invitrogen, Carlsbad, CA, USA), 2 mg/ml fungin (Invivogen, San Diego, CA, USA)]. All samples were processed 2 – 4 h after donation, apart from during the time course experiments. To investigate the impact of transport temperature and time to processing on $CD3^+$ T cell yields, samples from 34 men were incubated at either 4 °C (n=12), room temperature (n=10) or 37 °C (n=12) prior to processing. Samples were divided into three equal volumes and $CD3^+$ T cells extracted at 4, 12 and 24 h post ejaculation. Seminal mononuclear cells (SMCs) were isolated using Ficoll-Histopaque (Sigma-Aldrich, Egham, Runnymede, UK) density gradient centrifugation. Alternatively, samples were centrifuged at 1000×g for 10 min and seminal supernatant was removed. The pelleted fraction from semen was then digested with 0.4 mg/ml Pronase (Roche, Mannheim, Germany) for 15 min at 37 °C, followed by addition of cold PBS (GIBCO, Invitrogen). Samples were then filtered though 40 µM cell strainers (BD Biosciences, Plymouth, UK) and filtrates were centrifuged at 380×g for 10 min. Resuspended cell pellets were subjected to either $CD3^+$ or $CD45^+$ T cell selection using anti-CD3 or anti-CD45 beads according to the manufacturer's specifications (Miltenyi Biotec Inc, Auburn, CA, USA). $CD3^+$ cell counts were performed on a Guava cell counter using anti-CD3 PE monoclonal antibody (Guava Technologies, Hayward, CA, USA). In order to account for interference from autofluorescence as a result from residual sperm cells, an unstained

sample was used to measure sample autofluorescence and subtracted to obtain the number of $CD3^+$ cells in the sample.

2.2.3 Flow-cytometric analysis of seminal leukocytes

Functional T cells were assessed intracellularly by applying a monoclonal antibody panel: anti-CD3 APC, anti-CD4 FITC, anti-CD8 PerCP-Cy5.5, anti-IFN-γ PE (all BD Biosciences, San Diego, CA, USA). All antibodies were pre-titered to determine optimal staining amounts. To investigate the ability of seminal T cells to produce IFN-γ, cells were stimulated by the addition of 0.025 µg/ml PMA and 1 µg/ml ionomycin (Sigma Aldrich, St. Louis, MO, USA) for 4 h at 37 °C. Brefeldin A (10 µg/ml; Sigma Aldrich, St. Louis, MO, USA) was added to both PMA/ionomycin stimulated and unstimulated controls. Surface and intracellular staining of cells was performed for 20 min at room temperature. For intracellular staining, cells were permeabilized using CytoFix/CytoPerm (BD Biosciences, San Diego, CA, USA). Cells were fixed in Cell Fix (BD Biosciences, San Diego, CA, USA) and samples acquired on a FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analysed using FlowJo software v8.5.3 (Tree Star Inc, Ashland, OR, USA). To assess viability of T cells in semen, cells were stained with a green reactive amine dye ('GrVid', Invitrogen Molecular Probes, Eugene, OR, USA) for 20 min at room temperature followed by surface staining for 20 min at room temperature with anti-CD3 APC, anti-CD4 PE and anti-CD8 PerCP-Cy5.5 monoclonal antibodies (all BD Biosciences, San Diego, CA, USA).

Further phenotypic assessment of leukocytes was performed by splitting samples into four fractions and applying the following monoclonal antibody panels: 1. anti-CD19 PerCP-Cy5.5 (B cell marker) and anti-CD24 PE (neutrophil marker); 2. anti-CD16 PerCP-Cy5.5 and anti-CD56 PE (NK cell markers); 3. anti-CD207 PE (Langerhans cell marker) and anti-CD209 PerCP-Cy5.5 (DC-SIGN-expressing cells); 4. anti-CD14 PE (monocytes/macrophages) and anti-CD209 PerCP-Cy5.5 (all BD Biosciences, San Diego, CA, USA). All four panels also contained anti-CD3 APC and a seminal marker, anti-hAP FITC (human intra-acrosomal protein) to exclude residual sperm cells. Surface and intracellular staining of cells was performed for 20 min at room temperature. For intracellular staining of hAP, cells were permeabilized using CytoFix/CytoPerm (BD Biosciences). Cells were fixed in Cell Fix (BD Biosciences) and samples acquired on a FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analysed using FlowJo software v8.5.3 (Tree Star Inc, Ashland, OR, USA).

2.2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The Mann–Whitney U test was applied for independent non-parametric sample comparisons, the Wilcoxon rank test was used for matched non-parametric comparisons and Spearman ranks correlation was applied for assessing non-parametric associations. All tests were two-tailed and p-values ≤ 0.05 were considered significant.

2.3 Results

2.3.1 Comparison of methods to isolate T cells from semen

To optimise T cell recovery from semen samples, $CD3^+$ T cell yields using different processing and isolation procedures were investigated (Fig 2.1). Four methods were compared, namely Ficoll-Histopaque gradient separation, pronase digestion, pronase digestion followed by cell straining, and pronase digestion followed by cell straining and subsequent CD3 magnetic bead isolation. $CD3^+$ T cell numbers in samples prior to any leukocyte isolation or enrichment technique were determined using a Guava cell counter and an anti-CD3 PE stain, as described in the Methods. Purification of mononuclear cells using Ficoll-Hypaque density gradient centrifugation resulted in a median of 20 600 $CD3^+$ T cells (IQR 13 120 – 79 600), which represented a five-fold significant decrease of $CD3^+$ T cells from a median count of 190 000 prior to Ficoll isolation (Figure 2.1A). When semen samples were subjected to proteolytic enzyme digestion with pronase to release mononuclear cells from seminal mucus, median $CD3^+$ T cell recovery increased significantly from a median of 158 000 (IQR 86 000 – 212 000) pre-pronase treatment to 302 000 (IQR 123 000 – 394 000) post-pronase treatment (Figure 2.1B). When a cell strainer was used to filter the sample, median $CD3^+$ T cell yields increased significantly from 146 250 (IQR 62 900 – 312 750) to 196 000 (IQR 178 500 – 491 000; Figure 2.1C).

Since T cells isolated from semen were going to be analysed by flow cytometry, it was important to remove residual sperm and germ cells, since these non-immune cells autofluoresce during flow cytometry and interfere with detection by target-specific antibodies (Hulspas *et al.*,, 2009). To address the issue of autofluorescence during flow cytometric analysis, positive magnetic bead selection of $CD3^+$ T cells was used. First incorporating the steps of pronase digestion and filtration, followed by magnetic bead isolation of $CD3^+$ T cells, resulted in a T cell population of 71% purity (IQR 56-98%; data no shown). Using bead separation resulted in three-fold lower yield compared to pre-bead separation (Figure 2.1D). Nevertheless, this significantly improved $CD3^+$ T cell yield nearly four-fold to a median of 75 740 (IQR 59 410 – 91 425) compared to Ficoll isolation alone ($p=0.013$; Figure 2.1D). Furthermore, when applying anti-CD3 magnetic bead selection, the seminal lymphocyte population became clearly distinguishable by flow cytometry (Figure 2.5A).

Thus, by combining three methods, namely pronase digestion, the use of cell strainers and CD3 magnetic bead isolation (as outlined in Figure 2.2), $CD3^+$ T cell yield was improved over using density gradient separation alone. Furthermore, by using magnetic bead selection, the seminal lymphocyte population could be distinguished by flow cytometric analysis.

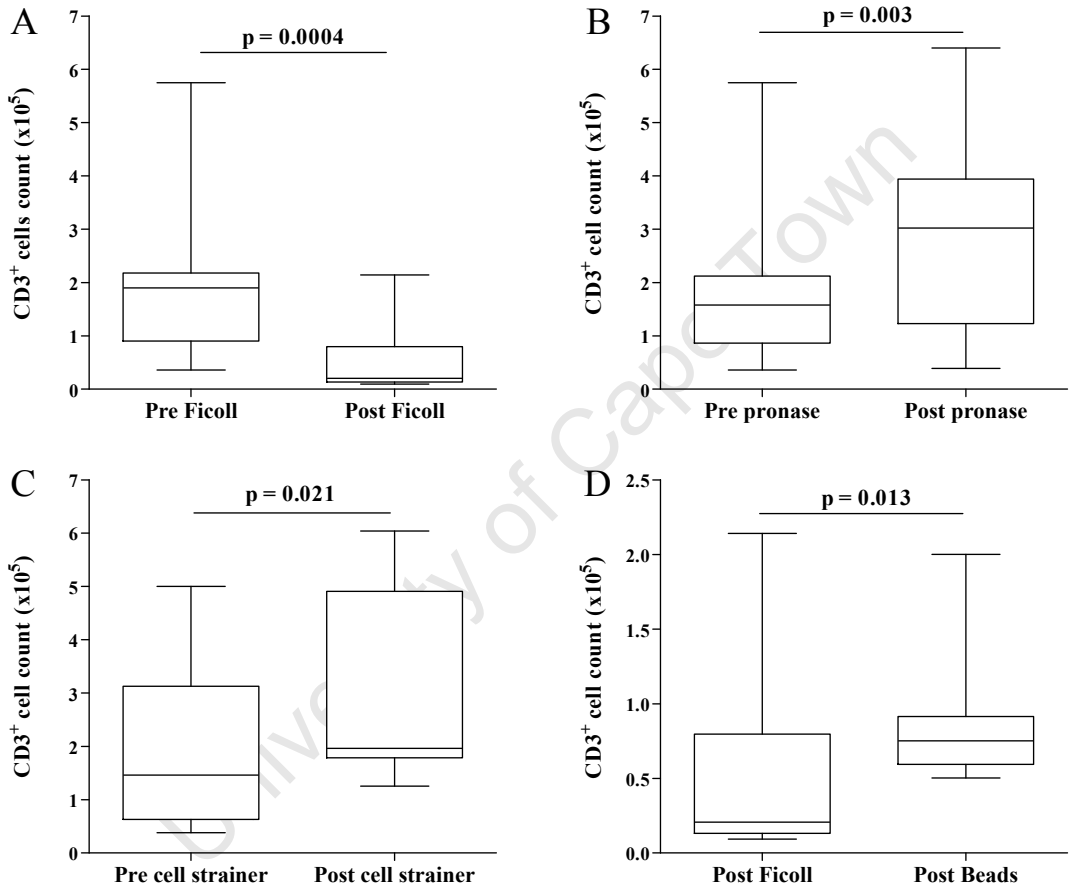


Figure 2.1 Isolation strategies for maximising $CD3^+$ T cell yields from semen. Semen samples were subjected to (A) Ficoll-Hypaque gradients (n=15), (B) pronase digestion (n=13) and (C) filtration through cell strainers (n=12) and (D) a comparison between Ficoll-Histopaque gradient (n=18) and anti-$CD3^+$ magnetic bead separation (n=16). Box-and-whisker plots indicate the median, 25th and 75th percentile. Wilcoxon matched pairs test was performed to compare matched groups and the Mann-Whitney U test was applied to compare unmatched groups. *p*-values < 0.05 were considered significant.

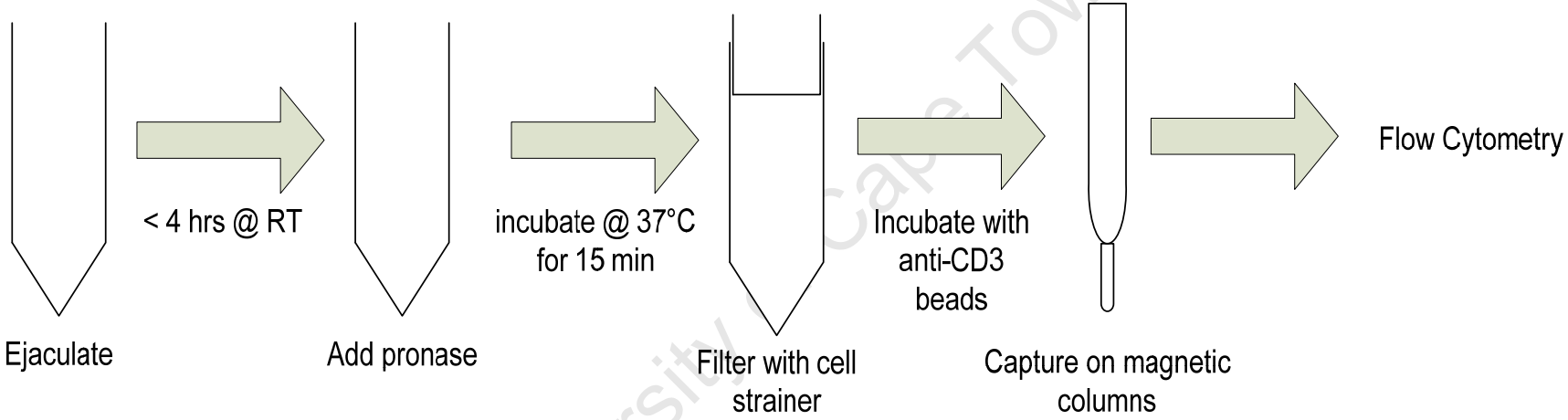


Figure 2.2 Schematic representation of seminal $CD3^+$ T cell isolation method. Samples were processed within 4 h of ejaculation by incubation at 37 °C with pronase. Subsequent filtration though cell strainers was followed by magnetic bead capture of $CD3^+$ T cells.

2.3.2 The influence of semen parameters on T cell yield

A range of characteristics were recorded for each semen sample collected (Table 2.1). From the 199 ejaculates collected in this study, ejaculate volumes ranged from 0.8 ml to 7.5 ml (median 3 ml, IQR 2-4 ml), and the median time from last ejaculation was 4 days (IQR 3-5 days). $CD3^+$ T cell yields were determined after pronase digestion and cell straining but prior to semen ejaculates being subjected to magnetic bead isolation. $CD3^+$ cell yields were weakly but significantly positively correlated with ejaculate volume (p=0.0004, r=0.25; Figure 2.3A). Furthermore, the number of days of abstinence prior to ejaculation had a profound influence on $CD3^+$ T cell yield, with a 54% higher cell yield in men who had abstained from ejaculation for ≥4 days compared to those that had abstained for only 2 days (p=0.043; Figure 2.3B). Consistent with this, there was a weak but significant positive correlation between days of abstinence and ejaculate volume (p=0.02, r=0.16; data not shown). Sperm count showed a significant association with $CD3^+$ T cell yield (Figure 2.3C; p=0.0002, r=0.26), however there was no direct association between sample volume and sperm count (Figure 2.3D). Samples were stratified according to raised versus normal viscosity by recording observed mucus in samples. Raised viscosity in samples resulted in a significantly lower $CD3^+$ T cell yield compared to samples with none to little (classified as normal) mucus present (p=0.0021; Figure 2.4A). The pH of samples had no significant influence on $CD3^+$ T cells yields (Figure 2.4B).

Thus, $CD3^+$ T cell yields can be improved if participants abstain from ejaculation for at least four days. Sample volume and sperm count may be used as an indicator of $CD3^+$ T cell yields.

Table 2.1 Characteristics of semen samples

Semen parameter (n=199)	Median (IQR)
Sample volume (ml)	3 (2 – 4)
Days abstinence	4 (3 – 5)
pH	7.5 (7.2 – 7.5)
Sperm count[a]	60 (30 – 84)

[a]Million viable spermatozoa/ml

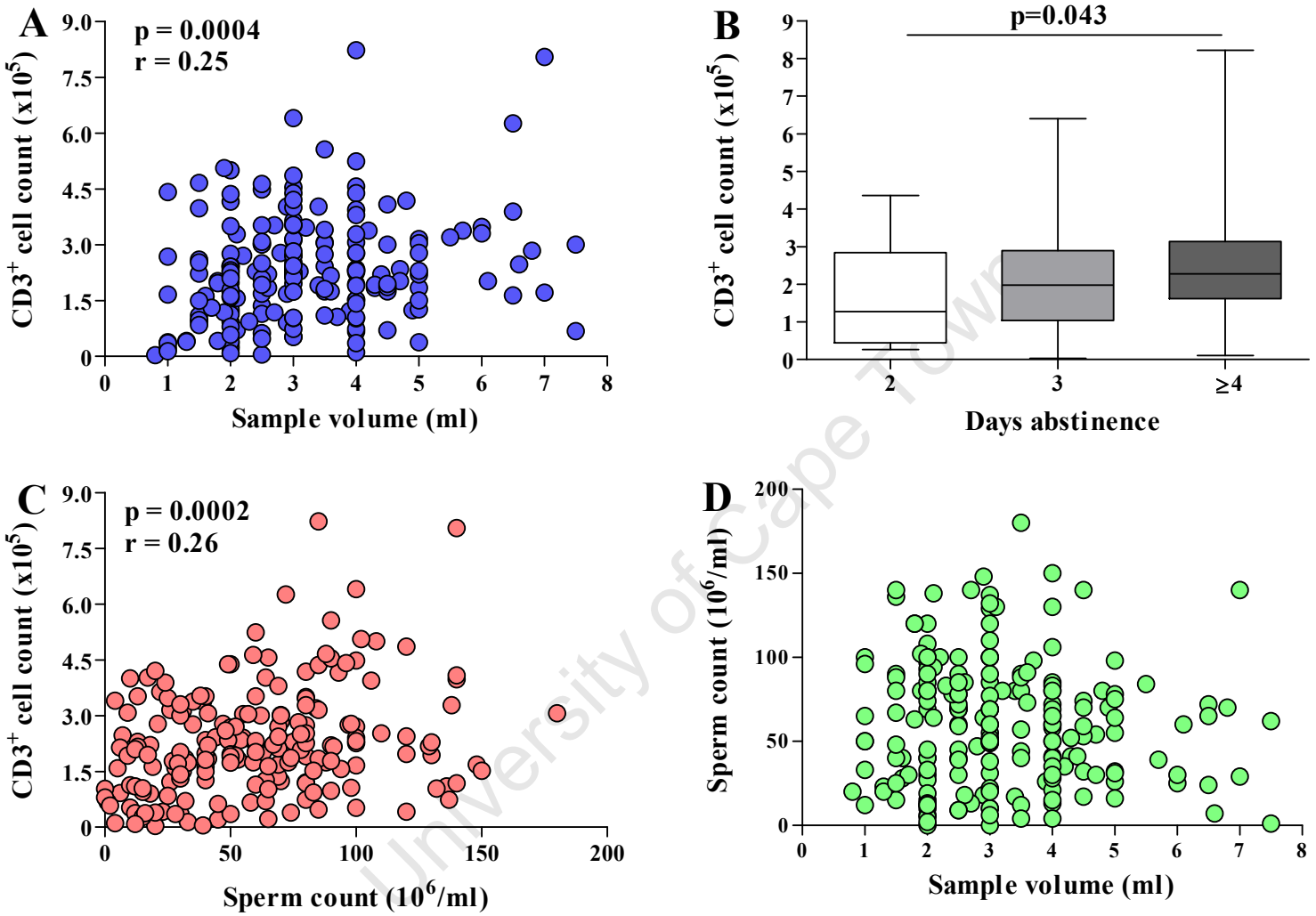


Figure 2.3 The relationship between CD3$^+$ T cell yields and clinical parameters. Shown are (A) a correlation between sample volume and CD3$^+$ T cell yields (n=199), (B) comparison between CD3$^+$ T cell yields after 2 days (n=12), 3 days (n=81) and longer than 4 days of abstinence (n=104), (C) correlation between sperm count and CD3$^+$ T cell yields (n=199) and (D) correlation between sperm count and sample volume (n=199). Box-and-whisker plots represent the median, 25th and 75th percentiles. *p*-values ≤ 0.05 were considered significant. Spearman correlations were performed, and the Mann-Whitney U test was applied to compare groups.

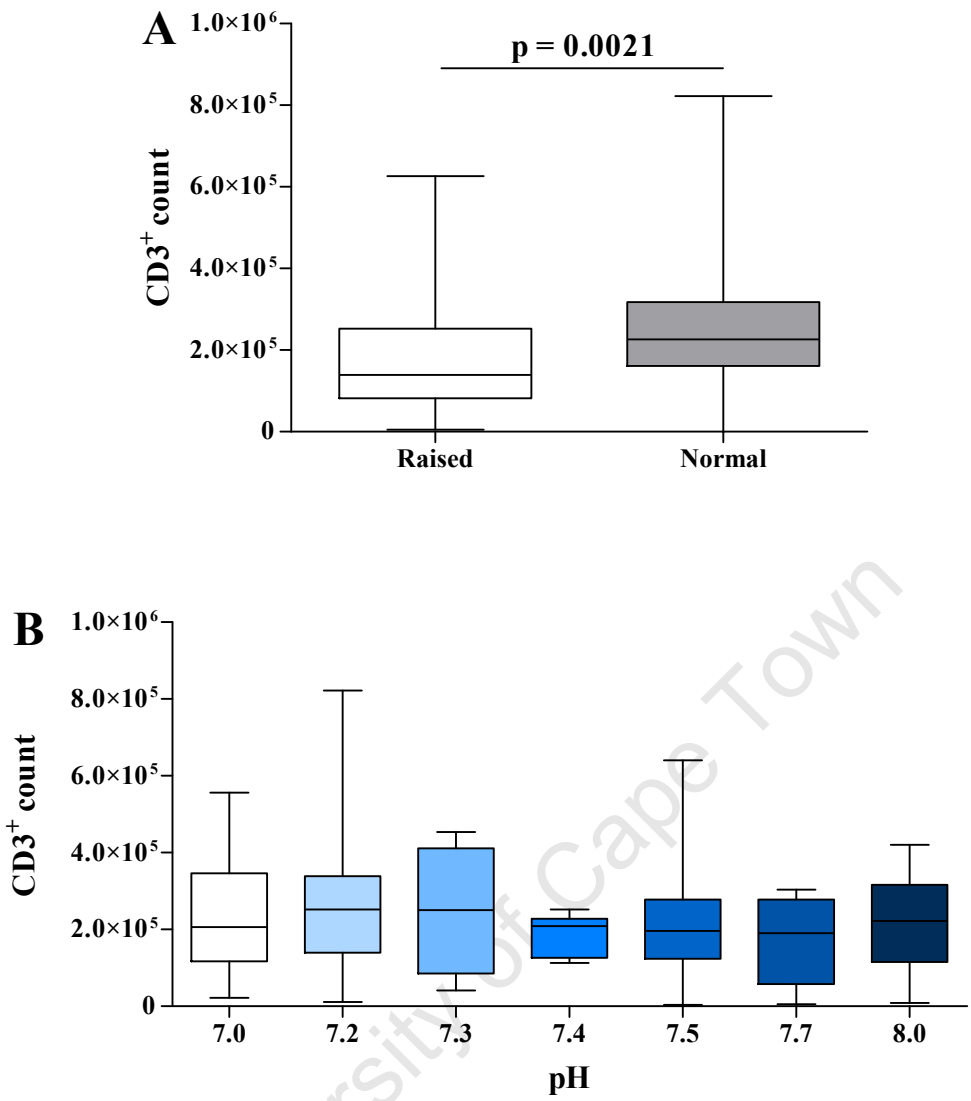


Figure 2.4 Viscosity and pH of semen samples. Shown are (A) the differences in $CD3^+$ T cell yields between semen samples with observed viscosity (raised) and those without (normal) and (B) A comparison of $CD3^+$ T cell yields at different sample pH levels. Box-and-whisker plots represent the median, 25th and 75th percentiles. *p*-values ≤ 0.05 were considered significant.

2.3.3 Functional capacity and viability of CD4$^+$ and CD8$^+$ T cells isolated from semen

Seminal CD4$^+$ and CD8$^+$ T cells, isolated by CD3$^+$ magnetic bead separation, were investigated for their ability to produce IFNγ following stimulation with the mitogen PMA/ionomycin, using intracellular cytokine staining and flow cytometry. Representative plots are shown in Figure 2.5B and C. Of the 61 semen samples analysed, 35/61 (58%) samples produced IFNγ, while 26/61 (42%) failed to respond to PMA/ ionomycin stimulation. Of the semen samples that responded to PMA/ionomycin stimulation, CD4$^+$ T cells produced a median frequency of 8.7% IFNγ, whilst CD8$^+$ T cells produced a higher median of 30.4% IFNγ (Table 2.2; Figure 2.5C).

The viability of T cells isolated from semen was tested in 18 samples using an amine reactive fluorescent dye ('GrVid') followed by flow cytometric analysis. As shown in Table 2.3, median viabilities for CD4$^+$ T cells were 64% (IQR 47.6-78%), and 71% for CD8$^+$ T cells (IQR 45.2-84.9%).

In summary, using the technique described to isolate T cells from semen, viable and functional cells able to produce cytokine in response to mitogen were obtained. The low to moderate viability obtained may influence the functional ability of the cells.

Table 2.2 IFNγ production from stimulated CD4$^+$ and CD8$^+$ T cells

Cell subset	IFNγ production[a] (IQR)		Fold increase over background
	Background	PMA/Ionomycin	
CD4	0 (0 – 0.44)	8.66 (3.28 - 20)	8.66
CD8	0 (0 – 1.02)	30.4 (10 – 48.4)	30.4

[a]Median IFNγ percentage with interquartile range

Table 2.3 Viability of T cell subsets

Cell subset	Percentage viable[a] (IQR)
CD4	64.2 (47.6 – 78)
CD8	71.3 (45.2 – 84.9)

[a]Median of frequencies with interquartile range (IQR)

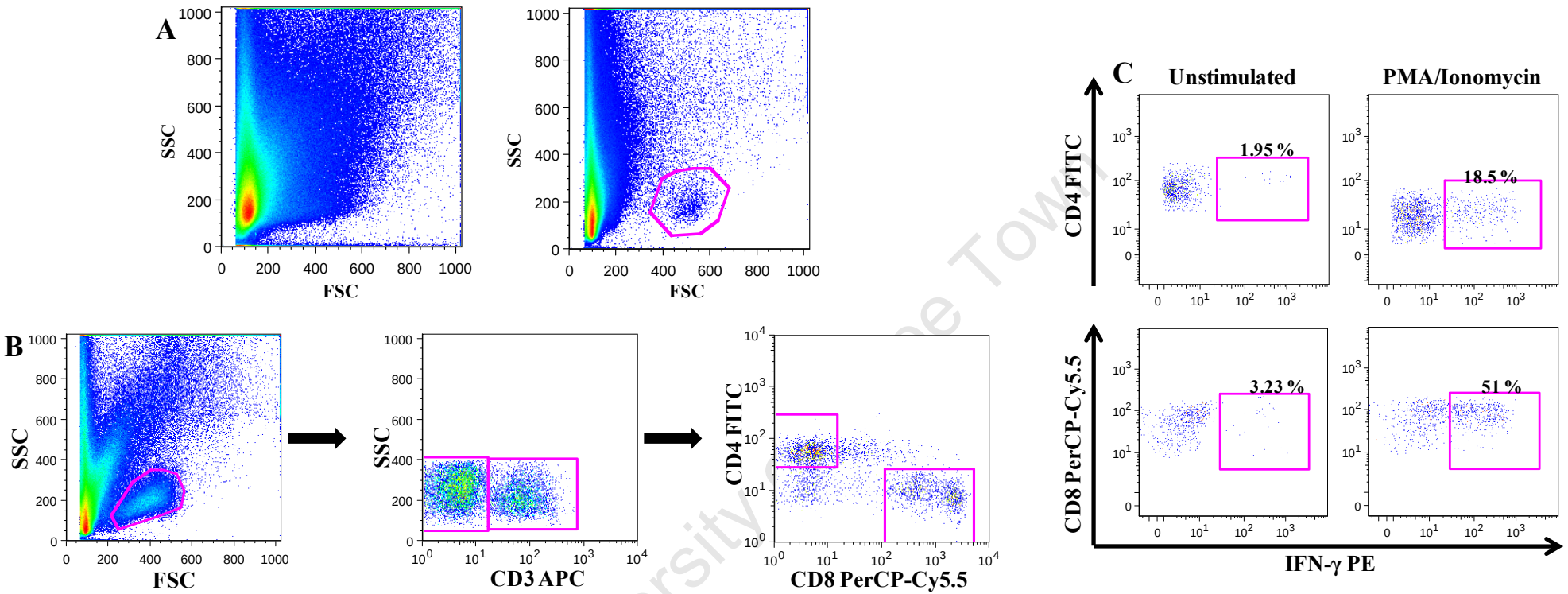


Figure 2.5 The impact of anti-CD3$^+$ isolation on flow cytometric visualisation of T cells and characterization of functional T cells. Shown are flow cytometry plots before and after anti-CD3 magnetic bead capture (A), gating strategy for seminal lymphocytes, CD3$^+$, CD4$^+$ and CD8$^+$ T cells (B) and IFNγ production by unstimulated versus PMA/ionomycin-stimulated CD4$^+$ and CD8$^+$ T cells (C).

2.3.4 Influence of semen processing time and temperature on T cell yields

In order to determine whether T cell yields from semen are affected by procedures prior to cell isolation, the influence of the time between ejaculation and processing of samples, as well as the impact of processing temperature on $CD3^+$ T cell yields, was investigated. Each sample was divided into equal fractions and each fraction was either processed at 4, 12 or 24 hours post ejaculation, or at 4 ^oC, room temperature or 37 ^oC for the varying time points. As before, $CD3^+$ T cell yields were calculated using a Guava cell counter.

To investigate the influence of processing time, 34 samples were processed at 4 h, 12 h and 24 h post ejaculation (Table 2.4 and Figure 2.6). Regardless of the processing temperature, there was a steady decrease in the $CD3^+$ T cell yield with longer processing times. At a processing time of 4 h post ejaculation, the median $CD3^+$ T cell yield was 274 960 (IQR 147 050 – 426 000). At 12 h post ejaculation, the $CD3^+$ T cell yield decreased by approximately 100 000 to 175 500 (IQR 55 470 – 258 650). By 24 h post ejaculation, the median $CD3^+$ T cell yield had decreased significantly from the 4 h processing time to 62 700 (IQR 31 030 – 162 100; $p<0.0001$).

To investigate storage temperature on $CD3^+$ cell yield, 10 samples were processed after incubation at room temperature (RT), while 12 samples each were processed after incubation at either 4 °C or at 37 °C (Table 2.4). When processing the samples within 4 h of ejaculation, the storage temperature did not affect the median $CD3^+$ yields, and the same was true when comparing the 12 h time point for the different storage temperatures. In contrast, for longer storage times (24 h), greater cell loss was prevented if the sample was stored at 37 °C, with a median $CD3^+$ T cell yield of 153 860 (40 050 – 184 550), compared 38 390 (IQR 7905 – 107 850) $CD3^+$ T cells remaining at 4 °C. This represented a 75% loss and was significantly less than the $CD3^+$ T cell yield at 37 °C ($p=0.046$; Figure 2.6).

Thus, it appears that optimal T cell yields from semen require processing within 4 h of ejaculation, regardless of the storage temperature investigated here. When it becomes necessary to store samples for longer periods of time before they are processed, storage at 37 °C results in a significantly greater yield of $CD3^+$ T cells from semen.

Table 2.4 Comparison of $CD3^+$ T cell yields at three different time points and storage temperatures after ejaculation

Temperature	Yield[a] (IQR)			p value[b]
	4 h	12 h	24 h	
4°C (n = 12)	274 960 (145 950 – 424 200)	173 900 (49 950 – 253 450)	38 390 (7905 – 107 850)	**0.0005**
RT[c] (n = 10)	229 600 (69 055 – 381 850)	142 860 (50 115 – 260 200)	67 300 (43 140 – 144 250)	**0.002**
37°C (n = 12)	265 000 (173 310 – 473 500)	180 100 (106 200 – 307 250)	153 860 (40 050 – 184 550)	**0.0005**
Total (n = 34)	274 960 (147 050 – 426 000)	175 500 (55 470 – 258 650)	62 700 (31 030 – 162 100)	**<0.0001**

[a]Median $CD3^+$ T cell count with interquartile range (IQR)

[b]Wilcoxon matched pair analysis between 4 h and 24 h timepoints

[c]RT = Room Temperature

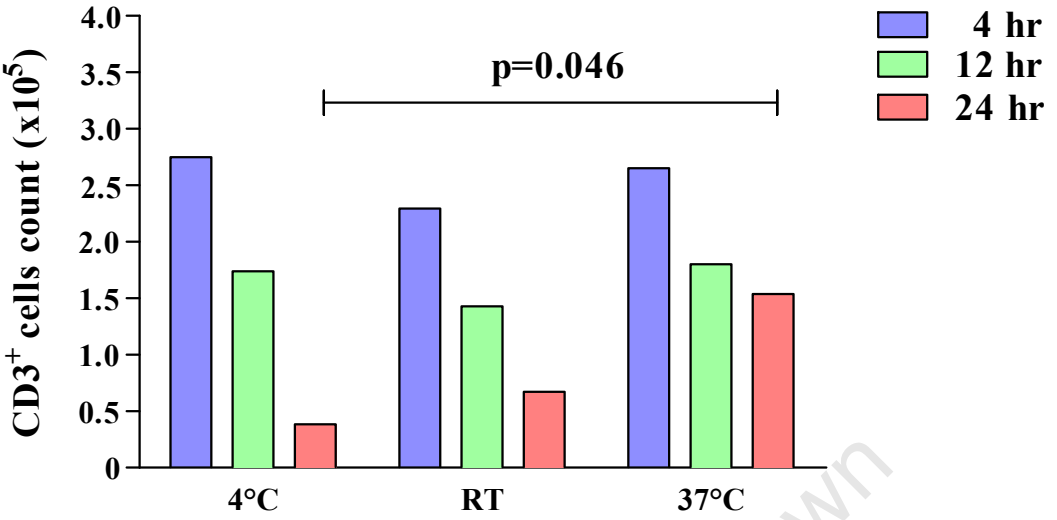


Figure 2.6 The impact of processing time and temperature on $CD3^+$ T cell yields.

$CD3^+$ T cell yields were compared after incubation of samples at either 4 °C (n=12), room temperature (RT; n=10) or 37 °C (n=12) for either 4 h, 12 h or 24 h post ejaculation. Bars represent median values. *p*-values $\leq$ 0.05 were considered significant and the Mann Whitney U test was applied to compare groups.

2.3.5 Characterisation of immune subsets in semen

In order to characterise the range of immune subsets in semen beyond T cells, sixteen semen samples were processed as described earlier and isolated with CD45-specific magnetic beads, followed by staining with phenotypic markers for T cells, B cells, monocytes, neutrophils, Langerhans cells, dendritic cells and NK cells (Figure 2.7).

The most prominent immune cells in semen were DC-SIGN$^+$ leukocytes, with a median number of 447 931 (IQR 274 758 – 676 459) per semen sample. (Table 2.5 and Figure 2.8). This was followed by neutrophils, with a median number of 42 308 (IQR 6 038 – 99 824). Next, B cells were enumerated at a median of 28 878 B cells (IQR 1 882 – 62 621) per semen sample, and monocytes at a median of 3 558 (IQR 1 110 – 9 147). The relative median number of T cells was 18-190 fold less than either neutrophils or DC-SIGN$^+$ cells, at 2 393 (IQR 1 275 – 4 758), indicating that these are a relatively minor immune cell population in semen, approximately equivalent to the number of monocytes, at 3 558 (IQR 1 110 – 9 147). Langerhans cells made up the second smallest subset tested, at a median of 1514 (563 – 4 925) cells per sample. The smallest subset was NK cells at a median of 1054 cells per sample, although their numbers were highly variable between samples (10 – 8 154).

In summary, DC-SIGN$^+$ leukocytes represent the major immune cell type found in semen, and large numbers of neutrophils and B cells are present. T cells represent a relatively minor population in semen.

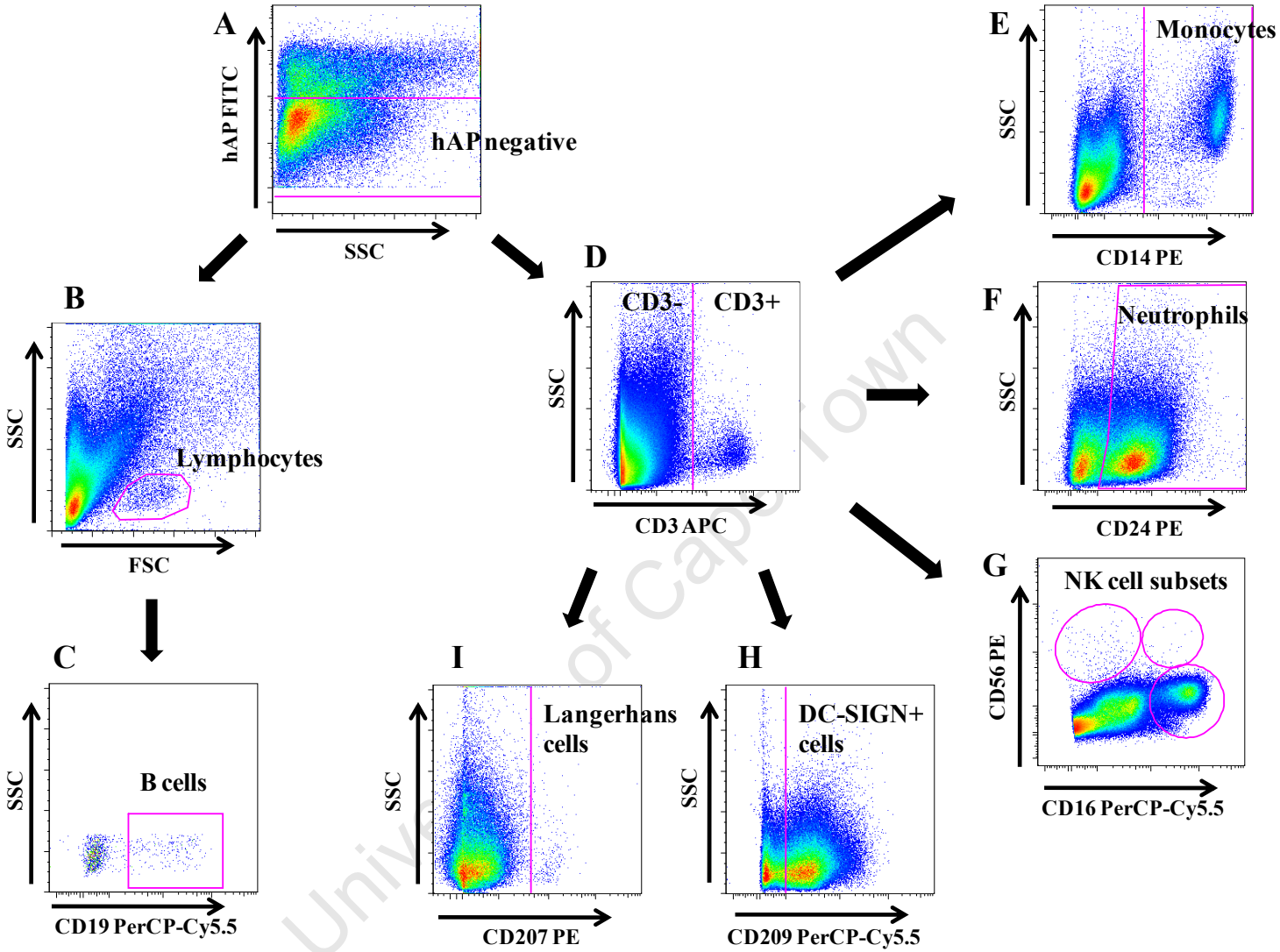


Figure 2.7 Gating strategy for immune cell subsets in semen. Cells were first gated on (A) spermatozoa-negative population (using human acrosomal protein), followed by either (B) lymphocytes and (C) B cells, or (D) CD3-negative population, followed by either (E) monocytes, (F) neutrophils, (G) NK cells, (H) DC SIGN+ cells or (I) Langerhans cells.

Table 2.5 Immune cell subsets in semen

Specificity	Subset	Yield[a] (IQR)
NK cells	$CD56^+$, $CD16^+CD56^+$	1054 (10 – 8 154)
Langerhans cells	$Langerin^+/CD207^+$	1 514 (563 – 4 925)
T cells	$CD3^+$	2 393 (1 275 – 4 758)
Monocytes	$CD14^+$	3 558 (1 110 – 9 147)
B cells	$CD19^+$	28 878 (1 882 – 62 621)
Neutrophils	$CD24^+$	42 308 (6 038 – 99 824)
DC $SIGN^+$ cells	$CD209^+$	447 931 (274 758 – 676 459)

[a]Medians with interquartile range (IQR)

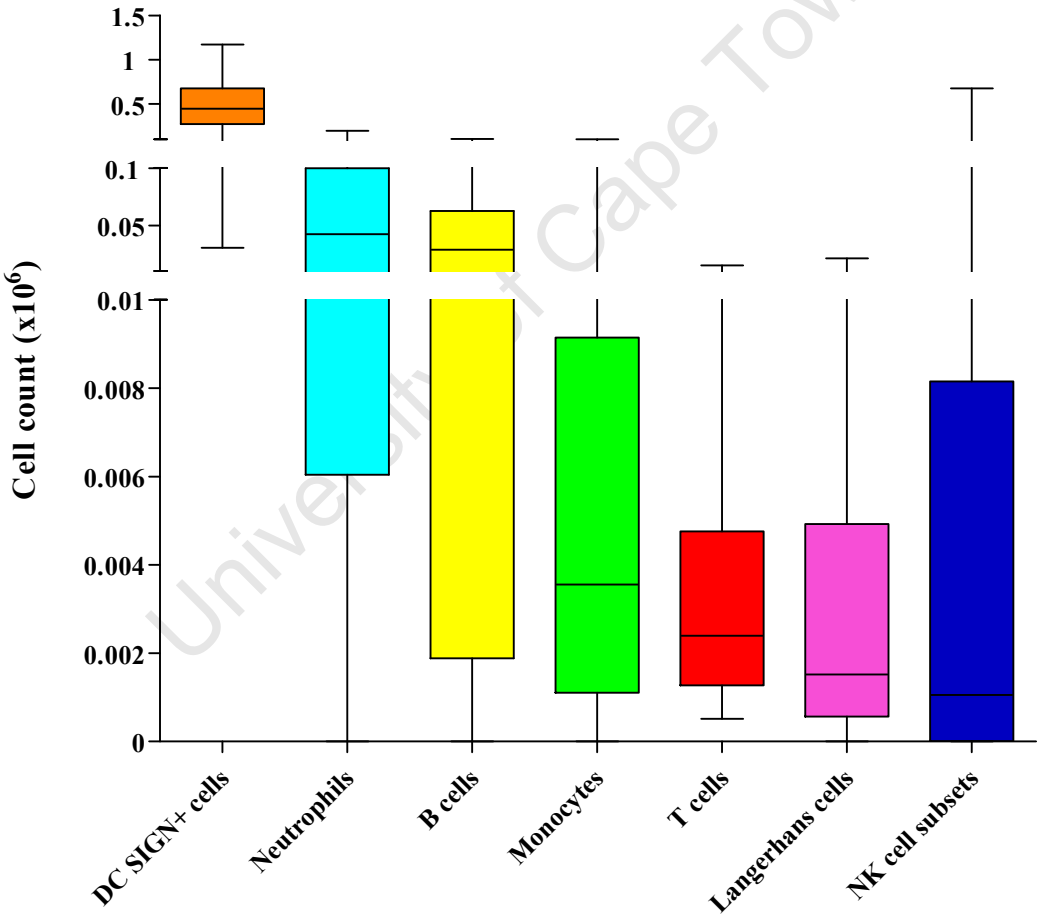


Figure 2.8 Immune cell subsets in semen. Shown are flow cytometric cell counts for DC $SIGN^+$ cells, neutrophils, B cells, monocytes, T cells, Langerhans cells and NK cell subsets in n=16 semen samples. Box-and-whisker plots represent the median, 25^{th} and 75^{th} percentiles. *p*-values ≤ 0.05 were considered significant.

2.4 Discussion

Optimised, standardised protocols for isolating and evaluating viable lymphocytes from the male genital tract are needed, in order to improve our understanding of HIV pathogenesis, and to assess HIV vaccine responses at the male genital mucosa. The aim of this study was to optimise a method for the isolation of $CD3^+$ T cells from semen to maximise functional $CD3^+$ T cell recovery while simultaneously minimising interfering fluorescence from residual sperm and germ cells during flow cytometric analysis. Various methodologies were compared to isolate T cells from semen, namely gradient separation, enzymatic digestion, sample filtering and magnetic bead isolation. It was found that pronase digestion (to dissolve leukocyte-trapping endogenous seminal proteins) and the use of cell strainers (to filter resulting debris) resulted in improved $CD3^+$ T cell yields compared to gradient separation, whilst selection of $CD3^+$ cells using magnetic beads resulted in improving seminal lymphocyte differentiation by flow cytometry. This method allowed for the subsequent functional analysis of $CD4^+$ and $CD8^+$ T cells.

To date, no studies have compared methods for optimising leukocyte isolation from semen. Ficoll-Histopaque density gradient centrifugation is commonly used for PBMC isolation from peripheral blood. However, this technique resulted in the loss of up to 90% of $CD3^+$ T cells. This finding was consistent with a previous study by Focacci *et al.,* who showed that almost 80% of leukocytes were lost subsequent to Ficoll separation (Focacci *et al.,* 1997). Upon ejaculation, semen coagulates form a dense network of fibres that can trap sperm and other cells. The principle proteins responsible for semen coagulates are semenogelin I and II (de Lamirande 2007). In the present study, the use of pronase to liberate trapped lymphocytes in semen resulted in significantly higher $CD3^+$ T cell yields. Use of cell strainers subsequent to pronase treatment was effective in filtering the digested protein fragments and improving T cell yields. After cell straining, residual sperm and germ cells in samples often obfuscated seminal lymphocyte populations during flow cytometry. Therefore magnetic beads were applied to positively select for $CD3^+$ T cells or $CD45^+$ leukocytes.

For robust assessment of immunity, for example in an HIV vaccine trial, isolation of functional cells is important. In response to mitogen stimulation, approximately 60%

of semen samples produced IFN-γ, and the frequency of responsive cells was between 30-35-fold above background. Although this study did not investigate the presence of antigen-specific immunity, the presence of HIV-specific T cells in semen from HIV-infected men has been shown previously (Quayle *et al.,* 1998, Sheth *et al.,* 2005). This finding, coupled with studies showing that HIV-specific T cells are capable of trafficking between blood and mucosal surfaces, including the male genital tract (Musey *et al.,* 2003a, Huang *et al.,* 2006), supports the possibility that candidate HIV vaccines (even those applied systemically) may be capable of eliciting T cell immunity in the male genital tract. Methods such as that described in this Chapter may be applied in clinical trials to assess such responses.

Semen sample volume and increased time of abstinence from ejaculation were found to significantly predict $CD3^+$ T cell yield. Because clinical trial sites may not be physically associated with laboratories capable of processing biological specimens, the impact of time to processing on yield and viability of lymphocytes from mucosal samples such as semen is crucial. In the present study, a shorter time to processing and a sample storage temperature of 37 °C prior to processing were identified to be critical for preserving $CD3^+$ T cells in semen. Although sample processing within 4 h was optimal, when samples were stored for a period of 24 h prior to processing, cell loss was greatly reduced by incubation at 37 °C, compared to at 4 °C or room temperature. A study performed in our laboratory on comparison of incubation of cervical cytobrush samples at 4°C, 37 °C or room temperature, found that detection of Cytomegalovirus/Epstein Barr virus/Influenza virus (CEF)-specific functional responses from T cells were highly significantly increased after 24 h at 37°C compared to the other two temperatures (Liebenberg *et al.,* 2011).

A shortcoming of the present study was that the maximum $CD3^+$ T cell yields and viability obtained were relatively low, at a median of approximately 250 000 cells after isolation, 75 000 following T cell enrichment with magnetic beads, and 2 400 T cells per semen sample subsequent to flow cytometric analysis. A previous studies using flow cytometry reported similar $CD3^+$ T cell counts (2 862 $CD3^+$ T cells) as the present study (Sheth *et al.,* 2005). Whilst our $CD3^+$ T cell yield obtained was the result of optimised procedures in terms of storage time, temperature and isolation method tested, it still limits subsequent analysis of immune function to just a few assays with a

few stimulations, making detailed functional analysis difficult. Additional techniques for measuring antigen-specific responses other than by flow cytometry, such as the IFN-γ ELISPOT assay, could be used prior to the T cell enrichment bead step, as the interfering fluorescence would not pose a problem. To address the low cell numbers, future studies could also explore short-term polyclonal expansion methods to increase T cell numbers, as performed previously with T cells isolated from genital tract samples (Huang *et al.,* 2006, Quayle *et al.,* 1997, Bere *et al.,* 2010a, Bere *et al.,* 2010b).

A further aim of this study was to characterise the immune milieu of the male genital tract by enumerating a range of immune cells subsets in semen. Previous studies have shown the presence of leukocyte populations other than T cells in semen and that the most prevalent of these are granulocytes and monocytes/macrophages (Wolff & Anderson 1988, Ball *et al.,* 1999, Politch, Mayer & Anderson 2009). In the present study, neutrophils and monocytes made up a substantial portion of the leukocyte population assayed. This is to be expected as neutrophils migrate to sites of recurring inflammation (Furze & Rankin 2008) like the mucosal surfaces. The present study shows that B cells are also abundant in semen, whereas previously they have been shown in semen at low to non-detectable levels (Ball *et al.,* 1999). The presence of B cells in semen suggests that the male genital tract may have the capacity to produce humoral responses to pathogens, and it remains to be seen whether HIV-specific antibodies, including neutralising antibodies, are present locally. The importance of Langerhans and DC-SIGN$^+$-DCs have been demonstrated in the transmission of HIV-1 (reviewed in de Witte *et al.,* 2007). Although the presence of Langerhans and DC-SIGN$^+$ cells has not conclusively been shown in semen, they are present in the foreskin and the epithelial layer of the urethral meatus of the penis (McCoombe & Short 2006), implying that these cells can be deposited in semen. In the present study, the largest population of leukocytes were found to be DC-SIGN$^+$. In addition to dendritic cells, DC-SIGN is expressed on neutrophils and a subset of macrophages. Regardless, this high prevalence of DC-SIGN$^+$ leukocytes in semen has serious implications for potential HIV-1 acquisition in the male genital tract. It may indicate that the susceptibility of the male genital tract to HIV is substantially higher than previously thought. NK cells are normally not abundant in tissues of the MGT or semen (Pudney & Anderson 1995), and few NK cells were found in the present study.

In summary, this Chapter describes an optimised method for the isolation of $CD3^+$ T cells from semen that can be used in immune assays to investigate the function of these cells in response to stimulation. The data presented here may be useful in the establishment of standardised protocols for testing the ability of candidate vaccines to elicit mucosal cellular immunity in the male genital tract, or other studies seeking to characterise aspects of immune cells in the male genital tract, as discussed in the following Chapter.

CHAPTER 3

T-cell activation in the male genital tract

Contents

3.1 Introduction

Sexual transmission of HIV-1 is the major route of infection and the mucosal surfaces of the male and female genital tracts therefore play an important role (Royce *et al.,* 1997, Simon *et al.,* 2006). Semen serves as the main vector for HIV transmission from men to their partners during sexual transmission, via cell-free or cell associated virus (Krieger *et al.,* 1995, Vernazza 2005).

HIV transmission is influenced by the concentration of virus in semen. When semen contains 100 000 HIV-1 RNA copies, the probability of transmission increases to 1 in 100 acts of intercourse, from 3 in 10 000 acts when semen contains 1000 HIV-1 RNA copies (Chakraborty *et al.,* 2001). The concentration of HIV-1 RNA in semen is influenced by the stage of HIV infection. In fact, it has been shown that seminal viral load peaks at 30 days post infection (Pilcher *et al.,* 2007), which elevates the risk of HIV-1 acquisition by a partner to maximum levels during acute infection. Although it has been shown that antiretroviral therapy reduces the concentration of HIV-1 in semen (Coombs *et al,* 2003), differential penetration of antiretroviral drugs into the male genital tract (MGT) can result in the establishment of HIV viral reservoirs (Zhang *et al.,* 1998; Ghosn *et al.,* 2004b, Smith *et al.,* 2007, Craigo *et al.,* 2004). Persistent viral replication due to local reservoirs of HIV-1 infection can lead to intermittent viral shedding in semen of infected men (Krieger *et al.,* 1995; Eyre *et al,* 2000, Bujan *et al.,* 2002), which in turn may lead to transmission of HIV-1.

Other factors may enhance HIV-1 transmission to a partner by elevating levels of viral replication in the MGT and subsequent viral shedding in semen. Primary among these are sexually transmitted infections (STIs), such as HSV-2 and gonorrhoea. Gonococcal infection elevates seminal viral loads (Sadiq *et al.,* 2005b, Taylor *et al.,* 2003), while HIV genital shedding has been shown to associate with HSV-2 replication (Baeten *et al.,* 2004, Mole *et al.,* 1997, Schacker *et al.,* 2002a, Serwadda *et al.,* 2003). Apart from enhancement of infection with higher viral shedding, increased risk of infection may also take place by other means. A seminal study by Munch *et al.,* found that amyloid fibrils present in semen may form complexes called SEVI (semen-derived enhancer of virus infection) that may trap HIV-1 virions and potentially enhance transmission (Munch *et al.,* 2007). The availability of infectable target cells within the

MGT can influence HIV acquisition via the penile route. The foreskin provides ideal target cells for enhanced acquisition of HIV-1 in the MGT (Ganor *et al.,* 2010). Circumcision has been shown in a number of randomized trials to have a protective benefit against HIV infection (Auvert *et al.,* 2005, Bailey *et al.,* 2007, Gray *et al.,* 2007). In addition, target cells in other parts of the MGT may facilitate transmission and spread of HIV subsequent to viral entry.

Immune activation is one of the main contributors to the pathogenesis of HIV disease. This is manifested in various ways, from increased T cell proliferation to increased expression of surface activation markers such as HLA-DR and CD38 (Ho *et al.,* 1993, Kestens *et al.,* 1994). Elevated CD38 expression in the context of HIV-1 infection was first described on $CD8^+$ T cells (Giorgi & Detels 1989). Since then, numerous studies in blood have shown that $CD8^+CD38^+$ can be used to predict the rate of HIV disease progression independent of plasma viral load and $CD4^+$ T cell counts (Giorgi *et al.,* 1993, Liu *et al.,* 1997, Bofill *et al.,* 1996). CD38 expressing $CD4^+$ T cells have also been shown to be elevated in HIV-infection (Carbone *et al.,* 2000, Hunt *et al.,* 2003). Two-thirds of gut mucosal $CD4^+$ T cells express CD38 during acute and early HIV-1 infection (Mehandru *et al.,* 2007) and in semen of HIV-infected men, frequencies of $CD8^+CD38^+$ memory T cells were significantly higher compared to healthy men (Lo Caputo *et al.,* 2003). The chemokine receptor CCR5 is the major co-receptor for HIV-1 (Alkhatib *et al.,* 1996, Zhu *et al.,* 1993, Roos *et al.,* 1992), and thus the availability of $CCR5^+$ T cells in the genital tract has specific importance for acquisition of HIV-1 through sexual contact. The levels of $CCR5^+$ T cells in the genital tract of healthy women are substantially higher compared to blood (Hladik *et al.,* 1999), as would be predicted for an effector site such as the genital tract, where $CD4^+$ $CCR5^+$ cells have been found in the foreskin and other epithelial layers of the penis (McCoombe & Short 2006).

Thus the genital tract of healthy sexually active adults seems to be the ideal target for HIV-1 acquisition and transmission, with elevated levels of CCR5-expressing $CD4^+$ T cells. In fact, not only are CCR5 levels elevated, a recent study found both $CD4^+$ and $CD8^+$ T cells expressing either the activation marker CD69 alone or together with CCR5, were significantly increased in the genital tract of HIV-uninfected Kenyan women (Cohen *et al.,* 2010) compared to genital activation levels in their counterparts

in the United States. The authors postulate that despite controlling for confounders such as genital co-infections, sexual behaviour and menstrual phase, it may be that genital immune activation is the result of differences in host genetics or systemic infections. Higher levels of systemic immune activation have also been reported in HIV-seronegative Africans (Kassu *et al.,* 2001, Clerici *et al.,* 2000, Eggena *et al.,* 2005). Investigating the status and impact of immune activation in the male genital tract of healthy and HIV-infected African men is an important unaddressed issue.

The aim of this chapter was to investigate T cell activation in the male genital tract, to understand more about the role it may play in both acquisition of HIV infection in uninfected men, and onward transmission of HIV in those already infected. T cell activation was examined in semen using the marker CD38, and CCR5 expression was used to identify $CD4^+$ target cells. T cells from HIV-infected, HIV-uninfected and ARV-treated men were examined, and genital tract activation levels were compared to those in blood. The impact of seminal and plasma viral loads on T cell activation was examined. Lastly, since a portion of participants formed part of an HIV-discordant couples cohort, T cell activation was examined in the semen and blood of HIV exposed but sero-negative men.

3.2 Materials and Methods

3.2.1 Study participants

Twenty-six ARV naïve and 12 ARV-treated HIV-infected men, along with 42 HIV-uninfected men were enrolled from the Empilisweni Clinic in Athlone, Cape Town, South Africa. All men gave written informed consent, and the Research Ethics Committee of the University of Cape Town approved all aspects of the study (HREC REF: 258/2006). Seventy-one of the 80 participants were part of 602 couples that were enrolled in a study investigating Human Papillomavirus (HPV) infection in HIV-discordant and concordant couples. Of the 38 HIV-infected men enrolled in the present study, 20 belonged to HIV-concordant couples and 16 were part of HIV-discordant couples. There were 14 HIV-uninfected men with HIV-uninfected partners and 21 HIV-uninfected men had HIV-infected partners. For 9 men, the HIV-status of the partner was unknown.

CD4 counts were performed by FlowCARE PLG kits (Beckman Coulter, Inc., Brea, CA) according to the manufacturer's protocol. CD4 counts were available for 21 of the 38 HIV-infected men within 2 months of sampling. For the HIV-infected men, the time of infection was not known. For ARV-treated men, neither the time on treatment nor the antiretroviral regimen was known.

3.2.2 Sample collection and processing

Ejaculates were collected in sterile specimen jars containing 6 ml of transport media [RPMI-1640 medium supplemented with 5 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (GIBCO®, Invitrogen™, Carlsbad, CA, USA), 2 mg/ml fungin® (Invivogen, San Diego, CA, USA)] following voluntary self masturbation. Semen samples were processed by optimal methods as established and described in Chapter 2 (Section 2.2.2). Whole blood was collected in ACD Vacutainer tubes (BD Biosciences, Plymouth, UK) by venipuncture. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, Egham, Runnymede, UK) and LeucoSep centrifuge tubes (Greiner Bio-one; Frickenhausen, Germany). Briefly, whole blood was layered on Ficoll and subsequent to 15 min of centrifugation at 1000 x g, the resulting plasma

and buffy layers containing PBMCs were harvested. Blood and seminal plasma was stored at -80°C for viral load determination as well as cytokine measurements (Chapter 4). $CD3^+$ cell counts were performed on a Guava cell counter using anti-CD3 PE monoclonal antibody (Guava Technologies, Hayward, CA, USA).

3.2.3 Flow-cytometric analysis

Both seminal mononuclear cells (SMCs) and PBMCs (2 million cells/sample) were split into two fractions and stained with the following two monoclonal antibody panels: 1. anti-CD3 PE, anti-CD4 FITC, anti-CD8 PerCP-Cy5.5 and anti-CCR5 APC or 2. anti-CD3 PE, anti-CD4 FITC, anti-CD8 PerCP-Cy5.5 and anti-CD38 APC (all BD Biosciences, San Diego, CA, USA). All antibodies were pre-titered to determine optimal staining dilutions. Surface staining of cells was performed for 20 min at room temperature. Cells were fixed in Cell Fix (BD Biosciences, San Jose, CA, USA) and samples acquired on a FACSCalibur. All events were collected for semen, whereas 500 000 events were collected for stained PBMC. Data were analysed using FlowJo software v8.5.3 (Tree Star Inc, Ashland, OR, USA). The gating strategy used to detect $CD8^+$ and $CD4^+$ T cells expressing CD38 and CCR5 started by applying a lymphocyte gate based on forward and side scatter, $CD3^+$ T cells were then gated on, followed by $CD4^+$ and $CD8^+$ gates. Each T cell subset was then examined for expression of CD38 or CCR5, depending on the panel used to stain with. To set gates for CD38 and CCR5, fluorescence minus one (FMO) controls were used. Subsequent to analysis, samples with $CD38^+$ and $CCR5^+$ event counts of 10 or less were excluded from the study.

3.2.4 Quantification of HIV RNA in semen and blood

Plasma and seminal HIV-1 RNA concentrations (copies/ml) were quantified using NucliSENS EasyQ® HIV-1 (version 2.0) [bioMérieux SA, Lyon, France] according to the manufacturer's protocol. The assay had a lower limit of detection of 70 copies of HIV-1 RNA/ml and a linear range of detection up to 10×10^6 copies of HIV-1 RNA/ml. For semen viral loads, values were adjusted according to the dilution factor according to the transport medium and the volume of the semen sample, and expressed as RNA copies/ml of semen.

3.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0® (GraphPad Software, San Diego, CA, USA). The Mann–Whitney U test was applied for

independent non-parametric sample comparisons, the Wilcoxon rank test was used for matched non-parametric comparisons and Spearman ranks correlation was applied for assessing non-parametric associations. Kruskal-Wallis test with Dunn's post test was used for non-parametric sample comparisons of three unmatched groups or more. All tests were two-tailed and p-values ≤ 0.05 were considered significant.

3.3 Results

3.3.1 Clinical characteristics of study participants

Forty-two HIV-uninfected men and 38 HIV-infected men were recruited into this study (Table 3.1). Twelve of the HIV-infected men were receiving anti-retroviral treatment. The median age of the men was 42 years (range 23 – 58), and this did not differ significantly between the groups. Only 22 out of the 38 HIV-infected men had recent CD4 counts available. The median absolute CD4 count for ARV-naïve men was 356 cells/mm^3 (IQR 257 – 650 cells/mm^3). Although the median CD4 counts were lower in ARV-treated men, at 264 cells/mm^3, this was not significant (IQR 208 – 585 cells/mm^3).

The majority of men were enrolled from a larger study, and specific STI data were not available for the group of men studied in this chapter. However, within the larger cohort from which they were recruited, 23% of male participants reported having penile discharge and 11% reported the occurrence of genital ulcers within 6 months of enrolment. HPV prevalence among men in the larger cohort was found to be 58% (49% in HIV-uninfected and 77% in HIV-infected men) [Mbulawa *et al.,* 2010]. At enrolment, male participants reported that they were with their partners for an average of 6 years and were having an average of 8 sex acts per month. Sixty-four percent of men reported using condoms with their partners and 93% of men were circumcised.

3.3.2 T cell yields and ratios in semen of HIV-infected and uninfected men

A recent study (Politch *et al*, 2009) provided immunohistological evidence that CD4$^+$ T cells are depleted in semen, implying that CD4$^+$ T cell depletion described in mucosal sites such as the gastrointestinal tract may also be occuring in the male genital tract. Here, flow cytometry was used to enumerate CD4$^+$ and CD8$^+$ T cells in blood and semen of all participants. The median CD3$^+$ T cell yields were lower in the semen of HIV-infected and ARV-treated compared to HIV-uninfected men, although this was not significant. Median CD4$^+$ T cell yields in semen were higher in HIV-uninfected compared to infected men and significantly so compared to ARV-treated men (p=0.039; Table 3.2). In contrast, median CD8$^+$ T cell yields were higher in the semen of HIV-infected and ARV-treated men compared to HIV-uninfected men. CD4/CD8

ratios were significantly lower in HIV-infected and ARV-treated than uninfected men (p=0.0011; Table 3.2).

3.3.3 Viral loads in semen and plasma

Viral loads were measured in matched plasma and seminal fluid of HIV-infected participants (Table 3.1 and Figure 3.1). Among the 26 untreated HIV-infected men, 25 had a detectable plasma viral load, whilst 19 had a detectable seminal viral load. ARV treatment successfully suppressed viral replication in both semen and blood to below detectable levels in the majority (10/12) men (Figure 3.1). Interestingly, two out of 12 ARV-treated men had detectable plasma viral load despite suppression of seminal virus replication, and a further two men had detectable virus in semen, despite plasma viral suppression. In one of these cases, the viral load was amongst the highest measured in semen, at 60 200 copies/ml.

As shown in Figure 3.2A, the median plasma viral load in HIV-infected men was 11000 RNA copies/ml (IQR undetectable – 300 000 RNA copies/ml), which was significantly higher than the median seminal viral load, at 1389 RNA copies/ml (IQR undetectable – 135 000 RNA copies/ml; p=0.039). A weak but significant positive correlation was found between viral loads in semen and blood (p=0.04, r=0.4; Figure 3.2B).

The relationship between CD4 counts in blood and seminal viral loads was investigated in untreated HIV-infected men. Although CD4 counts were available for just 13 men, there was a strong and highly significant inverse correlation between blood CD4 counts and seminal viral loads (p=0.0046, r=-0.75; Figure 3.3A). In blood, no such association was evident (data not shown). As shown in Figure 3.3B, the median seminal log viral load of 3.3 (IQR 2.4 – 4.0) for men with CD4 cell count > 350/mm^3 was lower than the median of 4.4 (IQR 3.4 – 5.4) for men with CD4 cell count ≤ 350. The median log plasma viral load of 1.8 (IQR 1.8 – 2.7) for men with CD4 cell count > 350/mm^3 was significantly lower than the median of 4.3 (IQR 2.8 – 4.5) for men with CD4 cell count ≤ 350 (p=0.011; Figure 3.3B).

Table 3.1 Clinical characteristics of participants

Characteristic	HIV+ ARV-naïve	HIV+ ARV-treated	HIV-uninfected
N	26	12	42
Age [years; median (IQR)]	39 (34-44)	43 (39-46)	44 (37-51)
CD4 count [cells/mm^3; median (IQR)]	356 (257-650)[a]	264 (208-585)[b]	-
Plasma viral load [RNA copies/ml; median (IQR)]	10200 (2250-40000)	LDL[c]	-
Number of men with detectable HIV RNA in plasma [N/Total (%)]	25/26 (96.2%)	2/12 (16.6%)	-
Genital tract viral load [RNA copies/ml; median (IQR)]	1389 (LDL[c]-20060)	LDL[c]	-
Number of men with detectable HIV RNA in semen [N/Total (%)]	19/26 (73.1%)	2/12 (16.6%)	-

[a]N = 13; CD4 counts were not available for the remainder of the participants
[b]N = 9
[c]LDL = lower than the detection level (70 HIV-1 RNA copies/ml)
[d]Two participants had plasma viral loads of 740 and 880 copies/ml; a different two participants had seminal viral loads of 414 and 60200 copies/ml

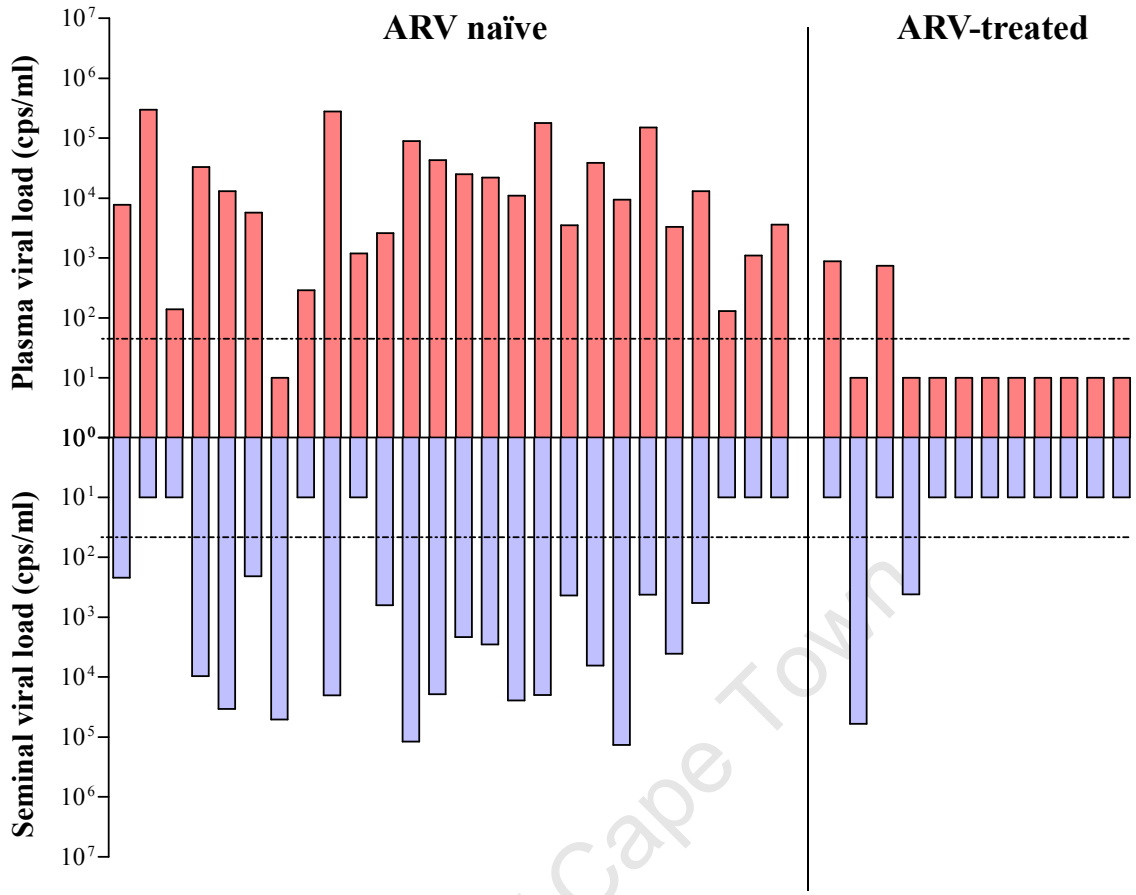


Figure 3.1 Matched viral loads in plasma and semen of HIV-infected men. Plasma viral loads (red) and seminal viral loads (blue) in HIV-infected ARV-naïve and ARV-treated men. Bars represent the matched viral loads (log scale) in both compartments. The dotted lines represent the lower limit of detection of the assay (70 HIV-1 RNA copies/ml).

Table 3.2 T cell counts and CD4:CD8 ratios in semen

T cell subset	Yields[a] (IQR)			p value[b]
	HIV+ ARV-naïve (n=26)	HIV+ ARV-treated (n=12)	HIV-uninfected (n=42)	
CD3	12190 (3825–29929)	10567 (5563–21391)	14069 (7489–43443)	ns
CD4	250 (69–1285)	91 (53–365)	330 (284–1120)	**0.039**
CD8	1201 (333–7997)	1146 (389–2129)	799 (284–3050)	ns
CD4:CD8 ratio	0.18 (0.12– 0.5)	0.13 (0.05–0.43)	0.71 (0.2–1.27)	**0.0011**

[a]Median of yields with interquartile range (IQR), semen samples adjusted for dilution factor

[b]Kruskal-Wallis with Dunn's post test

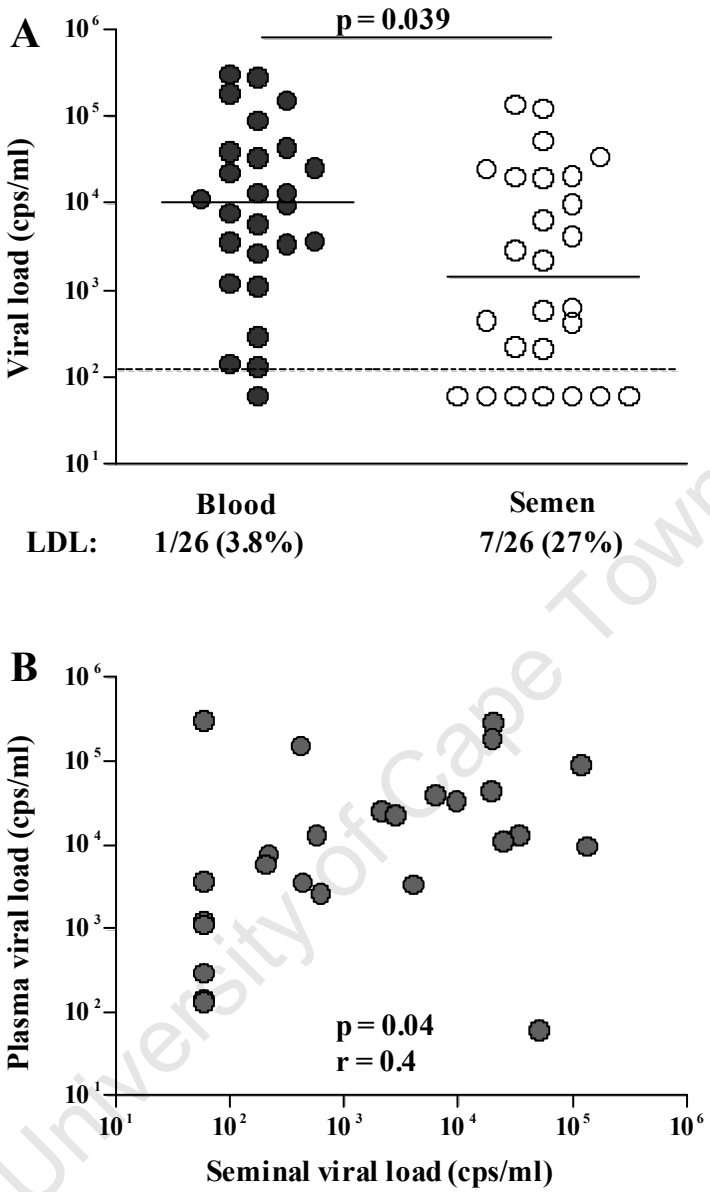


Figure 3.2 The association of viral loads in blood and semen of HIV-infected men. (A) Data points represent plasma viral load (filled circles) and seminal viral load (unfilled circles) from 26 HIV-infected untreated men. Bars represent medians and the dotted line indicates the lower limit of detection of the assay (70 HIV-1 RNA copies/ml). The Wilcoxon signed rank test was used to test for a significant difference between the groups. (B) Each data point represents the viral load of each participant in blood and semen. Statistical analysis was performed using the Spearman Rank Test and p-values ≤ 0.05 were considered significant. Spearman r-value is shown on the plot.

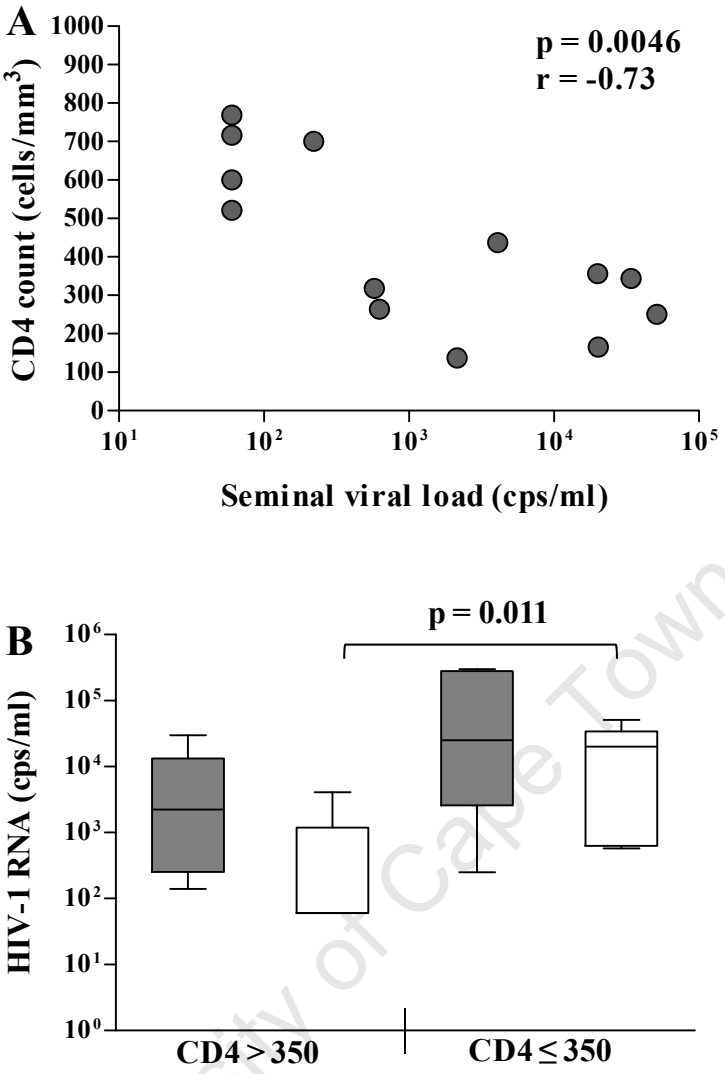


Figure 3.3 The association between viral loads and CD4 counts. (A) Each data point represents the matched seminal viral load and plasma CD4 count of 13 of the ARV-naïve men. Statistical analysis was performed using the Spearman Rank Test and p-values ≤ 0.05 were considered significant. Spearman r-value is shown on the plot. (B) Bar-and-whisker plots represent the median viral load in blood (filled bars) and semen (unfilled bars) separated into CD4 counts > 350 and CD4 counts ≤ 350. The Mann-Whitney U test was performed and p-values ≤ 0.05 were considered significant.

It was of interest to determine whether there were any differences in seminal viral loads in those men in a relationship with a partner who was HIV-infected (concordant) or uninfected (discordant). Within the group of 26 HIV-infected, untreated men that formed part of this study, 15 men were in concordant and 9 men were in discordant relationships. In the case of two men, the HIV-status of their partners was unknown, and they were thus excluded from this analysis. As shown in Figure 3.4, there were no significant differences between plasma or semen viral loads in HIV concordant and discordant men.

In summary, these results show that there were a positive correlation between viral loads in blood and semen, with median viral loads approximately one log higher in plasma compared to semen of HIV-infected, untreated men. ARVs successfully suppressed viral replication in the genital tract of 10/12 men, and two viral shedders (17%) were identified, despite suppressed plasma viral replication. Seminal viral load increased with decreasing blood absolute CD4 counts. Finally, there was no significant difference in seminal or plasma viral load between men in HIV-concordant relationships or those in HIV-discordant relationships.

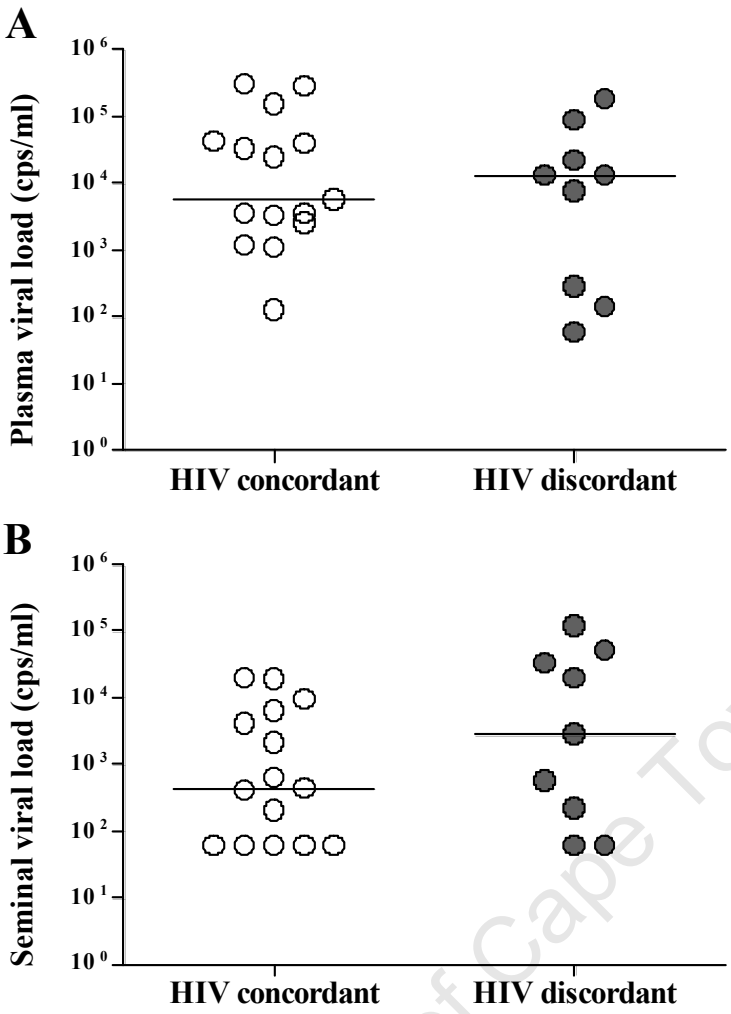


Figure 3.4 Viral loads in blood and semen of HIV-infected men in HIV-concordant and discordant relationships. (A) Plasma viral loads and (B) seminal viral loads of HIV-concordant (unfilled circles) and HIV-discordant (filled circles) men. The Mann-Whitney U test was performed and p-values ≤ 0.05 were considered significant.

3.3.4 Frequencies of activated T cells are higher in semen than blood

Four colour flow cytometry was used to determine the proportions of CCR5 and CD38 expressing-$CD4^+$ and $CD8^+$ T cells in blood and semen of HIV-infected and uninfected men, as a measure of immune activation and to enumerate potential target cells for HIV-infection. The gating strategy and representative flow plots are shown in Figure 3.5. Overall, the frequencies of activated T cells in semen were higher than those in blood (Figure 3.6). In HIV-uninfected men (Figure 3.6A), there were significantly more $CD8^+$ T cells expressing CCR5 ($p<0.0001$) and CD38 ($p<0.0001$) in semen compared to blood. $CD4^+$ T cells expressing CCR5 was also significantly increased in semen compared to blood ($p<0.0001$). In HIV-infected men the same pattern was maintained (Figure 3.6B). Both CCR5- ($p=0.0001$) and CD38-expressing $CD8^+$ T cells ($p=0.0002$) were significantly higher in semen than in blood, with only CCR5-expressing $CD4^+$ T cells reflecting the same significant increases in semen ($p=0.0003$). In ARV-treated men (Figure 3.6C), the number of $CD8^+$ T cells expressing CCR5 ($p=0.002$) and CD38 ($p=0.0029$) were significantly higher in semen than in blood.

These results indicate that semen contains greater numbers of activated CD4 and CD8 T cells, and $CD4^+CCR5^+$-expressing T cells than blood, regardless of HIV infection or treatment.

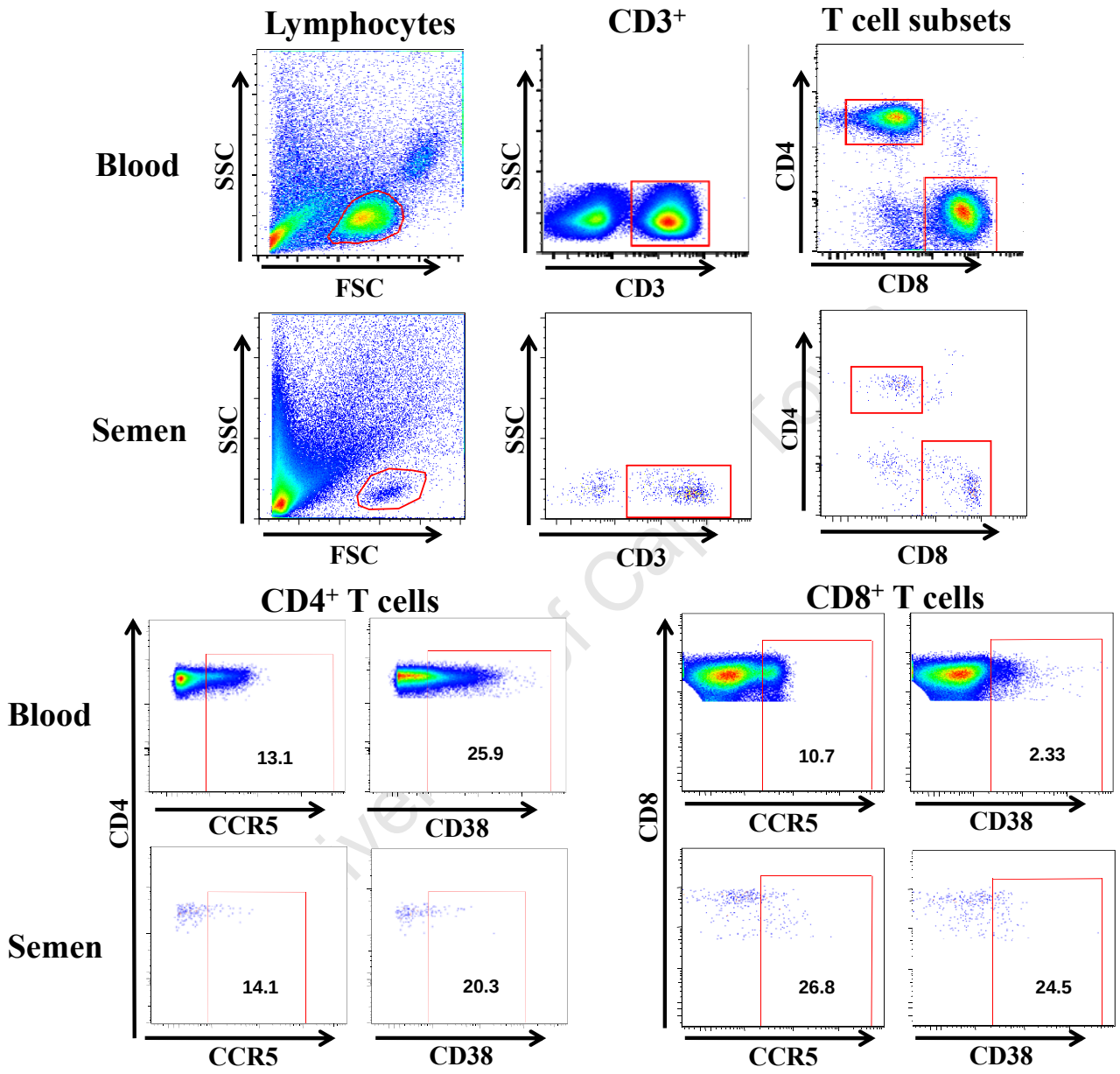


Figure 3.5 Gating scheme used for enumeration of activated T cells in blood and semen. Cells were first gated on lymphocytes, then $CD3^+$ T cells, followed by $CD4^+$/$CD8^+$ gating. Next $CD4^+$ and $CD8^+$ T cells expressing either CCR5 or CD38 were gated on.

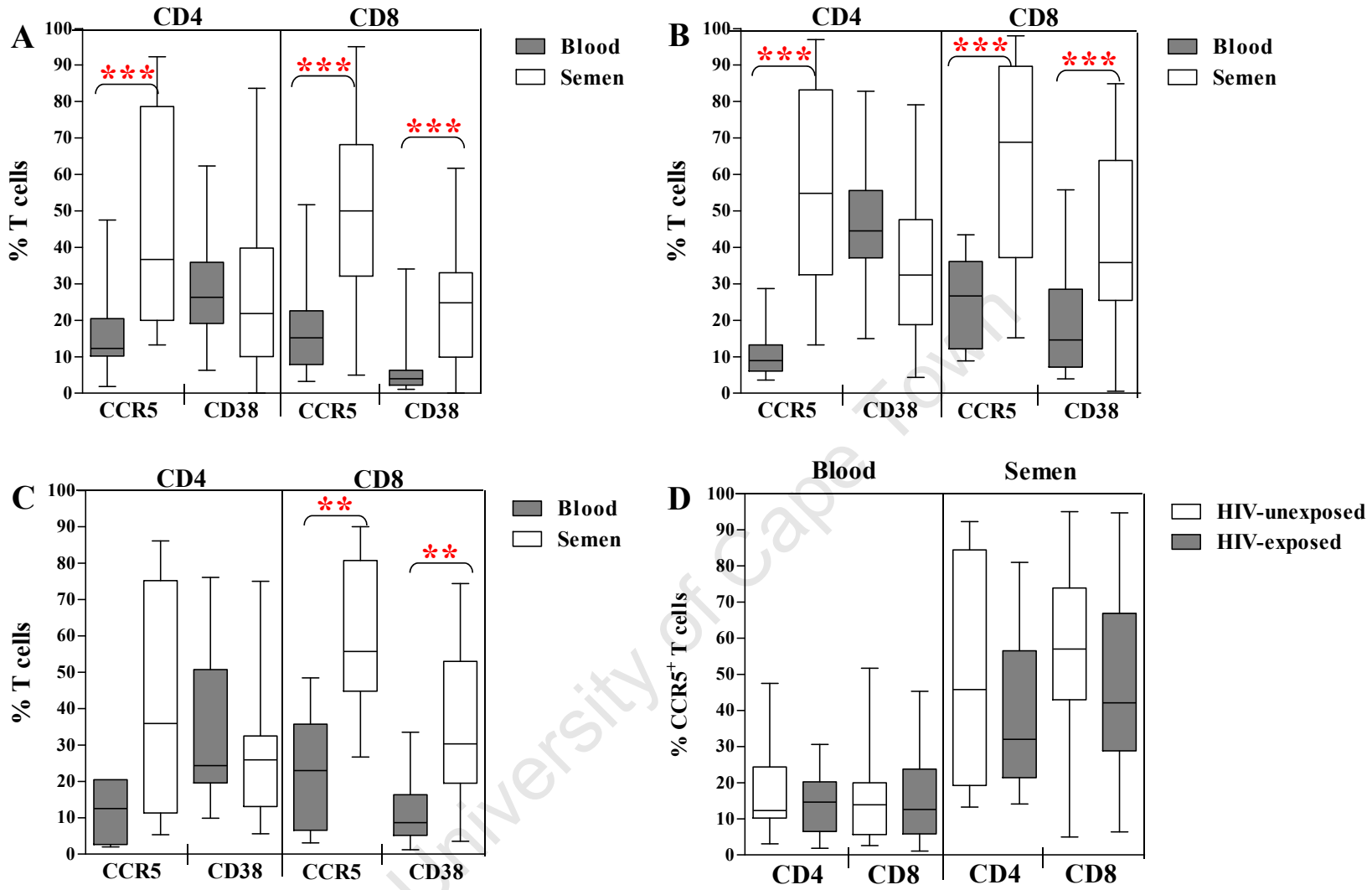


Figure 3.6 Activated T cells in blood and semen I. A comparison is shown between the frequencies of activated T cells in blood and semen of (A) HIV-uninfected, (B) HIV-infected and (C) ARV-treated men. In (D) a comparison of the frequency of $CCR5^+$ T cells between HIV-unexposed and HIV-exposed sero-negative men in blood and semen is shown. Each box-and-whisker plot indicates the median, IQR and 5-95% range (error bars). * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.0001$, using the Wilcoxon signed rank test (A – C) and Mann-Whitney U test (D).

3.3.5 Frequencies of activated T cells in HIV-infected and uninfected men

To further characterise cellular activation, a comparison of frequencies of activated T cell subsets were performed between the three patient groups in this study: HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men. The results are shown in Figure 3.7.

With the exception of the $CD4^+CCR5^+$ T cell subset, the frequencies of activation of all other immune subsets in both blood and semen were highest in HIV-infected (ARV-naïve) men, followed by HIV-infected (ARV-treated) men, and the lowest in HIV-uninfected men (Figure 3.7A and B). In blood, $CD4^+CD38^+$ T cell frequencies were significantly higher in HIV-infected (ARV-naïve) men than frequencies in HIV-uninfected men ($p=0.0003$). In the blood of HIV-infected (ARV-naïve) men, both CCR5-expressing) and CD38-expressing $CD8^+$ T cell frequencies were significantly higher than frequencies in HIV-uninfected men ($p=0.033$ and $p<0.0001$, respectively). In semen, $CD8^+CD38^+$ T cell frequencies in HIV-infected (ARV-naïve) men were significantly higher than frequencies in HIV-uninfected men ($p=0.006$). In blood, median frequencies of $CD4^+CCR5^+$ T cells, the target cells for HIV infection, were higher (though not significantly so) in HIV-uninfected compared to HIV-infected (ARV-naïve) men, whereas in ARV-treated men they were the lowest. Interestingly, the opposite was found in semen. A median of 19% more $CD4^+CCR5^+$ T cells were found in HIV-infected (ARV-naïve) men compared to both ARV-treated and HIV-uninfected men. Furthermore, the frequencies of $CD4^+CCR5^+$ T cells in semen of ARV-treated men were comparable to those of HIV-uninfected men.

To investigate the possible influence of HIV-infected partners on expression frequencies of CCR5 and CD38 on T cells in HIV-uninfected men, frequencies of immune subsets were compared between blood and semen in HIV-uninfected, exposed (n=21) and HIV-uninfected, unexposed men (n=21). In semen, although not significant, there was a consistent decrease in CCR5 expression on both $CD4^+$ and $CD8^+$ T cells in HIV-exposed compared to HIV-unexposed men. In blood, there were no differences between the expression frequencies of CCR5 in either $CD4^+$ or $CD8^+$ T cells (Figure 3.6D). There were no significant differences observed in frequencies of CD38 expressing T cells in both semen and blood of the groups compared.

Thus, when comparing HIV-uninfected, HIV-infected (ARV-treated) and HIV-infected (ARV-naïve) men, the highest frequencies of immune activation in semen were found in untreated HIV-infected men, and this was mirrored in blood. Frequencies of the target cells for HIV infection, $CD4^{+}CCR5^{+}$ T cells, were depleted from the blood of HIV-infected men; however, they were found in higher frequencies in the semen of these men, possibly indicating a greater influx of these cells due to factors such as inflammation or viral replication (or both), than destruction. Finally, although ARV-treatment effectively reduced T cell activation, it was not restored to levels found in the semen of HIV-uninfected men.

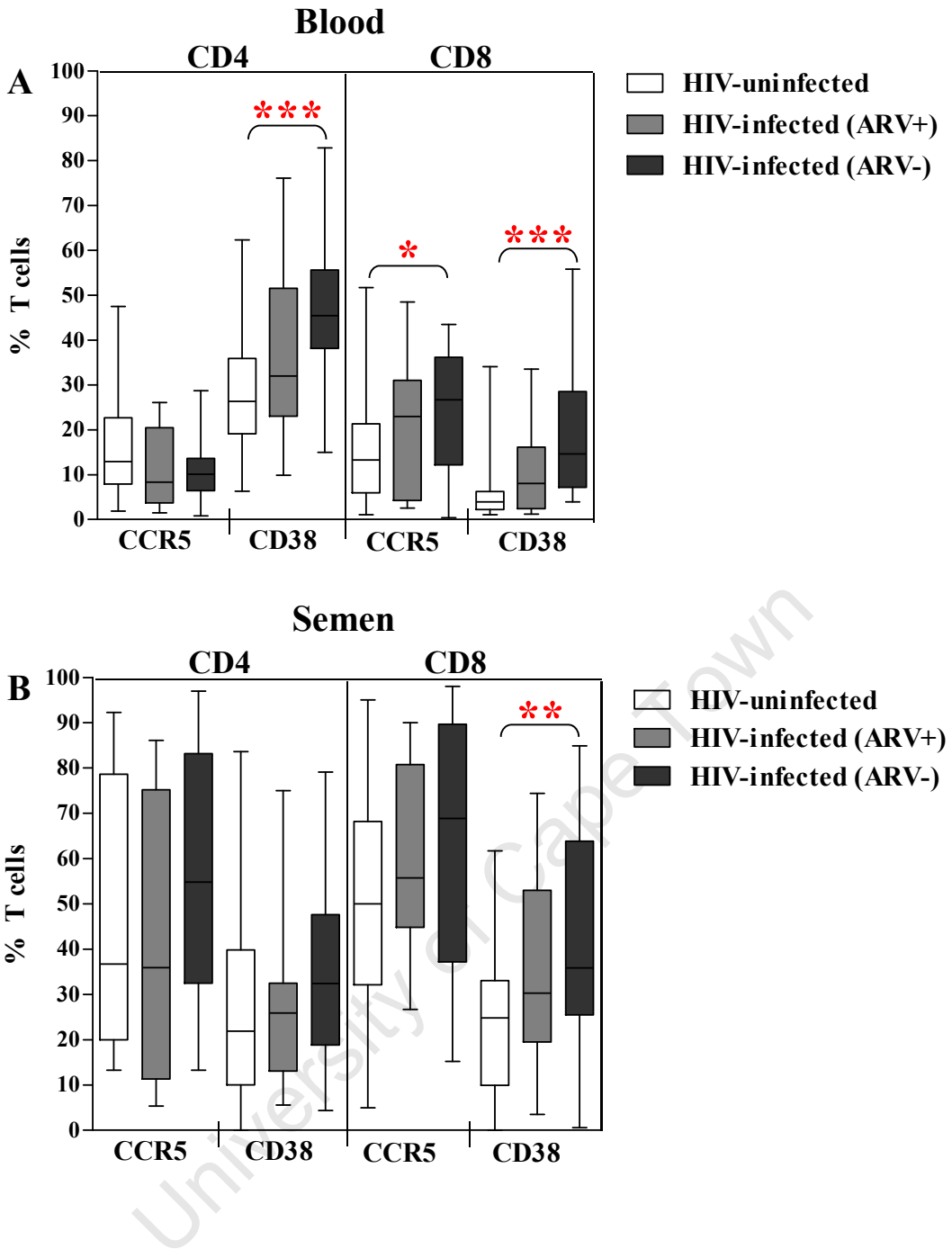


Figure 3.7 Activated T cells in blood and semen II. Frequencies of activated T cells in (A) blood and (B) semen of HIV-uninfected, HIV-infected (ARV+) and HIV-infected (ARV-). Each box-and-whisker plot indicates the median, IQR and 5-95% range (error bars). * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.0001$, using the Kruskal-Wallis test with Dunn's post test for the comparison of three or more unmatched groups.

3.3.6 The relationship between T cell activation in blood and semen

To investigate whether there was a relationship between activation in blood and semen, correlations between activated immune subsets in HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) participants were compared for the two compartments. As was to be expected, there was no correlation of $CD8^+CD38^+$ frequencies between blood and semen of HIV-uninfected men (Figure 3.8A). In contrast, there was a significant positive correlation between $CD8^+$ $CD38^+$ T cells between blood and semen of HIV-infected men ($p=0.0077$, $r=0.44$; Figure 3.8B). This association held true even when the ARV-treated subset was removed from the analysis. There were no significant associations of activated $CD4^+$ T cells between compartments, likely due to their deletion as target cells for HIV.

Next the relationship between $CD4^+$ and $CD8^+$ T cell activation, in blood and semen was examined. In blood, strong and significant associations between the frequencies of $CD4^+CD38^+$ and $CD8^+CD38^+$ T cells were found in HIV-uninfected ($p=0.0003$; $r=0.53$; Figure 3.9A), HIV-infected (ARV-treated) [$p=0.0053$; $r=0.75$; Figure 3.9B] and HIV-infected (ARV-naïve) men ($p=0.0002$; $r=0.66$; Figure 3.9C). As observed previously, a striking difference between HIV-infected and uninfected men was that the majority of HIV-infected men, expressed CD38 on both immune subsets at a frequency of > 30%, compared to less than a third of HIV-uninfected men. The strong positive association seen for CD38 expression in the two immune subsets in uninfected men was mirrored for CCR5 (Figure 3.9D), as well as HIV-infected (ARV-treated) men (Figure 3E). This relationship was predictably disrupted in the case of HIV-infected men (ARV-naïve), with the deletion of $CD4^+CCR5^+$ cells (Figure 3.9F). In semen, CD38 expression followed the same general pattern as in blood. Both HIV-uninfected and infected men showed significant positive associations between the frequencies of $CD4^+CD38^+$ and $CD8^+CD38^+$ T cells ($p<0.0001$, $r=0.68$ and $p=0.0089$, $r=0.53$, respectively; Figure 3.10 A and C). HIV-infected (ARV-treated) men followed a similar trend, however too few data points make this analysis unreliable (Figure 3.10 B and E). A strong and significant positive correlation between $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in semen was maintained in healthy men, similar to results from blood ($p<0.0001$, $r=0.73$; Figure 3.10D). However, in contrast to results from blood, HIV-infected men also had a strong positive correlation between $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in semen ($p=0.0026$, $r=0.68$, Figure 3.10F).

These results show that the frequencies of activated $CD8^+$ T cells correlate between semen and blood in HIV-infected men but not uninfected men. In agreement with blood, the frequencies of activated $CD4^+$ T cells correlated with frequencies of activated $CD8^+$ T cells in semen of both HIV-uninfected and infected men. Somewhat surprisingly, contrary to what was found in blood, the frequencies of CCR5 expression in semen also correlated strongly between CD4 and CD8 T cell subsets in HIV-infected men, once again indicating an additional driver of the influx of these cells into the genital tract.

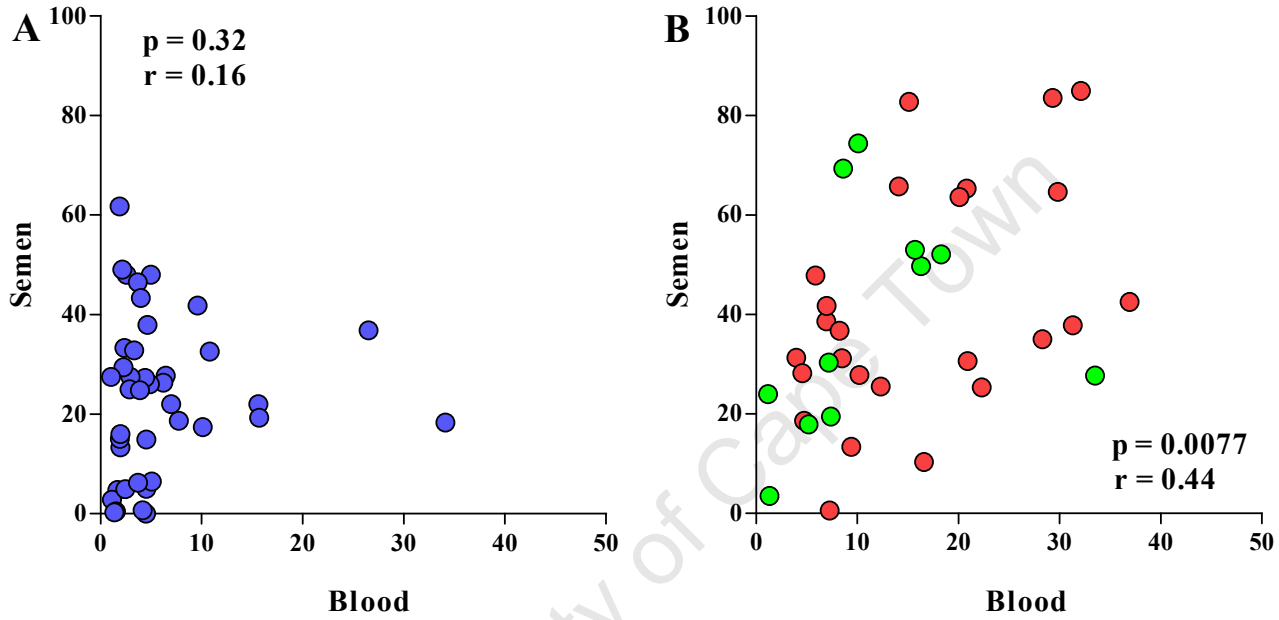


Figure 3.8 Association of activated CD8$^+$ T cells between blood and semen. Shown are correlations of CD8$^+$CD38$^+$ T cells between compartments in (A) HIV-uninfected [blue circles] and (B) HIV-infected, ARV naïve [red circles] + HIV-infected, ARV-treated [green circles] men. Each circle represents a matched CD8$^+$ T cell frequency of a participant in blood and semen. The Spearman rank test was used for statistical analysis and p-values ≤ 0.05 were considered significant.

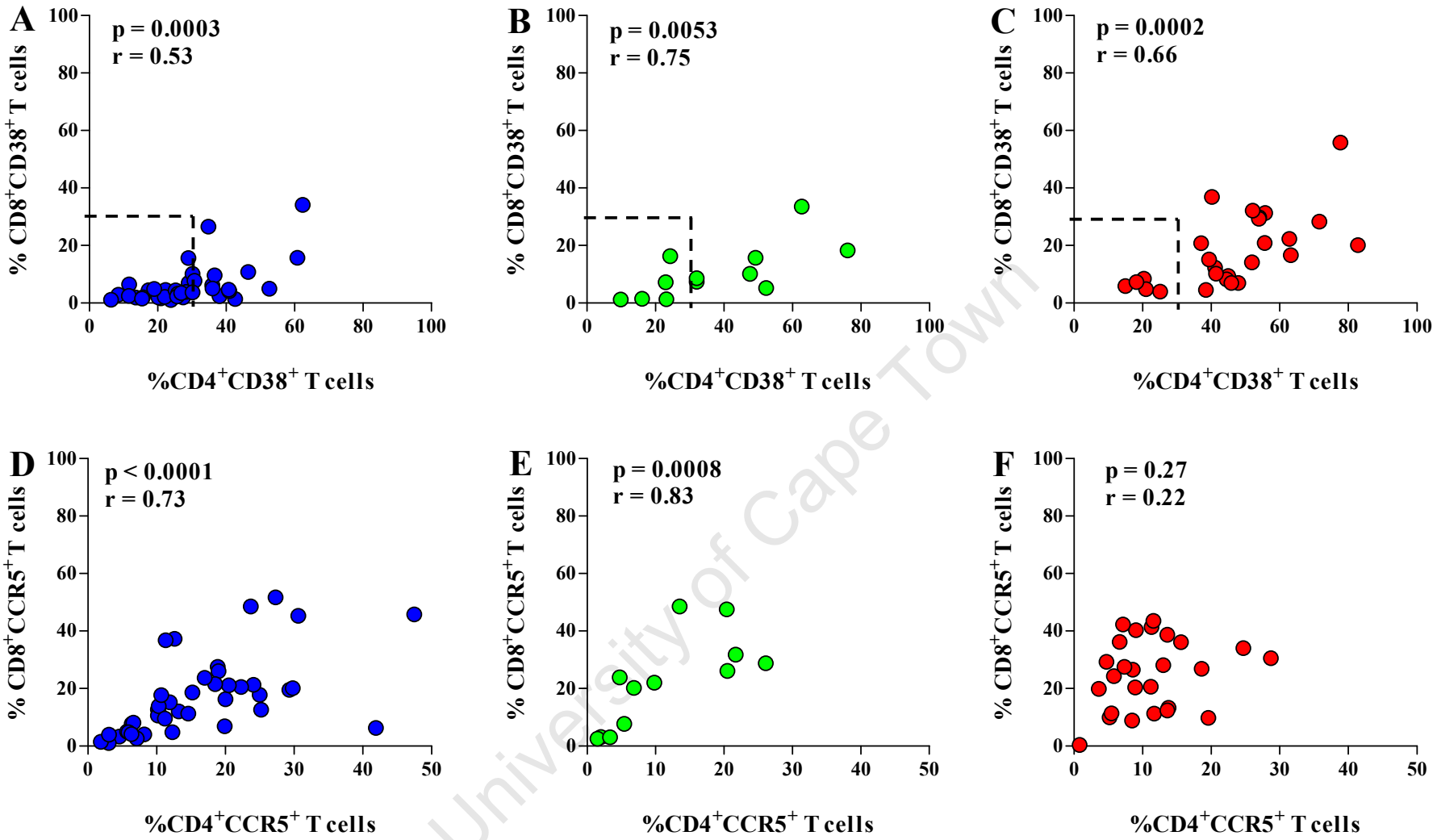


Figure 3.9 Association of activated CD4$^+$ T cells with activated CD8$^+$ T cells in blood. Shown are correlations of (A – C) CD4$^+$CD38$^+$ with CD8$^+$CD38$^+$ T cells and (D – F) CD4$^+$CCR5$^+$ with CD8$^+$CCR5$^+$ T cells of HIV-uninfected [blue circles], HIV-infected (ARV-treated) [green circles] and HIV-infected, ARV-naïve [red circles] men. Each circle represents a matched CD4$^+$ with CD8$^+$ T cell frequency of a participant in blood. Dashed lines indicate 30% frequency. The Spearman rank test was used for statistical analysis and p-values ≤ 0.05 were considered significant.

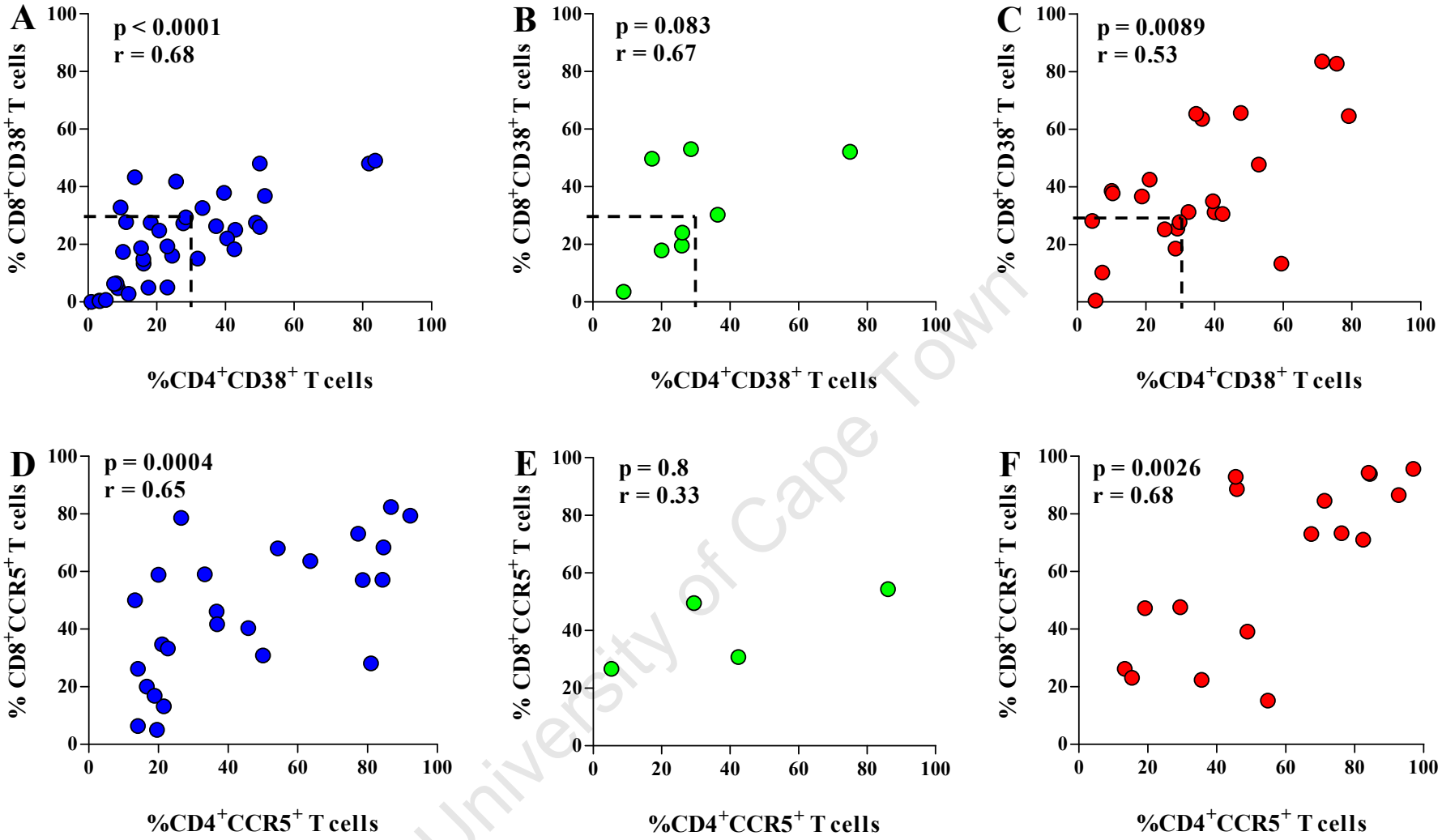


Figure 3.10 Association of activated CD4$^+$ T cells with activated CD8$^+$ T cells in semen. Shown are correlations of (A – C) CD4$^+$CD38$^+$ with CD8$^+$CD38$^+$ T cells and (D – F) CD4$^+$CCR5$^+$ with CD8$^+$CCR5$^+$ T cells of HIV-uninfected [blue circles], HIV-infected (ARV-treated) [green circles] and HIV-infected, ARV-naïve [red circles] men. Each circle represents a matched CD4$^+$ with CD8$^+$ T cell frequency of a participant in semen. Dashed lines indicate 30% frequency. The Spearman rank test was used for statistical analysis and p-values ≤ 0.05 were considered significant.

3.3.7 No association between semen viral load and T cell activation

To investigate the relationship between viral loads and T cell activation, activated T cell subsets were correlated with viral loads in both blood and semen. There were no significant correlations between activated $CD4^+$ or $CD8^+$ T cell subsets or CCR5-expressing cells and seminal and plasma viral loads in either compartment examined (data not shown).

To investigate the influence of viral loads on frequencies of activated T cells further, the viral loads of all HIV-infected men were divided into 3 groups, namely LDL (lower than the detection level), VL < 10^4 (viral load lower than 10 000 HIV-1 RNA copies/ml) and VL > 10^4 (viral load higher than 10 000 HIV-1 RNA copies/ml) [Figure 3.11]. In blood, a significant elevation in the frequency of activated $CD4^+$ T cells in men with viral loads > 10000 HIV-1 RNA copies/ml compared with men with viral loads < 10000 HIV-1 RNA copies/ml was found. No such a difference, however, was seen in semen. In fact, regardless of the concentration of viral load, neither the frequency of $CD4^+$ or $CD8^+$ T cells expressing CD38 differed significantly from each other. In blood, a significant decrease in the median frequency of HIV-1 target cells, $CD4^+CCR5^+$ expression was found in men with viral loads > 10^4 HIV-1 RNA copies/ml compared with men with viral loads < 10^4 HIV-1 RNA copies/ml ($p=0.032$; Figure 3.11A). In semen, the frequency of CCR5 expression did not follow the same pattern as was evident in blood. In fact, frequencies of T cells expressing CCR5 were notably higher, regardless of the level of viral load, in comparison to frequencies in blood.

In summary, although no significant associations were found between levels of activated T cells and viral loads in either compartment, elevated levels of activated $CD4^+$ T cells in blood were found to be associated with viral loads higher than 10 000 HIV-1 RNA copies/ml. Conversely, a decrease in the frequency of CCR5 expressing $CD4^+$ T cells were found to be associated with viral loads higher than 10 000 HIV-1 RNA copies/ml. Interestingly, no differences in activated cells were found in semen at different viral loads, likely indicating an influx of activated cells not linked to the level of virus.

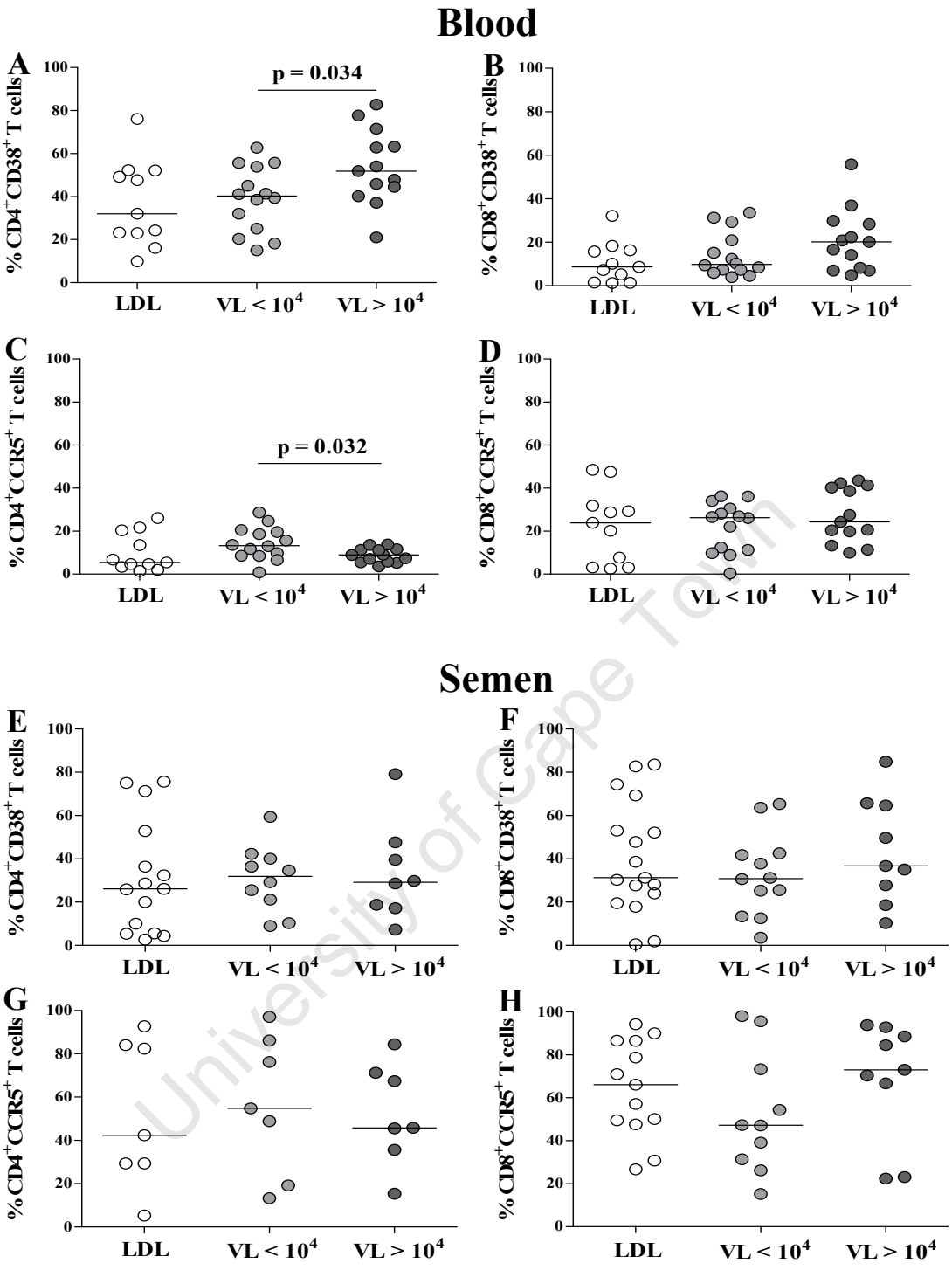


Figure 3.11 Influence of high viral loads on activated T cells in blood and semen. Graphs depict comparisons between men with plasma (A – D) and seminal (E – H) viral loads that are LDL (lower than detectable level), VL < 10^4 (viral load < 10 000 HIV-1 RNA copies/ml), VL > 10^4 (viral load > 10 000 HIV-1 RNA copies/ml) and (A & E) $CD4^+CD38^+$, (B & F) $CD8^+CD38^+$, (C & G) $CD4^+CCR5^+$, (D & H) $CD8^+CCR5^+$ T cells. Wilcoxon signed rank tests were performed and p-values ≤ 0.05 were considered significant.

3.3.8 T cell activation in exposed sero-negative (ESN) men

Within the group of HIV-uninfected men that were included in this study, 21/42 formed part of an established heterosexual discordant couples cohort. To investigate the influence of HIV exposure on T cell activation in exposed sero-negative (ESN) men, the frequencies of activated T cells in blood and semen of HIV-unexposed and ESN men were compared (Figure 3.12). There were no significant differences in either CCR5 or CD38 expression on $CD4^+$ or $CD8^+$ T cells in blood or semen between the two groups (Figure 3.12). In semen, although not significant, there were lower median frequencies of $CCR5^+$ $CD4^+$ and $CD8^+$ T cells in ESN men (Figure 3.12 C and D).

To investigate the impact of viral loads in HIV-infected partners on the frequencies of activated T cells of ESN men, plasma viral loads of HIV-infected partners were correlated with activated T cell subsets in blood and semen (Figure 3.13). In blood, there was a significant positive correlation between the plasma viral load of the infected partner and CCR5-expressing $CD8^+$ T cells ($p=0.028$, $r=063$; Figure 3.13B). A weak significant inverse correlation was observed when comparing frequencies of CD38-expressing $CD4^+$ T cells with plasma viral loads of the infected partners ($p=0.041$, $r=-0.59$; Figure 3.13C). There were no significant correlations between plasma viral loads of the infected partners and $CD4^+CCR5^+$ and $CD8^+CD38^+$ T cells (Figure 3.13A & D). When the same analysis was performed in semen, no significant correlations were evident (data not shown).

Thus, in semen there was a trend toward lower frequencies of CCR5 expressing $CD4^+$ and $CD8^+$ T cells in ESN men. Furthermore, in ESN men there was no relationship between plasma viral loads of the infected partners and activated T cells in semen. However, in the blood of ESN men, higher frequencies of CCR5-expressing $CD8^+$ T cells were related to higher viral replication in the plasma of the infected partner.

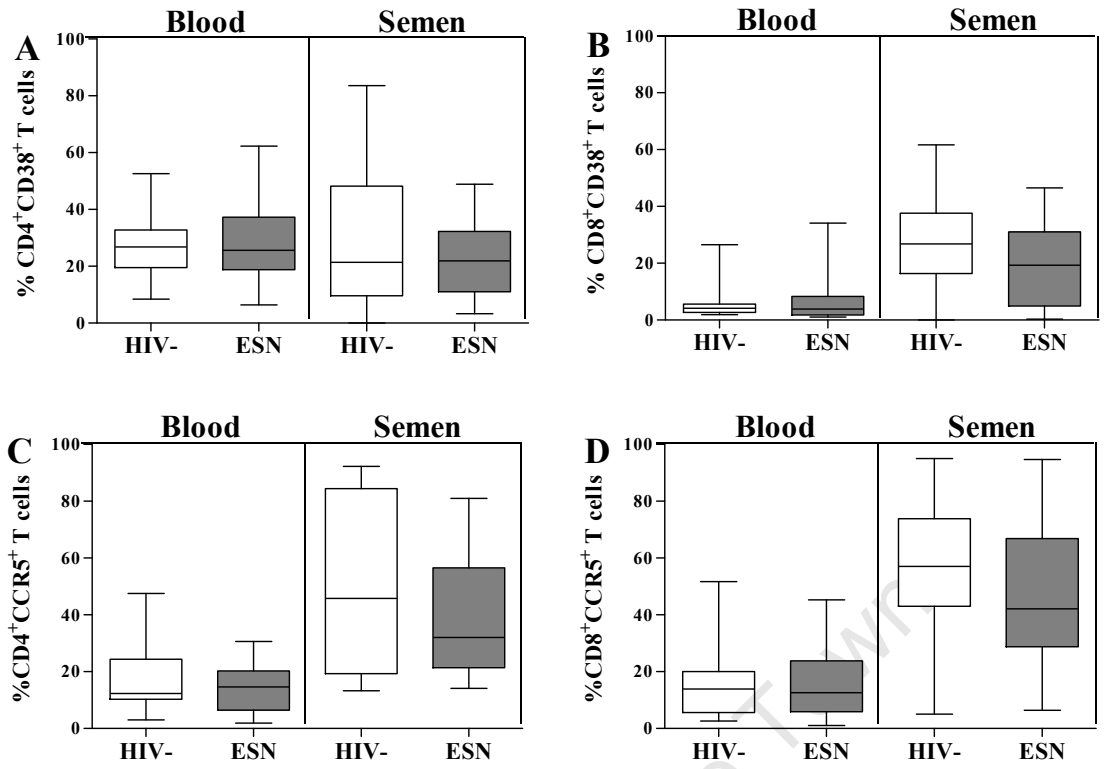


Figure 3.12 T cell activation in blood and semen of HIV-uninfected and exposed sero-negative men. Shown are comparisons of frequencies of (A) $CD4^+CD38^+$, (B) $CD8^+CD38^+$, (C) $CD4^+CCR5^+$, (D) $CD8^+CCR5^+$ T cells in blood and semen of HIV-unexposed (HIV-; unfilled bar) and exposed sero-negative (ESN) men (filled bars). Box-and-whisker plots show the median, IQR and 5-95% range (error bars). Mann-Whitney U tests were performed to detect significant differences between groups.

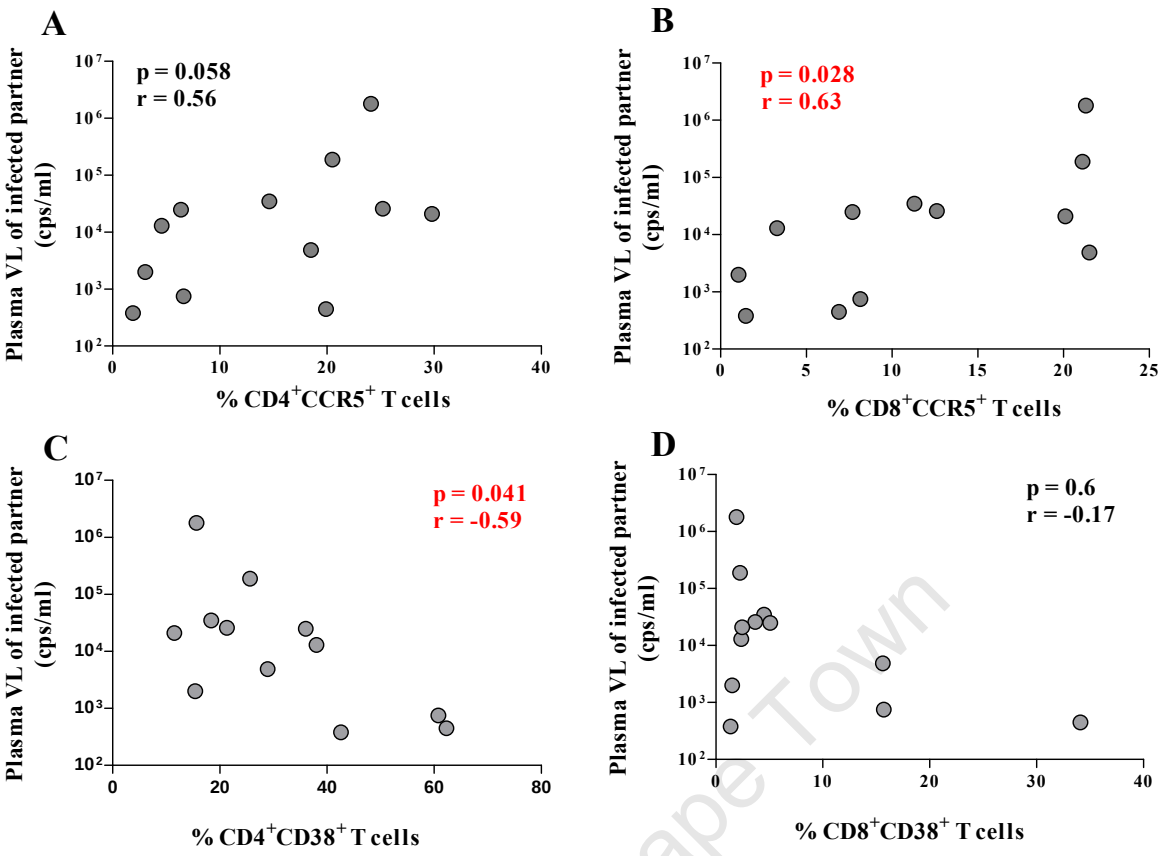


Figure 3.13 Impact of viral load on exposed sero-negative (ESN) men. Shown are correlations between plasma viral load of the infected partner and frequencies of (A) $CD4^+CCR5^+$, (B) $CD8^+CCR5^+$, (C) $CD4^+CD38^+$, (D) $CD8^+CD38^+$ T cells. Each circle represents a plasma viral load of the infected partner with the concomitant frequency of the relevant activated T cells in the ESN men. Spearman test was used and p-values < 0.05 were considered significant. Spearman Rho score is shown on each plot.

3.4 Discussion

HIV infection weakens and ultimately destroys the immune system by CD4 T cell depletion, and hyperactivation of the immune system contributes to this, in part by providing a steady supply of target cells for the virus to infect. In this study, immune activation and HIV replication were investigated in the male genital tract, using semen, and compared between HIV-uninfected and infected men, including a subset on ARV therapy. The major findings were as follows: (i) HIV replication was detected in the genital tract of the majority of HIV-infected (ARV-naïve) men studied. Although viral loads in semen were substantially lower than in plasma, there was a positive correlation between viral loads in the two compartments. ARVs successfully suppressed viral replication in the male genital tract in the majority of men on treatment; (ii) There was substantially greater immune activation in semen compared to blood, with semen containing greater numbers of activated CD4 and CD8 T cells, and CD4 CCR5-expressing T cells, regardless of HIV infection or treatment; (iii) The highest levels of immune activation in semen were found in HIV-infected (ARV-naïve) men, and this was mirrored in blood. Notably, although ARV-treatment reduced the numbers of activated T cells in semen, frequencies were not restored to those observed in HIV-uninfected men; (iv) Although frequencies of activated $CD8^+$ T cells correlated between compartments in HIV-infected men, there were no associations between immune activation and viral loads; (v) Finally, HIV-uninfected men in partnerships with HIV-infected women had elevated frequencies of $CD8^+CCR5^+$ T cells in their blood, but no differences were seen in the genital tract.

HIV was detected in the genital tract of 73% of infected (ARV-naïve) men in this study. A significant positive association was found between viral loads in blood plasma and semen. Previously, a number of studies have confirmed a correlation between viral load in blood and semen (Coombs *et al.,* 1998, Vernazza *et al.,* 1997, Bourlet *et al.,* 2001, Sheth *et al.,* 2004). These findings imply that there is movement of virus between blood and the genital tract. It has been shown that the male genital tract is a source for distinct seminal viral populations from blood (Smith *et al.,* 2007, Pillai *et al.,* 2005, Diem *et al.,* 2008), and a recent study by Anderson and colleagues suggested that these distinct viral populations are possibly supplemented by direct import of both infected $CD4^+$ T cells as well as virus from blood (Anderson *et al.,* 2010). It was also found that semen of HIV-infected (ARV-naïve) men contained

approximately 10-fold lower levels of detectable virus compared to plasma viral loads. This confirms the findings of a number of studies showing that HIV is less concentrated in semen than in blood plasma, with differences often exceeding 1 log (Dyer *et al.,* 1998, Pilcher *et al.,* 2007, Vernazza *et al.,* 1997, Stekler *et al.,* 2008).

While successful suppression of HIV in semen was evident in the majority of men on ARV treatment, HIV replication was found in 2/12 men. Both of these individuals had successful viral suppression in blood, and one individual's seminal viral load was 60200 copies/ml, third highest among HIV-infected men. Studies have shown that despite ARV-treatment, genital HIV shedding is evident in the genital tract of both men and women (Kaul *et al.,* 2008, Sheth *et al.,* 2009, Marcelin *et al.,* 2008). There are several factors that may contribute to suboptimal viral suppression in the genital tract. These include drug potencies, absorption, drug penetration (Kashuba *et al.,* 1999, Eron *et al.,* 2000) and non-adherence to drug regimens. Effective drug penetrance into the genital compartment has largely been found to be dependent on the ARV regimen used. In the female genital tract, studies have shown that nucleoside reverse transcriptase inhibitors (NRTIs) penetrate more efficiently than either non-nucleoside reverse transcriptase inhibitors (nNRTIs) or protease inhibitors (PIs)[Kwara *et al.,* 2008, Dumond *et al.,* 2007]. In semen, the ineffective penetrance of certain nNRTIs, such as efavirenz (Taylor *et al.,* 2001), highlights the necessity for drugs to achieve therapeutic concentrations within the male genital tract where they may potentially control local viral replication and thus subsequent transmission. In South Africa, the recommended first-line treatment consists of the NRTIs lamivudine and stavudine in combination with the nNRTIs efavirenz or nevirapine (Fairall *et al.,* 2008). If the two men that were shedding virus in semen were on first-line treatment that included efavirenz, it may explain the persistence of viral replication in the genital tracts of these men.

In this study, higher levels of seminal HIV were significantly associated with lower plasma CD4 counts. Although not significant, higher plasma HIV levels were associated with lower plasma CD4 counts as well. Similar results were found by Pilcher *et al.,* (Pilcher *et al.,* 2007). Recent studies have shown that ARVs are able to restore depleted $CD4^+$ T cells in the semen of ARV-treated men as well as at the cervix of ARV-treated women (Politch *et al,* 2009, Mkhize *et al.,* 2010). In the present study, the number of $CD4^+$ T cells in the semen of ARV-treated men was more than two-fold

lower than untreated men. Since the time on ARV treatment was not known, the reason for poor $CD4^+$ T cell recovery in semen remains speculative. Poor recovery could have been due to insufficient duration on ARV-treatment. This seems likely, since the median blood CD4 counts of the ARV-treated men at sampling (264 cells/mm^3) had not improved substantially over enrolment (212 cells/mm^3). The number of $CD8^+$ T cells was 1.5-fold higher in HIV-infected men compared to uninfected men. This may be due to an influx of $CD8^+$ T cells or depletion of $CD4^+$ T cells or a combination of both in the HIV-infected male genital tract. This was not related to local levels of viral replication. This observation was also made recently in the HIV-infected female genital tract (Nkwanyana *et al.,* 2009).

Previous studies have shown that HIV-infected individuals have significantly elevated levels of CD38 expression on both $CD4^+$ and $CD8^+$ T cells in blood (Hunt *et al.,* 2003, Eggena *et al.,* 2005, Benito *et al.,* 2004, Almeida *et al.,* 2007, Restrepo *et al.,* 2010) and in the genital tract of HIV-infected women (Biasin *et al.,* 2000). $CD4^+$ T cells expressing CCR5 are the main targets for HIV infection (Brenchley *et al.,* 2004, Mehandru *et al.,* 2004, Guadalupe *et al.,* 2003). Immune activation exacerbates the depletion of target cells in HIV infection by promoting surface expression of CCR5 (Wu *et al.,* 1997, Bleul *et al.,* 1997). Since the genital tract is an effector site, higher levels of CCR5-expressing T cells are to be expected, and CCR5-expressing cells have been shown to be present in high numbers in the genital tract of female SIV-infected macaques (Veazey *et al,* 2003) and in the genital mucosa of women (Hladik *et al.,* 1999). Indeed, higher levels of $CCR5^+$ $CD4^+$ and $CD8^+$ T cells in the genital tract of both HIV-infected and uninfected men were found, compared to numbers of these cells in blood. There were also significantly higher frequencies of $CD8^+CD38^+$ T cells in the semen of HIV-infected compared to uninfected men. CD38 up-regulation on $CD8^+$ T cells was previously reported in the semen of HIV-infected men (Lo Caputo *et al.,* 2003). Furthermore, frequencies of this subset of activated T cells were found to be correlated between blood and semen of HIV-infected men, suggesting that systemic immune activation may be used to predict levels in the genital tract. Interestingly, whilst frequencies of activated $CD8^+$ T cells in semen were significantly elevated in HIV-infection, frequencies of these cells, as well as CCR5-expressing $CD4^+$ T and $CD8^+$ T cells, were also significantly higher in the semen of HIV-uninfected men compared to blood. This implies a high degree of genital tract immune activation in HIV-uninfected men. Men in this study were not screened at the time of sampling for

STIs other than HIV, and the possibility of concurrent sub-clinical STIs elevating activation levels in the genital tract may be quite likely. This could lead to activation of resident genital T cells, as well as the recruitment of systemic T cells.

In individuals on ARV treatment, a rapid reduction in T cell activation in blood has been demonstrated (Giorgi *et al.,* 1998). However, T cell activation may persist in a proportion of individuals at levels higher than in healthy individuals despite years of ARV treatment (Hunt *et al.,* 2003). In the present study, although the ARV-treated subset was small, a reduction in activation levels to those of uninfected individuals was not achieved in either semen or blood, supporting previous findings that ARV treatment does not necessarily reduce levels of T cell activation to baseline levels. Yet, in the semen of the majority of men, ARV-treatment effectively suppressed viral replication. Furthermore, in the present study, there were no associations found between seminal viral load and either activated T cells or CCR5-expressing T cells. Although several studies showed distinct associations between immune activation and viral loads (Eggena *et al.,* 2005, Chun *et al.,* 2004), these studies were conducted in plasma only. Therefore, if viral replication does not necessarily drive immune activation in the male genital tract, what are the possible factors involved? Hunt and colleagues speculated that elevated immune activation despite suppressive ARV treatment may be due to ongoing antigenic stimulation from either low-level HIV replication, the presence of other infections or due to persistence of immune damage, such as gut damage leading to microbial translocation and immune activation (Hunt *et al.,* 2003). Although that study was performed in blood, the same findings may be relevant to the present study. In the semen of HIV-infected men, recurring STIs are even more likely to contribute or even drive immune activation, thereby masking the effects of local HIV replication, resulting in no discernable associations between immune activation and seminal viral loads. Since the male genital tract is a mucosal site, lack of any correlations with seminal viral loads may be because of high 'background noise' levels of $CD38^+$ T cells and CCR5 expression due to effector site homing, obscuring any direct relationship with viral load.

Recent findings from our laboratory have demonstrated a significant increase in plasma and cervical viral loads in HIV-concordant (HIV-infected couple) women when compared to HIV-discordant (HIV-uninfected partner) women was found (Jaumdally *et al.,* unpublished data). The present study showed that there were no significant

differences in viral loads between HIV-concordant and HIV-discordant men in either blood or semen. In the HIV-uninfected group of men, half of them were in HIV-discordant relationships, and were termed HIV-exposed seronegative (ESN) individuals. Whilst the majority of studies on ESN have focused on blood, one study demonstrated that frequencies of activated $CD8^+$ T cells in the semen of ESN men were significantly elevated above levels in the semen of healthy men (Lo Caputo *et al.,* 2003). Frequencies of CCR5 expression in the genital tract of ESN women were significantly enhanced above levels of activated $CD8^+$ T cells compared to healthy controls (Biasin *et al.,* 2000). A previous study also found significant correlations between activated $CD4^+$ and $CD8^+$ T cells in blood of ESN men and plasma viral loads in HIV-infected female partners were shown (Suy *et al.,* 2007). In the present study, no significant differences in seminal activation levels between ESN and unexposed men were found. The presence of HIV-specific $CD8^+$ T cells in ESN men has also been reported, and may be indicative of HIV exposure as a result of an unproductive infection leading to systemic specific immunity (Lo Caputo *et al.,* 2003). Although HIV-specific T cells were not enumerated in the present study, it was found that numbers of CCR5-expressing $CD8^+$ T cells in the blood of ESN men were significantly positively correlated with viral loads of their infected female partners, implying that there may be some level of exposure affecting up-regulation of CCR5 expression on T cells in blood. This finding is interesting and deserves further study in a larger, well-characterised cohort. A limitation of our study was that data on high risk exposures within discordant couples were not available for the men included in the study. At baseline, in the cohort as a whole, 64% of men reported using condoms with their regular partners, however, no data was available on the frequency of this condom usage.

This study had several limitations. At the time of semen donation, no STI screening was performed on the participants. The presence of undiagnosed STIs could have an enormous impact on immune activation and CCR5 expression on T cells in the genital tract, and indeed, high T cell activation in the semen of HIV-uninfected men provides evidence for this. In the HIV-infected men, the time of infection was also unknown. This is important if levels of seminal viral loads are to be compared with other studies. Furthermore, within the ARV-treated subgroup, the duration of treatment was not known. This study was a cross-sectional one, and longitudinal data would be ideal to assess the effects of ARV treatment on seminal levels of immune activation. Also,

only in two of the ARV-treated men were ARV regimens known. To properly assess the risk of HIV transmission from the male genital tract, the ARV regimen in seminal viral shedders would be useful to know. On the technical front, only a single activation marker (CD38) and CCR5 expression was assayed, since this study was limited to performing four-colour flow cytometry. Incorporation of additional markers such as Ki67 and HLA-DR may be useful in further characterising immune activation, using polychromatic flow cytometry. In addition, despite applying an optimised method, the limited numbers of T cells obtained from semen could lead to possible inaccuracies in assessing CD38 and CCR5 frequencies expressed on T cells; the minimum event number cut-off established for this study led to the exclusion of up to 40% of the samples from analysis, depending on the subset and marker studied.

In summary, this study identified a high degree of immune activation in semen, regardless of HIV infection. CCR5-expressing $CD4^+$ targets were abundant in HIV-uninfected men, which have implications for HIV acquisition. In addition, 3/4 of the HIV-infected untreated men in the cohort were shedding virus, which has implications for transmission. No direct relationship between T cell activation in the genital tract of HIV-infected men and viral replication was observed. This finding, together with the high degree of T cell activation in the semen of HIV-uninfected men implies that there may be ongoing inflammation due to other causes resulting in immune activation, which is investigated in the following chapter.

CHAPTER 4

Characterisation of the cytokine and chemokine milieu in semen of HIV-infected and uninfected men

Table of Contents

4.1 Introduction

Cells of the innate and adaptive immune response, as well as other non-hematopoeitic cells, secrete cytokines and chemokines in response to infection (Hofmann *et al.,* 2002). Cytokines mediate a host of immune functions, while chemokines are chiefly responsible for trafficking and recruitment of leukocytes to sites of inflammation (Glass *et al,* 2003). Unsurprisingly, several studies have described differing levels of cytokines and chemokines in plasma of HIV-infected individuals compared to those uninfected. Elevated plasma levels of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-8 (Clouse *et al.,* 1991; Herbein *et al.,* 1995, Herbein *et al.,* 1994, Karsten *et al.,* 1996, Olivetta *et al.,* 2003) as well as chemokines such as MIP-1α, MIP-1β and RANTES have been demonstrated in HIV-infected individuals (Olivetta *et al.,* 2003, Canque *et al.,* 1996, Muthumani *et al.,* 2000, Cremer, Vieillard & De Maeyer 2000, Kim *et al.,* 1999). Stacey and colleagues (Stacey *et al.,* 2009) recently described a plasma cytokine 'storm' in acute HIV infection, which was strikingly more intense compared with that induced by HCV or HBV infection. Early elevated levels of MCP-1, TNFα, IL-8, IL-6, IL-18 and IFNγ described in this study may detrimentally affect disease outcome in HIV infection. In a comprehensive study examining 30 cytokines, our group (Roberts *et al.,* 2010) identified a range of plasma cytokines during acute HIV infection that were associated with higher viral set points or greater CD4 loss, namely IL-7, IL-15, IL-1α and eotaxin.

In the genital tract of vaginally SIV-infected macaques, pro-inflammatory cytokines were induced early on in infection, positively correlating with viral replication likely due to the recruitment of target cells (Abel *et al.,* 2005). In the genital tract of women, previous studies have associated HIV infection with increased inflammation at the cervix (Gumbi *et al.,* 2008, Belec *et al.,* 1995, Thea *et al.,* 1996, Zara *et al.,* 2004). HIV-infected women had upregulated IL-6, IL-10, IL-12, IFNγ and TNFα in the cervix as well as in plasma (Biasin *et al.,* 2000). In addition, IL-1β, IL-8 and RANTES were elevated (Iqbal *et al.,* 2005, Nkwanyana *et al.,* 2009), while MIP-1α levels were significantly lower at the cervix of HIV-infected compared to uninfected women (Iqbal *et al.,* 2005). During acute HIV infection, our group found elevated levels of IL-6, IL-8 and IL-12 in the female genital tract (Bebell *et al.,* 2008). Interestingly, this study

suggested that greater mucosal inflammation resulted in higher CD4 loss. Whilst different studies have measured different cytokines using a range of technologies with differing sensitivities, it does appear from multiple studies that HIV induces higher levels of pro-inflammatory cytokines in the female genital tract.

Fewer studies have comprehensively characterised the immune milieu of the male genital tract in the context of HIV infection. In healthy, fertile men, examination of 23 cytokines and other mediators of inflammation in semen indicated a broad array of cytokines, with TGFβ, IL-7, SDF-1α, MCP-1 and IL-8 present at high concentrations (Politch *et al.,* 2007). Using a comprehensive approach similar to what this chapter will describe, semen levels of IL-1β, IL-4, IL-6, IL-7, IL-8, GM-CSF and MCP-1 were all shown to be up-regulated in HIV-infection (Anderson *et al.,* 2010). A few studies have shown positive associations between seminal viral load and increased levels of IL-1β and RANTES (Storey *et al.,* 1999, Berlier *et al.,* 2006a). These results imply that elevated levels of pro-inflammatory mediators in the male genital tract during HIV infection may be as a result of local viral replication, which in turn exacerbate the activation of potential target cells.

This study describes a comprehensive investigation of the cytokines and chemokines present in the semen of HIV-infected and uninfected men, using multiplex technology. Cytokine/chemokine levels were compared to those in blood in order to identify differences between compartments. The influence of ARV treatment on cytokine/chemokine levels was also investigated. Since many immune regulators act in networks, how HIV infection might influence or disrupt the relationships between cytokines was examined. Using data described in Chapter 3, the relationship between levels of cytokines and chemokines and viral loads as well as cellular activation was assessed. The relationship between viral replication, T cell activation and genital tract inflammation is discussed, as well as the implications of these findings for HIV transmission and acquisition.

4.2 Materials and Methods

4.2.1 Study participants and sample collection

The cohort of study participants used to generate the data in this chapter is the same as that described in Chapter 3. Briefly, this consisted of 26 ARV-naïve and 12 ARV-treated HIV-infected men, along with 28 HIV-uninfected men, from the Empilisweni Clinic in Athlone, Cape Town, South Africa. Ejaculates were collected in sterile specimen jars following voluntary self masturbation, and blood was collected in ACD Vacutainer tubes. After sample processing, seminal fluid and and blood plasma were stored at -80°C.

4.2.2 Measurement of cytokines and chemokines

Twenty-one cytokines and chemokines were measured in seminal and blood plasma from HIV-uninfected and HIV-infected men using High Sensitivity Human and Human Cytokine Milliplex® MAP kits (Millipore Corporation, St. Charles, Missouri, USA). The following analytes were measured with the High Sensitivity Human Cytokine kit: Interleukin (IL)-1β, IL-2, IL-6, IL-7, IL-12p70, Granulocyte-macrophage (GM)-CSF, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The sensitivity of this kit ranged between 0.05 and 0.46 pg/ml for each of the cytokines measured (Table S1). The following analytes were measured with the Human Cytokine kit: IL-1α, IL-8, IL-12p40, IL-15, Eotaxin, Fractalkine, Granulocyte colony stimulating factor (G-CSF), Monocyte chemotactic protein (MCP)-1, Macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES. The sensitivity of this kit ranged between 0.2 and 10.5 pg/ml for each of the cytokines measured (Table S1). Samples were thawed and filtered by centrifugation using 0.2 μm cellulose acetate filters (Sigma, USA) prior to cytokine/chemokine measurements. For each kit, two plates were used, with all seminal plasma samples (undiluted) assayed on a single plate, and matching blood plasma samples on a separate plate. To monitor variation between plates, quality controls, included with each kit, were used. To control for backgrounds, assay buffer alone was used and values subtracted from all readings. Each assay was performed on separate days, while all quality controls and background values were within acceptable ranges. Data were collected using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories Inc., Hercules, California, USA) in the laboratory of Prof Gerhard Walzl, University of Stellenbosch. A 5 PL regression formula was used to calculate sample concentrations from the standard curves. Data was analysed using BIO-plex manager

software (version 4). Cytokine concentrations below the lower limits of detection were reported as the midpoint between the lowest concentration and zero for each cytokine measured, as described previously (Bebell *et al.,* 2008). IL-1α was undetectable in blood plasma samples and was subsequently excluded from any comparative analysis between semen and blood.

4.2.3 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) and STATA™ version 10 (StataCorp, Texas, USA). Mann Whitney U test was used for unmatched comparisons. Wilcoxon Signed Rank test was used to compare cytokine levels in men matched semen and blood. Non-parametric Spearman correlation was used for correlations. Linear regression was used to evaluate the relationships between cytokine levels and viral load in blood and semen, and activated T cell subsets. Multivariate regression was used to adjust for the use of ARVs and viral loads. P-values ≤ 0.05 were considered significant. For comparisons of frequencies of cytokines/chemokines between blood and semen, semen:blood ratios, HIV-uninfected, ARV naïve and ARV-treated men, as well as all correlations of cytokines/chemokines between each other within each compartment, P-values were adjusted using the False Discovery Rate (FDR) step-down procedure in order to reduce false positive results when multiple comparisons were made (Columb & Sagadai 2006).

Principal component analysis (PCA) was used to determine whether all the variables (in this case the cytokines and chemokines) in three groups (categorized according to whether there was a detectable viral load or not) clustered in these groups. It is a useful tool for reducing the number of observed variables to a smaller dataset that account for most of the variance in the larger dataset. Hierarchical cluster heat maps were used to visualise the concentrations of the cytokines and chemokines within groups. The analyte concentration level is generally indicated by red for high expression and green for low expression. Relationships between analytes are represented by a tree whose branch lengths reflect the degree of similarity between them, as is assessed by a pairwise similarity function. In supervised clustering, analytes are classified with respect to known reference vectors, whereas in unsupervised clustering, no predefined reference vectors are used (Eisen *et al.,* 1998). In the present study, unsupervised clustering was performed.

4.3 Results

4.3.1 Detection of cytokines and chemokines in semen and blood

Twenty pro-inflammatory, regulatory and adaptive cytokines and chemokines were measured in paired blood and semen samples in 28 HIV-uninfected and 38 HIV-infected men, of whom 12 of the latter were on ARV treatment. Descriptive statistics are provided for cytokines and chemokines assayed in seminal plasma (Table 4.1) and blood plasma (Table 4.2), and the frequencies of detection and concentrations of the 20 analytes are represented graphically in Figure 4.1A and B. Of the 20 cytokines and chemokines investigated in the study, 14 were classified as pro-inflammatory (IL-1β, IL-6, Il-8, IL-12p40, IL-12p70, TNFα, Eotaxin, Fractalkine, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β and RANTES). IL-10 was classified as regulatory, whereas IFNγ, IL-2, IL-7 and IL-15 were classified as adaptive cytokines based on their function. Overall, for the set of cytokines and chemokines tested, concentrations were higher in semen than in blood for the majority of cytokines (15/20). Analytes were also more frequently detected in semen compared to blood (96% vs 81% detectable observations). On the whole, and somewhat surprisingly, median concentrations of cytokines and chemokines were generally comparable in semen between HIV-infected and uninfected groups, and the same was true for blood (Fig 4.1C and D). This indicates a homogeneity within compartments that is relatively conserved, regardless of HIV infection.

In seminal plasma, all 20 cytokines and chemokines assayed were detectable in 46% of samples, whereas 10 out of 20 cytokines were detectable in 93% of samples (Table 4.1). Six analytes were detected in all samples, namely MCP-1, RANTES, Fractalkine, G-CSF, IL-15 and TNF-α. MCP-1 was present in the highest concentrations in the seminal plasma of all groups, at a median of 14 110 pg/ml in HIV-uninfected men, 9805 pg/ml in HIV-infected men, and 17 720 pg/ml in HIV-infected ARV-treated men (Table 4.1 and Figure 4.1C). In addition, one cytokine and three chemokines were detected in high concentrations in all three groups: IL-8 (median 1141 pg/ml), Fractalkine (median 802 pg/ml), RANTES (median 370 pg/ml) and IL-7 (median 715 pg/ml). IL-1α, IL-6, Eotaxin, MIP-1β, G-CSF, IL-10 and IL-15 were detected in all

Table 4.1 Descriptive statistics of cytokines and chemokines in seminal plasma of HIV-uninfected, HIV-infected and ARV-treated men.

Cytokines/chemokines Pro-inflammatory	HIV-uninfected[a]		HIV-infected, ARV-naïve[b]		ARV-treated[c]	
	% Positive	Median (IQR)	% Positive	Median (IQR)	% Positive	Median (IQR)
IL-1α	92	16.3 (5.5 – 32.9)	100	17.2 (5.3 – 79.3)	100	17.4 (6.5 – 82)
IL-1β	86	0.25 (0.08 – 0.4)	81	0.17 (0.05 – 0.69)	75	0.32 (0.044 – 1)
IL-6	96	32.5 (6.8 – 118.3)	100	22 (8.7 – 122)	92	33.4 (7.3 – 147.2)
IL-8	95	1 486 (241 – 2 771)	100	1 110 (643 – 2 115)	100	1 141 (540.5 – 2 947)
IL-12p40	7	2.8 (1.3 – 3.5)	19	1.9 (1.4 – 3.5)	50	3.1 (1.78 – 9.85)
IL-12p70	43	0.05 (0.03 – 1.9)	35	0.036 (0.02 – 0.99)	58	1.2 (0.036 – 2.57)
TNF-α	100	1.82 (0.79 – 4.5)	100	1.2 (0.57 – 3.95)	100	1.88 (0.27 – 5.9)
Eotaxin	62	23 (4.4 – 38.9)	85	16.3 (8.6 – 37.9)	92	28.2 (23.3 – 41.3)
Fractalkine	100	802.9 (333 – 1 636)	100	655.8 (251 – 1 489)	100	875.6 (250 – 1 019)
MCP-1	100	14 110 (3 908 – 31 028)	100	9 805 (3 211 – 18 029)	100	17 720 (7 486 – 27 430)
MIP-1α	22	3.15 (1.3 – 4.9)	54	7.5 (2.3 – 36)	75	15.7 (5.5 – 86.4)
MIP-1β	90	78.1 (31.9 – 112.2)	100	53 (35.4 – 171.6)	100	83.4 (30.2 – 195.8)
RANTES	100	370.9 (189.2 – 649.3)	100	266.5 (98.6 – 818.3)	100	419.5 (178.6 – 1 239)
G-CSF	100	29.5 (12.2 – 60.5)	100	21.2 (7.5 – 84.3)	100	20 (12.9 – 58.1)
GM-CSF	93	2.4 (0.65 – 7.35)	100	2.9 (1.6 – 6.2)	92	5.05 (2.1 – 14.7)
Regulatory						
IL-10	86	27.1 (7.1 – 52.4)	77	10.9 (2.02 – 17.5)	67	7.7 (0.8 – 24.5)
Adaptive						
IFN-γ	89	6.3 (2.7 – 21.9)	96	5.6 (1.9 – 10.2)	92	9.8 (0.78 – 40.5)
IL-2	57	0.4 (0.03 – 2.56)	58	0.2 (0.026 – 1.22)	58	1.6 (0.022 – 3.52)
IL-7	96	786.4 (395.9 – 1 930)	100	715.8 (347 – 1 865)	100	524.4 (414 – 2 011)
IL-15	100	27.8 (8.8 – 45.4)	100	27.6 (10.1 – 64.8)	100	30.7 (27.6 – 39.5)

[a]N = 28; [b]N = 26; [c]N = 12

Table 4.2 Descriptive statistics of cytokines and chemokines in blood plasma of HIV-uninfected, HIV-infected and ARV-treated men.

Cytokines/chemokines	HIV-uninfected[a]		HIV-infected, ARV-naïve[b]		ARV-treated[c]	
Pro-inflammatory	**% Positive**	**Median (IQR)**	**% Positive**	**Median (IQR)**	**% Positive**	**Median (IQR)**
IL-1β	86	0.15 (0.04 – 0.33)	100	0.26 (0.012 – 0.59)	92	0.09 (0.012 – 0.35)
IL-6	100	6.5 (4 – 12.3)	100	6.6 (4 – 14.1)	100	5.5 (3.2 – 9.2)
IL-8	80	2.3 (0.27 – 4.7)	92	2.5 (1.07 – 3.7)	67	2.8 (0.04 – 9.2)
IL-12p40	50	5.4 (0.47 – 44.7)	38	0.47 (0.47 – 46.1)	42	0.47 (0.47 – 3.07)
IL-12p70	29	0.01 (0.01 – 0.17)	38	0.01 (0.01 – 1.16)	17	0.01 (0.01 – 0.01)
TNF-α	100	5.9 (4.6 – 7)	100	10.1 (7.02 – 12.5)	100	7.6 (6.4 – 9.8)
Eotaxin	100	89.1 (37.8 – 182.8)	100	89.2 (59 – 195.1)	100	49.8 (44.2 – 68.3)
Fractalkine	75	42.6 (11.1 – 102.3)	81	42.6 (18.8 – 82.4)	42	0.59 (0.59 – 42.6)
MCP-1	100	216.7 (174.1 – 301.1)	100	221.3 (168.4 – 364.7)	100	220.3 (155.2 – 303.8)
MIP-1α	40	1.95 (1.95 – 9.52)	35	1.95 (1.95 – 7.24)	25	1.95 (1.95 – 5.9)
MIP-1β	68	8.7 (1.5 – 12.7)	69	3.01 (1.5 – 12.7)	50	5.1 (1.5 – 16.1)
RANTES	100	2 809 (1 997 – 3 156)	100	2 663 (2 080 – 3 207)	100	2 637 (2 483 – 3 332)
G-CSF	100	25.8 (15.5 – 47.8)	100	40.02 (24.6 – 53.2)	100	23.3 (15.5 – 30.7)
GM-CSF	43	0.01 (0.01 – 0.86)	62	0.26 (0.01 – 0.69)	50	0.02 (0.01 – 0.69)
Regulatory						
IL-10	100	1.26 (0.88 – 2.02)	100	2.02 (1.26 – 2.8)	100	1.07 (0.88 – 1.5)
Adaptive						
IFN-γ	82	0.86 (0.37 – 2.9)	85	2.4 (0.86 – 7.8)	83	1.21 (0.37 – 1.33)
IL-2	54	0.1 (0.01 – 0.98)	81	0.47 (0.05 – 1.39)	50	0.08 (0.01 – 0.86)
IL-7	86	3.12 (1.27 – 7.44)	100	3.13 (1.5 – 5.26)	100	1.91 (0.54 – 4.9)
IL-15	50	0.37 (0.25 – 1.25)	42	0.25 (0.25 – 1.25)	17	0.25 (0.25 – 0.25)

[a]N = 28; [b]N = 26; [c]N = 12

three groups assayed at concentrations < 100 pg/ml. IL-12p40, TNFα, GM-CSF, MIP-1α and IFNγ were present at < 10 pg/ml, whereas IL-1β, IL-12p70 and IL-2 were present in concentrations < 1 pg/ml in all three groups assayed.

In blood plasma, all 20 cytokines and chemokines assayed were detectable in 41% of samples, whereas half the analytes were detectable in 81% of samples (Table 4.2). RANTES was present in the highest concentrations in the plasma of HIV-uninfected, infected and ARV-treated men (median of 2 809, 2 663 and 2 637 pg/ml, respectively; Figure 4.1D). MCP-1 was detected in moderately high concentrations in all three groups (median 220 pg/ml), although this represented a 5-fold lower concentration than in semen. Eotaxin, Fractalkine, and G-CSF were detected in all three groups at concentrations < 100 pg/ml. IL-6, IL-7, IL-8, IL-10, TNFα, IFNγ, MIP-1α and MIP-1β were present at < 10 pg/ml, whereas IL-1α, IL-1β, IL-2, IL-12p40, IL-12p70, IL-15 and GM-CSF were present in concentrations < 1 pg/ml in all three groups assayed, if at all.

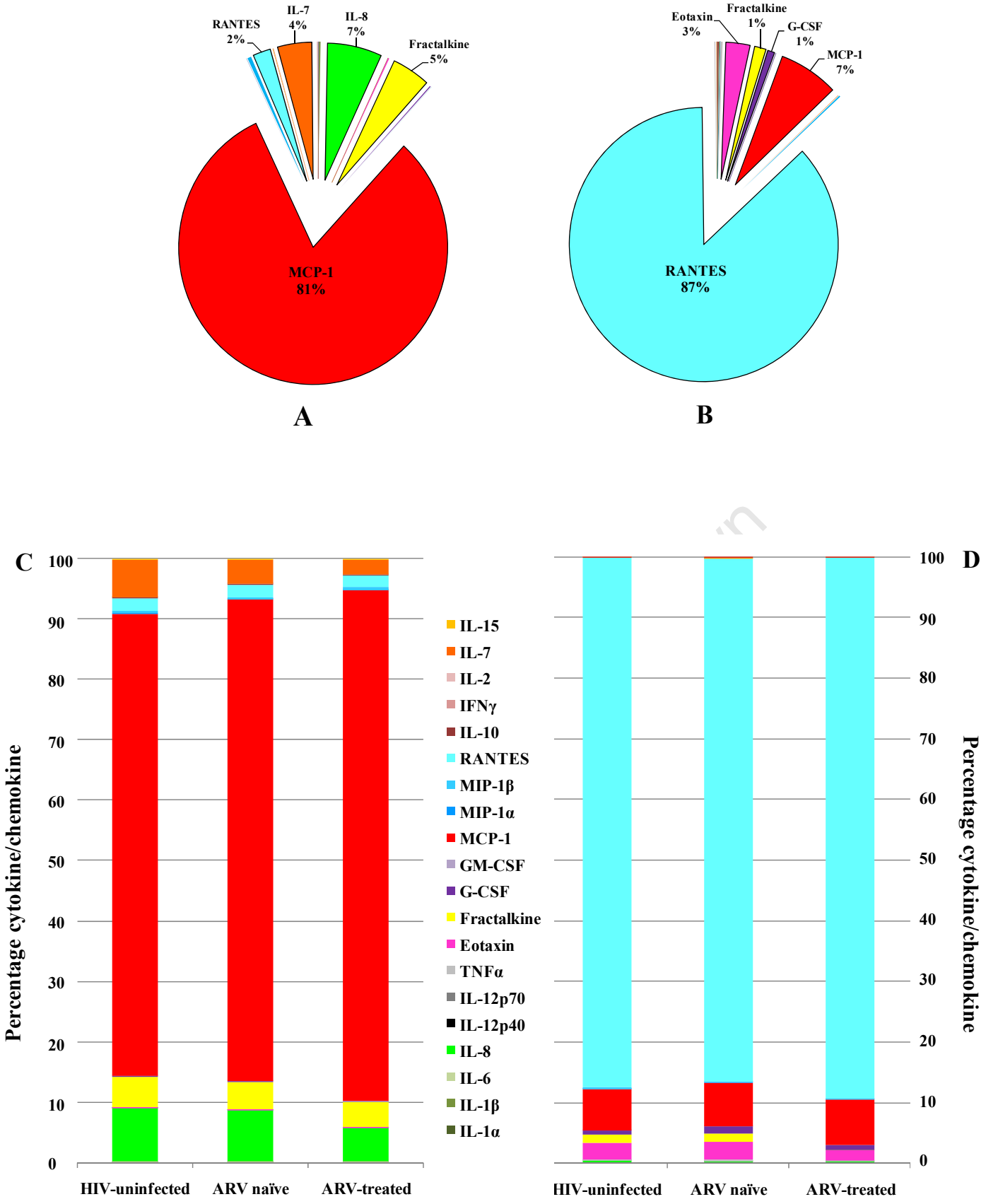


Figure 4.1 Detection of cytokines and chemokines in semen and blood. Frequency of detection of all analytes in (A) semen and (B) blood. A 100% stacked bar graph of median concentrations (pg/ml) of cytokines/chemokines in semen (C) and blood (D) of HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men.

4.3.2 The cytokine and chemokine milieu in semen and blood differ substantially

To assess whether the levels of cytokines and chemokines detected differed between semen and blood, matched comparisons were performed (Table 4.3). In HIV-uninfected men, 14 out of 19 (74%) cytokines/chemokines assayed differed significantly between semen and blood, whereas in HIV-infected men 10 out of 19 (53%) and in ARV-treated men 8 out of 19 (42%) differed significantly between semen and blood. Specifically, in the semen of HIV-uninfected men, concentrations of IL-12p70 and IFNγ were significantly higher than in blood, whereas IL-12p40 concentrations were significantly higher in blood compared to semen of HIV-uninfected men (Table 4.3). Eotaxin and RANTES levels were significantly higher in blood compared to semen of both HIV-uninfected and infected men (Table 4.3). In the semen of HIV-uninfected, infected and ARV-treated men, levels of IL-6, IL-8, Fractalkine, MCP-1, MIP-1β, GM-CSF, IL-7 and IL-15 were significantly higher in semen compared to blood (Table 4.3).

To assess whether seminal plasma has a distinct immunological profile relative to blood plasma, semen:blood ratios of analytes were calculated (Figure 4.2). Ratios at concentrations 5-fold and higher were empirically considered meaningful. A subset of cytokines and chemokines, IL-8, Fractalkine, GM-CSF, MCP-1, MIP-1β, IL-7 and IL-15, were concentrated in the semen of all three groups (HIV-uninfected, HIV-infected and ARV-treated men), with median levels 5 to approximately 600 fold greater than in blood. Conversely, the concentration of RANTES in all three groups was more than 5-fold higher in blood than in semen. Some differences were observed between HIV-infected and uninfected men. In the semen of HIV-uninfected men, IL-6 concentrations were >5 times higher than in blood, while in blood Eotaxin concentrations were >5 times higher than in semen. In the blood of HIV-infected men, TNFα and Eotaxin concentrations were >5 times higher than in semen, whereas in semen of ARV-treated men, IL-12p70 and IFNγ concentrations were >5 times higher than in blood. Although there were several differences in levels of semen:blood ratios between the three groups investigated, controlling for multiple comparisons showed that only IL-10 semen:blood ratios remained significantly higher in HIV-uninfected compared to infected men ($p=0.034$; Figure 4.2).

In summary, the levels of the majority of cytokines and chemokines in semen differed significantly from levels in blood. Furthermore, the composition of the major

detectable cytokines and chemokines in semen were not substantially different between HIV-uninfected and infected men.

Table 4.3 Comparison of cytokine/chemokines between blood and semen of HIV-infected and HIV-uninfected men.

Cytokines/chemokines		HIV-uninfected	HIV-infected	ARV-treated
Pro-inflammatory:	**IL-1β**	ns[a]	ns	ns
	IL-6	0.0038[b]	0.02	0.013
	IL-8	<0.0019	<0.0019	0.0095
	IL-12p40	0.027[c]	ns	ns
	IL-12p70	0.032	ns	ns
	TNF-α	0.046	ns	ns
	Eotaxin	<0.0019	<0.0019	ns
	Fractalkine	<0.0019	<0.0019	0.0095
	MCP-1	<0.0019	<0.0019	0.0095
	MIP-1α	ns	ns	ns
	MIP-1β	<0.0019	<0.0019	0.0095
	RANTES	<0.0019	<0.0019	ns
	G-CSF	ns	ns	ns
	GM-CSF	0.0032	<0.0019	0.023
Regulatory:	**IL-10**	ns	ns	ns
Adaptive:	**IFN-γ**	0.0043	ns	ns
	IL-2	ns	ns	ns
	IL-7	<0.0019	<0.0019	0.0095
	IL-15	<0.0019	<0.0019	0.0095

Wilcoxon matched pair analyis, p-values adjusted for multiple comparisons by false discovery rate (FDR) step-down procedure

[a]ns: non-significant

[b]Red: cytokine/chemokine levels in semen higher than in blood

[c]Blue: cytokine/chemokine levels in blood higher than in semen

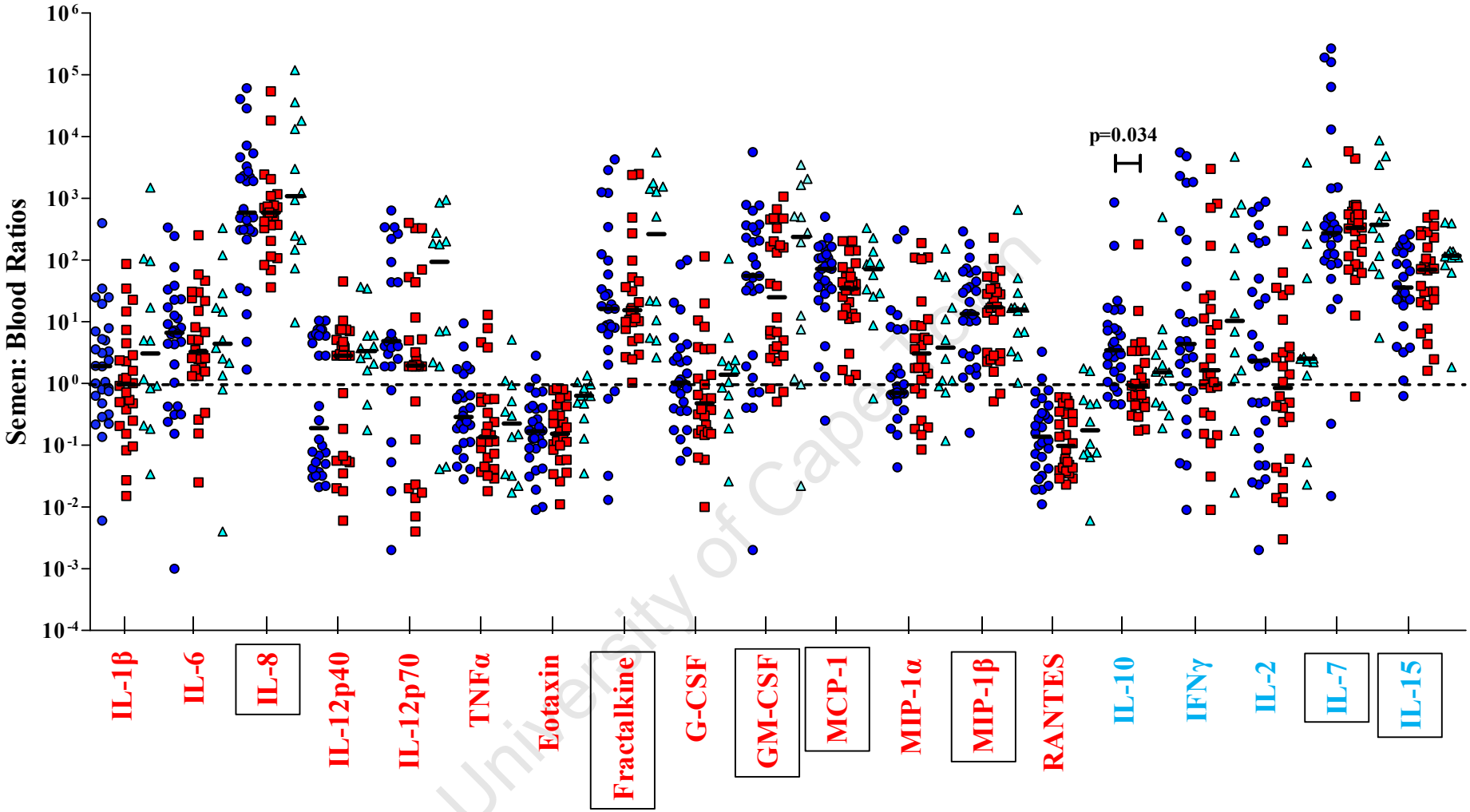


Figure 4.2 Graphic representation of semen:blood ratios of 19 cytokines and chemokines in HIV-uninfected (blue circles) [n = 28], HIV-infected (ARV-naïve) (red squares) [n = 26] and ARV-teated (green triangles) [n = 12] men. The dashed line illustrates an equivalent semen:blood cytokine/chemokine ratio. Boxed cytokines/chemokines have median semen:blood ratios >5 in all three groups. Cytokine/chemokine labels in red are considered pro-inflammatory and those in blue are regulatory/adaptive. Mann-Whitney t tests adjusted for multiple comparisons by false discovery rate (FDR) step-down procedure. p-values ≤ 0.05 was considered significant.

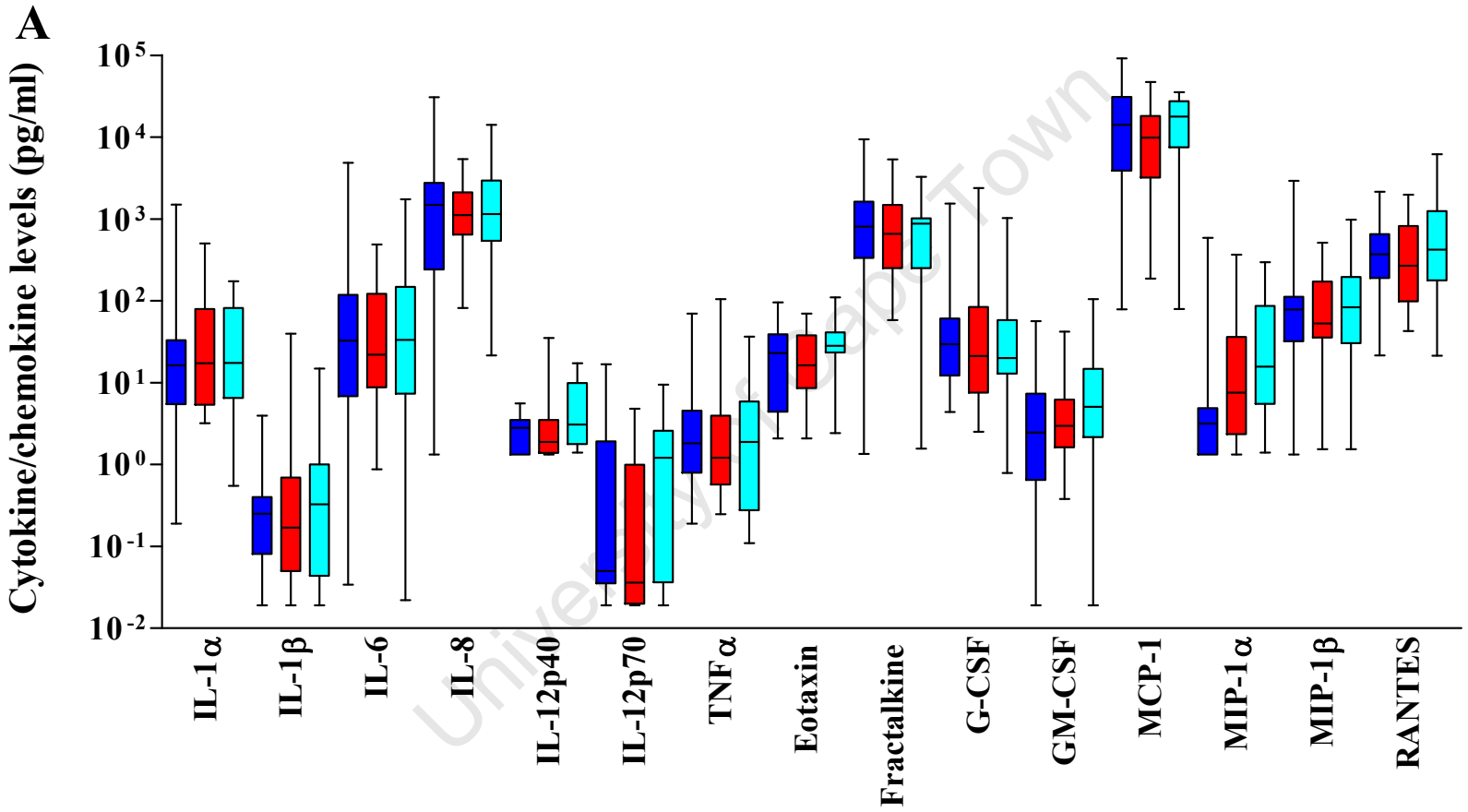
4.3.3 Characterisation of cytokines and chemokines in semen and blood of HIV-uninfected and infected men

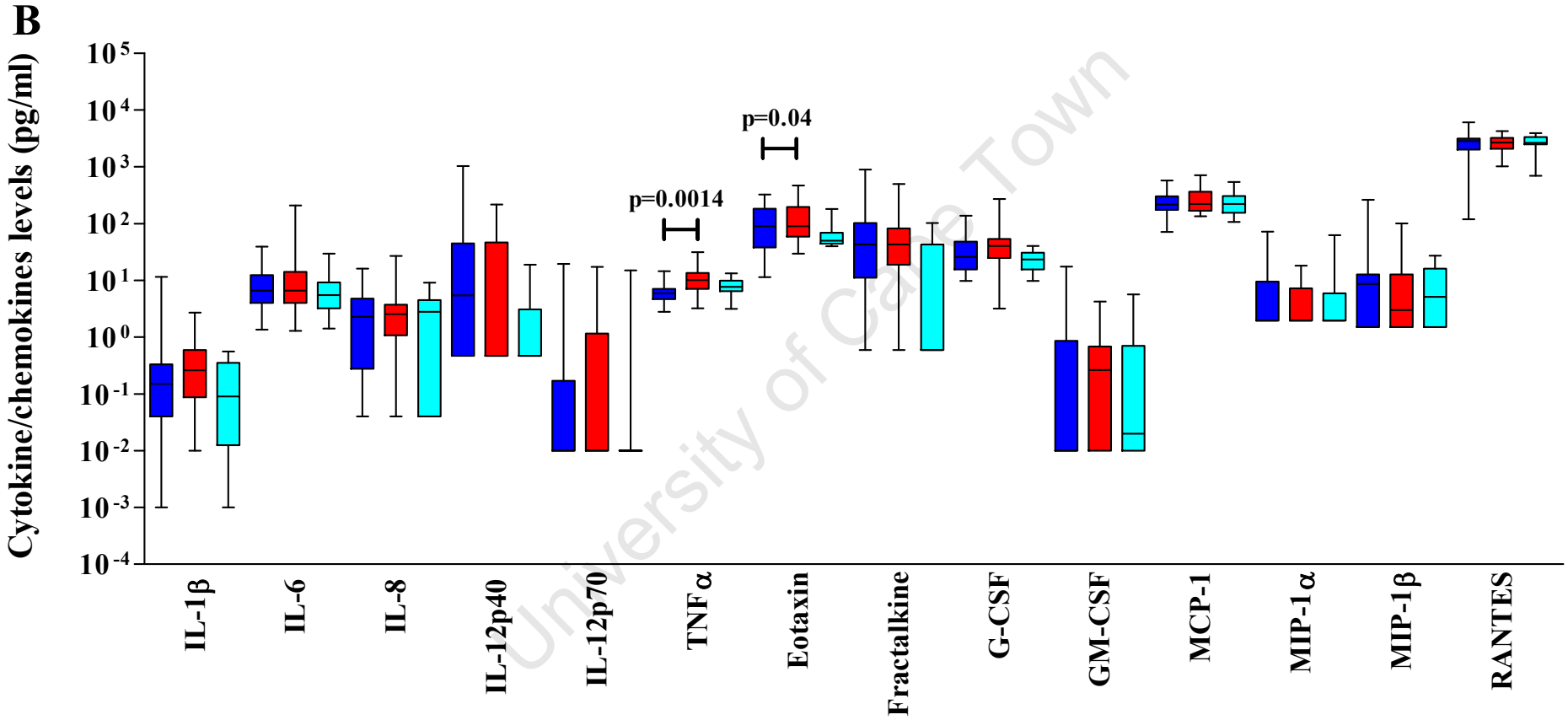
Next, differences in quantities of cytokines/chemokines in semen and blood between HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men were investigated. These data are presented graphically in Figure 4.3, with the indicated p values adjusted for multiple comparisons. The results from univariate analyses are summarised in the Appendix, Table S2 and S3.

Of note, there was a large degree of heterogeneity in the levels of the majority of cytokines and chemokines in semen compared to blood (Figure 4A and C compared to Figure 4B and D). For example, median levels of IL-6 were between 1 and 100 pg/ml in both compartments (Figure 4A and B). Variation in IL-6 levels in blood between individuals were within this range, however this was not the case in semen, where individual variation spanned five orders of magnitude, from less than 10^{-1} to almost 10^{4} pg/ml. Univariate analysis revealed significantly lower levels of the chemokine MIP-1α in the semen of HIV-uninfected men compared to HIV-infected ARV-naïve (p=0.045) and ARV-treated men (p=0.0076; Table S2). In contrast, IL-10 levels were significantly higher in HIV-uninfected compared to HIV-infected (ARV-naïve) men. In blood, both TNFα (p=0.0001) and IL-10 levels (p=0.017) were significantly lower in HIV-uninfected compared to HIV-infected men, whereas Eotaxin (p=0.0087), Fractalkine (p=0.025), G-CSF (p=0.0077), IL-10 (p=0.025) and IFNγ levels (p=0.021) in blood were significantly higher in HIV-infected (ARV-naïve) men compared to those on treatment (Table S3). After adjusting for multiple comparisons, there were no significant differences in levels of cytokines or chemokines in semen between the three groups investigated (Figure 4.3A and C). In blood, however, TNFα and Eotaxin remained significantly higher in HIV-infected (ARV-naïve) compared to HIV-uninfected men (p=0.0014 and p=0.04, respectively; Figure 4.3B). Among regulatory and adaptive cytokines and chemokines, both IL-10 (p=0.042) and IFNγ levels (p=0.05) remained significantly higher in the blood of HIV-infected (ARV-naïve) compared to HIV-infected (ARV-treated) men.

Thus, comparison of the concentrations of 19 cytokines and chemokines in the semen in HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men failed to detect significant differences in any analytes between these groups, after

adjustment for multiple comparisons. The extensive intra-cytokine variation between individuals and the number of cytokines measured are likely causes of this observation.





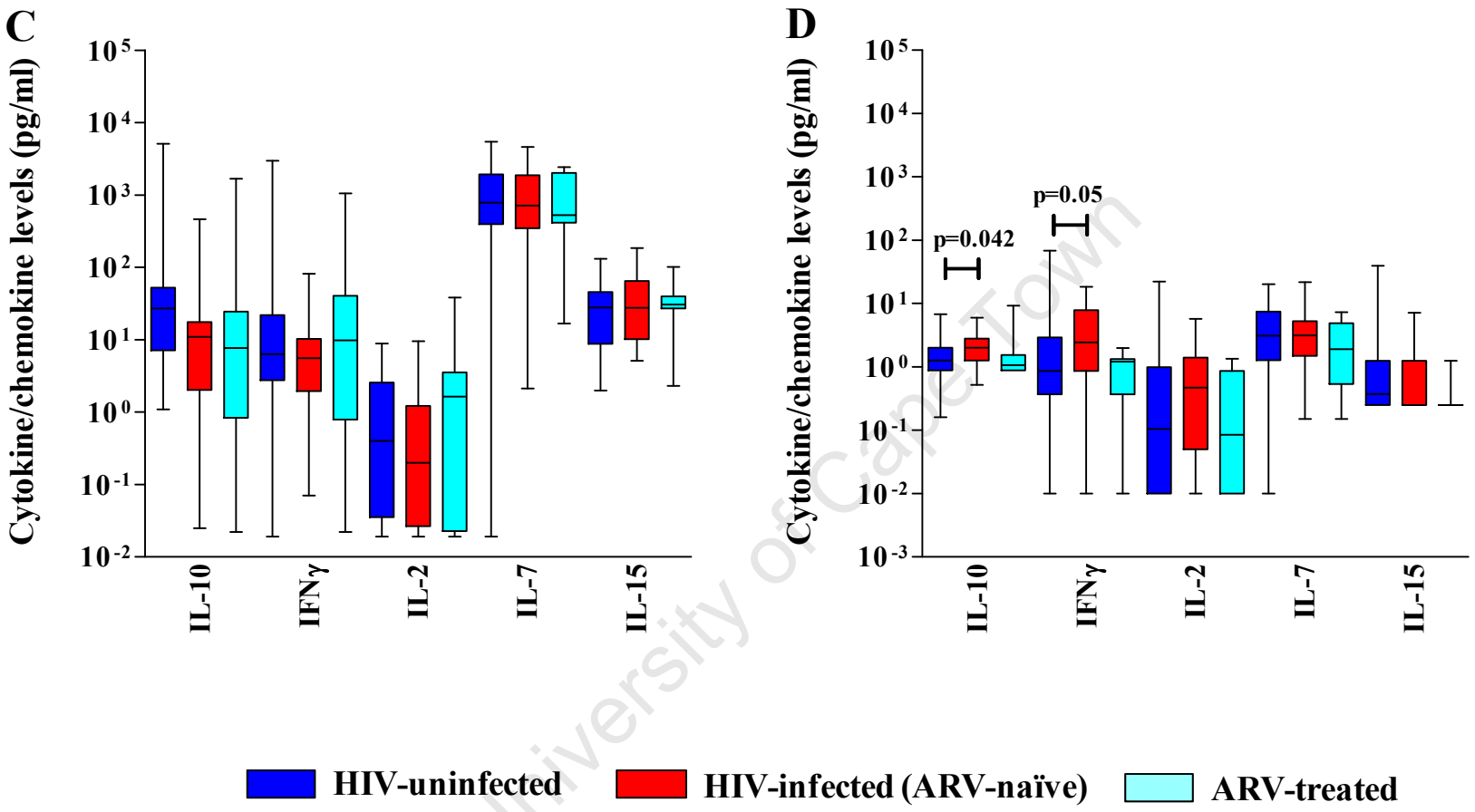


Figure 4.3 Levels of pro-inflammatory mediators in (A) semen and (B) blood and levels of regulatory and adaptive cytokines and chemokines in (C) semen and (D) blood of HIV-uninfected, HIV-infected (ARV-naïve) and ARV-treated men. Box-and-whisker plots reperesents the median, IQR and 5-95% range (error bars). Mann-Whitney t-tests were adjusted for multiple comparisons by the false discovery rate (FDR) step-down procedure. p-values ≤ 0.05 were considered significant.

4.3.4 HIV infection leads to dysregulation of pro-inflammatory and other mediators in the male genital tract

Cytokines and chemokines within the body work in synergy to not only elicit immune responses, but to establish gradients at sites of infection attracting immune cells (Fivenson *et al.,* 1997). Participating cell types, together with the cytokines and chemokines they produce, may form a cytokine network that can be highly disease-specific. To illustrate this interaction between cytokines and chemokines in semen better, 17 cytokines and chemokines assayed in the semen of healthy men were used to design a network using Ingenuity Pathway Analysis software (Figure 4.4). This analysis revealed an intricate network of relationships between the cytokines measured. In this network, NFκB upregulation is influenced by IL-6, IL-8, IL-12p40, IL-12p70, IFNγ and TNFα levels, while upregulation of NFκB in turn results in enhanced production of these pro-inflammatory mediators. Interestingly, both IL-10 and TNFα have an effect on HLA-DR and IL-10, IL-6 and IFNγ influences MHC Class II expression.

To determine whether HIV infection had any impact on relationships in the cytokine network, correlations between cytokines and chemokines in the semen of HIV-uninfected men (Table 4.4) were compared to the same correlations in HIV-infected men (Table 4.5). After adjustment for multiple comparisons correlations of all cytokines and chemokines within the semen of HIV-uninfected men showed that 114/190 (60%) were significantly positively correlated with each other,. The four correlations that showed the strongest correlations were IL-8 versus RANTES, IL-2 versus Fractalkine, IL-2 versus IL-1β and IL-7 versus IFNγ (all: p=0.0095; r=0.66). The cytokines and chemokines that showed the highest number of significant positive correlations with other cytokines and chemokines were fractalkine (16 other cytokines), IL-6, IL-10, IL-15 and MIP-1β (15 other cytokines). In stark contrast to HIV-uninfected men, in semen from HIV infected men only 56/190 (29.5%) relationships between cytokine/ chemokine pairs remained significant (Table 4.5). In contrast to semen, substantially fewer correlations (~10%) are evident in the blood of HIV uninfected men, however in HIV-infected men the frequency of correlations decrease to ~4% (data not shown) reflecting the situation in semen. These analyses reveal a highly dysregulated cytokine network in semen as a result of HIV infection.

IL-10 is a key immunoregulatory cytokine primarily involved in an anti-inflammatory capacity. To investigate the relationship between IL-10 and key Th1 cytokines IFNγ and TNFα, ratios of these cytokines were compared between HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men (Figure 4.5). In blood both IL-10:IFNγ and IL-10:TNFα ratios did not differ significantly between HIV-uninfected, infected or ARV-treated men (Figure 4.5A). In semen, there was a trend towards higher IL-10:IFNγ ratios in HIV-uninfected than both HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men. IL-10:TNFα ratios were significantly higher in HIV-uninfected compared to HIV-infected men ($p=0.0012$; Figure 4.5B). These data further demonstrate the dysregulated nature of cytokine networks in semen in HIV infection.

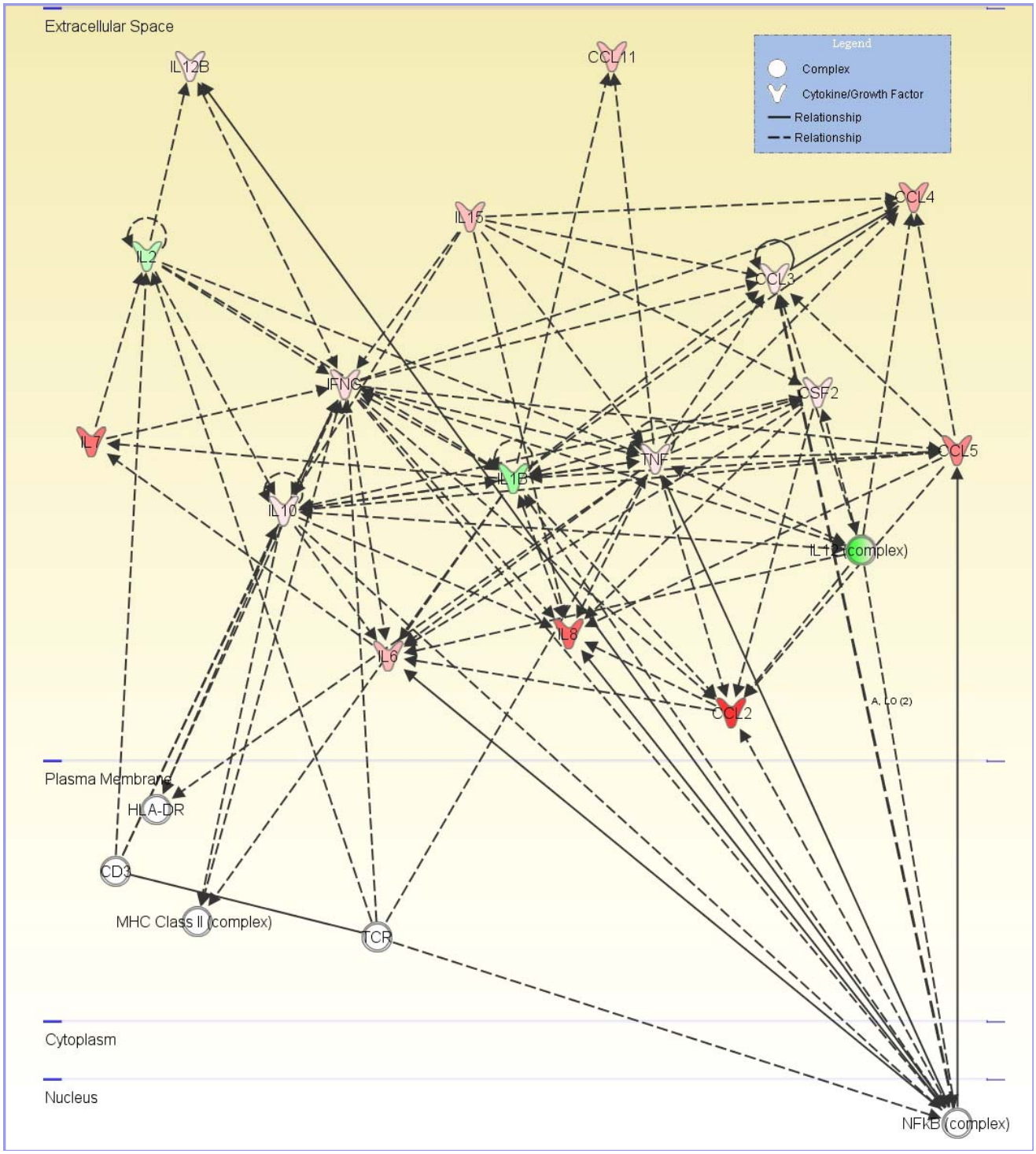


Figure 4.4 Schematic representation of relationships between cytokines and chemokines in semen. Shown here is a network based on 17 cytokines and chemokines in semen assayed in this study in relation to cellular and extra-cellular location. CCL2 = MCP-1; CCL3 = MIP-1α; CCL4 = MIP-1β; CCL5 = RANTES; CCL11 = Eotaxin and IL12B = IL12p40. Also shown is the NFκβ-complex in the nucleus, membrane bound MHC Class II complex, TCR, CD3 and HLA-DR. Dotted lines represent one-way and solid lines represent reciprocal relationships/effects between cytokines and chemokines. The intensity of the red icons is proportional to the rise in concentration while the intensity of green icons is proportional to the fall in concentration of cytokines and chemokines.

Table 4.4 Correlations of cytokines and chemokines in semen of HIV-uninfected men. Cytokines and chemokines in red are pro-inflammatory while blue are regulatory and adaptive. CCL11 = Eotaxin, CX3CL1 = Fractalkine, CCL2 = MCP-1, CCL3 = MIP-1α, CCL4 = MIP-1β and CCL5 = RANTES. Spearman Rank test was used and resulting p-values were adjusted for multiple comparisons using false discovery rate (FDR) step-down procedure. Significant p-values ≤ 0.01 are in yellow cells and p-values ≤ 0.05 are in lavender cells.

HIV-	IL-1α	IL-1β	IL-2	IL-6	IL-7	IL-8	IL-10	IL-12p40	IL-12p70	IL-15	IFNγ	TNFα	GM-CSF	CCL11	CX3CL1	G-CSF	CCL2	CCL3	CCL4	CCL5
IL-1α	-																			
IL-1β	ns	-																		
IL-2	ns	p=0.0095 r=0.66	-																	
IL-6	ns	p=0.017 r=0.6	p=0.013 r=0.64	-											p ≤ 0.05					
IL-7	ns	p=0.03, r=0.52	p=0.016 r=0.61	ns	-										p ≤ 0.01					
IL-8	ns	ns	ns	p=0.015 r=0.62	ns	-														
IL-10	ns	p<0.019 r=0.74	p<0.019 r=0.86	p<0.019 r=0.8	p=0.014 r=0.64	ns	-													
IL-12p40	ns	ns	ns	ns	p=0.03, r=0.51	ns	p=0.036 r=0.49	-												
IL-12p70	ns	ns	ns	ns	ns	ns	ns	p=0.049 r=0.46	-											
IL-15	ns	ns	p=0.025 r=0.56	p=0.016 r=0.61	p<0.019 r=0.67	p=0.03, r=0.52	p=0.023 r=0.57	p<0.019 r=0.71	p=0.034 r=0.5	-										
IFNγ	ns	p<0.019 r=0.71	p<0.019 r=0.8	p<0.019 r=0.76	p=0.0095 r=0.66	ns	p<0.019 r=0.89	ns	ns	p=0.037 r=0.48	-									
TNFα	ns	p<0.019 r=0.75	p<0.019 r=0.69	p<0.019 r=0.76	ns	ns	p<0.019 r=0.85	ns	ns	ns	p<0.019 r=0.73	-								
GM-CSF	ns	p=0.033 r=0.51	p=0.027 r=0.53	p=0.016 r=0.62	p=0.017 r=0.6	ns	p=0.013 r=0.64	ns	p=0.024 r=0.55	p=0.033 r=0.5	p=0.013 r=0.64	p=0.03, r=0.52	-							
CCL11	ns	ns	p=0.031 r=0.51	p=0.017 r=0.6	ns	ns	p=0.023 r=0.57	p=0.014 r=0.63	ns	p<0.019 r=0.73	p=0.023 r=0.57	ns	p=0.017 r=0.6	-						
CX3CL1	ns	ns	p=0.0095 r=0.66	p=0.016 r=0.61	p<0.019 r=0.74	ns	p<0.019 r=0.67	p=0.023 r=0.57	p=0.023 r=0.55	p<0.019 r=0.78	p<0.019 r=0.75	p=0.034 r=0.5	p=0.002 r=0.58	p<0.019 r=0.68	-					
G-CSF	ns	p=0.023 r=0.57	p=0.025 r=0.56	p=0.023 r=0.54	p=0.033 r=0.5	ns	p<0.019 r=0.76	ns	ns	ns	p<0.019 r=0.67	p<0.019 r=0.73	ns	p=0.034 r=0.5	p=0.024 r=0.55	-				
CCL2	ns	ns	ns	p=0.025 r=0.55	ns	p=0.024 r=0.55	ns	p=0.024 r=0.55	p=0.034 r=0.5	p<0.019 r=0.72	ns	ns	p=0.024 r=0.55	p<0.019 r=0.8	p=0.015 r=0.63	ns	-			
CCL3	p=0.027 r=0.52	ns	p=0.023 r=0.54	p=0.034 r=0.5	ns	ns	p=0.013 r=0.66	p=0.025 r=0.56	ns	p=0.036 r=0.49	ns	p=0.023 r=0.54	p=0.027 r=0.39	p=0.024 r=0.56	p=0.023 r=0.41	p=0.023 r=0.57	ns	-		
CCL4	ns	ns	p=0.015 r=0.62	p<0.019 r=0.72	p=0.04, r=0.47	p=0.046 r=0.47	p<0.019 r=0.74	p=0.037 r=0.49	ns	p=0.019 r=0.59	p=0.017 r=0.6	p=0.025 r=0.56	p=0.024 r=0.55	p=0.037 r=0.48	p=0.04, r=0.47	p=0.015 r=0.63	ns	p=0.013 r=0.65	-	
CCL5	ns	ns	p=0.023 r=0.54	p=0.016 r=0.61	p<0.019 r=0.78	p=0.0095 r=0.66	p=0.023 r=0.54	p=0.024 r=0.56	p=0.027 r=0.53	p<0.019 r=0.83	p=0.024 r=0.56	ns	p=0.016 r=0.62	p=0.015 r=0.62	p<0.019 r=0.83	ns	p=0.013 r=0.65	ns	p=0.033 r=0.5	-

Table 4.5 Correlations of cytokines and chemokines in semen of HIV-infected men. Cytokines and chemokines in red are pro-inflammatory while blue are regulatory and adaptive. CCL11 = Eotaxin, CX3CL1 = Fractalkine, CCL2 = MCP-1, CCL3 = MIP-1α, CCL4 = MIP-1β and CCL5 = RANTES. Spearman Rank test was used and resulting p-values were adjusted for multiple comparisons using false discovery rate (FDR) step-down procedure. Significant p-values ≤ 0.01 are in yellow cells and p-values ≤ 0.05 are in lavender cells.

HIV+	IL-1α	IL-1β	IL-2	IL-6	IL-7	IL-8	IL-10	IL-12p40	IL-12p70	IL-15	IFNγ	TNFα	GM-CSF	CCL11	CX3CL1	G-CSF	CCL2	CCL3	CCL4	CCL5
IL-1α	-																			
IL-1β	p<0.019 r=0.59	-																		
IL-2	ns	p<0.019 r=0.6	-																	
IL-6	ns	ns	p=0.019 r=0.45	-											p ≤ 0.05					
IL-7	ns	ns	p=0.035 r=0.49	ns	-										p ≤ 0.01					
IL-8	ns	ns	ns	p<0.019 r=0.77	ns	-														
IL-10	ns	p=0.021 r=0.52	ns	p<0.019 r=0.63	ns	ns	-													
IL-12p40	ns	ns	ns	ns	ns	ns	ns	-												
IL-12p70	ns	p=0.019 r=0.52	p<0.019 r=0.57	ns	ns	ns	ns	ns	-											
IL-15	ns	ns	ns	ns	p<0.019 r=0.79	p=0.019 r=0.57	ns	ns	ns	-										
IFNγ	ns	ns	p<0.019 r=0.64	p=0.019 r=0.54	ns	ns	p<0.019 r=0.65	ns	p=0.019 r=0.58	ns	-									
TNFα	p=0.019 r=0.53	p<0.019 r=0.76	p=0.038 r=0.48	p<0.019 r=0.83	ns	p=0.019 r=0.53	p<0.019 r=0.69	ns	ns	ns	p<0.019 r=0.6	-								
GM-CSF	ns	p=0.05, r=0.45	p=0.019 r=0.53	p=0.048 r=0.46	ns	ns	ns	ns	p=0.019 r=0.53	ns	p=0.019 r=0.58	ns	-							
CCL11	ns	ns	ns	ns	ns	p=0.047 r=0.46	ns	ns	p=0.019 r=0.55	p=0.019 r=0.55	ns	ns	ns	-						
CX3CL1	ns	ns	ns	ns	p<0.019 r=0.68	ns	ns	ns	ns	p<0.019 r=0.81	ns	ns	ns	p<0.019 r=0.63	-					
G-CSF	p=0.035 r=0.49	p=0.019 r=0.54	ns	ns	ns	ns	ns	ns	ns	ns	ns	p<0.019 r=0.64	ns	ns	ns	-				
CCL2	ns	ns	ns	p=0.03, r=0.5	ns	p<0.019 r=0.75	ns	ns	ns	p=0.019 r=0.56	ns	ns	ns	p=0.021 r=0.51	ns	ns	-			
CCL3	p=0.019 r=0.54	p<0.019 r=0.64	ns	p=0.045 r=0.47	ns	ns	ns	ns	ns	ns	ns	p<0.019 r=0.6	ns	ns	ns	p=0.021 r=0.51	ns	-		
CCL4	ns	p<0.019 r=0.6	ns	p<0.019 r=0.79	ns	p<0.019 r=0.71	ns	ns	ns	ns	ns	p<0.019 r=0.72	ns	ns	ns	p=0.019 r=0.58	p=0.019 r=0.52	p=0.019 r=0.56	-	
CCL5	ns	ns	ns	ns	p<0.019 r=0.74	ns	ns	ns	ns	p<0.019 r=0.74	ns	ns	ns	ns	p<0.019 r=0.76	ns	ns	ns	ns	-

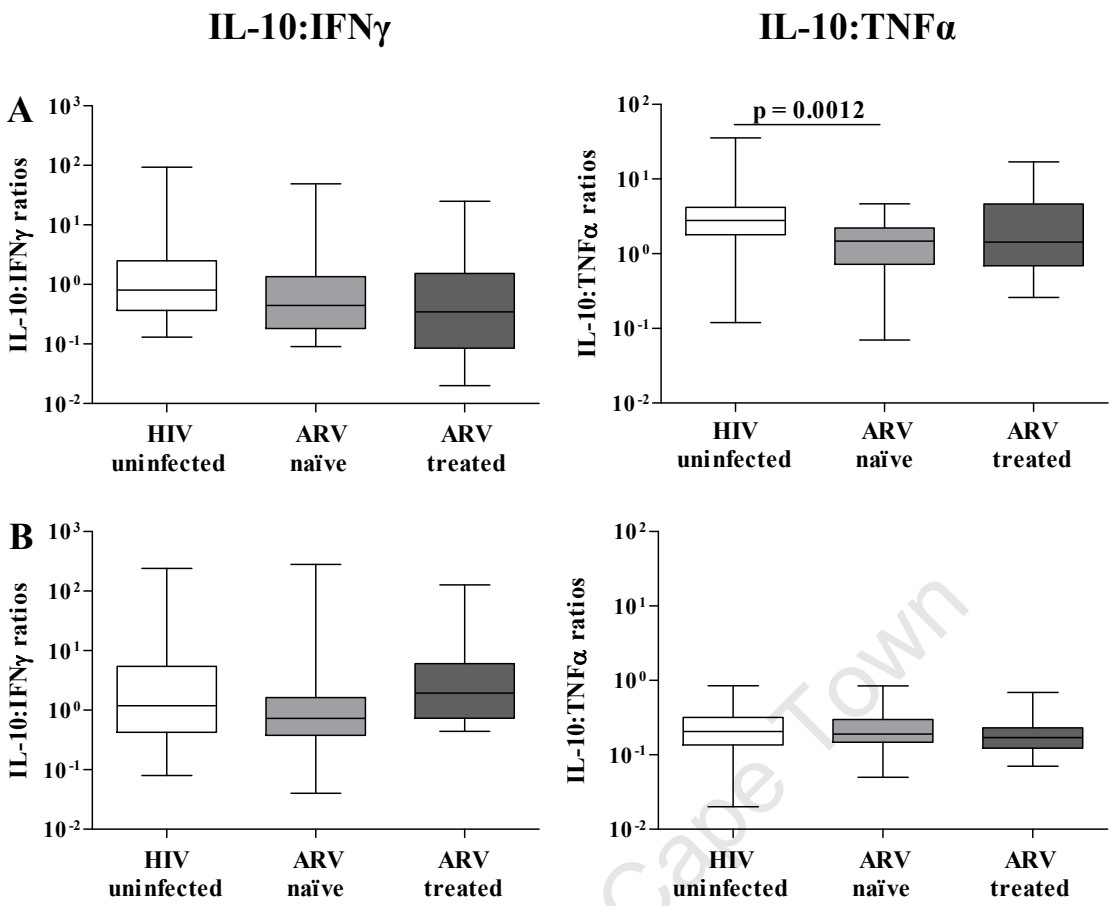


Figure 4.5 Relationship between regulatory and pro-inflammatory cytokines. Ratios between regulatory cytokine, IL-10 and IFNγ or TNFα in (A) blood and (B) semen of HIV-uninfected, HIV-infected (ARV-naïve) and ARV-treated men. Box-and-whisker plots reperesents the median, IQR and 5-95% range (error bars). Mann-Whitney t tests were used to compare ratios. A p-value of < 0.05 was considered significant.

4.3.5 The relationship between cytokines/chemokines and viral loads

To investigate whether there was an association between viral loads and cytokine and chemokine levels in blood and semen, the first approach was to perform univariate analysis by correlating cytokine and chemokine concentrations with matched viral load levels in blood and semen, respectively. This analysis is shown in Table 4.6, and significant correlations are depicted in Figure 4.6. TNFα in both blood and semen were significantly positively correlated with both plasma viral load (r=0.53, p=0.0054) and seminal viral load (r=0.55, p=0.013). Furthermore, seminal viral load was significantly positively correlated with G-CSF (r=0.61, p=0.0057), IL-10 (r=0.61, p=0.006) and IFNγ (r=0.57, p=0.01). No association with viral load was detected for the remainder of the analytes measured in blood or semen. After adjusting for multiple comparisons, however, none of these associations remained significant.

To investigate the influence of seminal viral load on cytokine and chemokine levels in seminal plasma, a univariate logistic regression analysis was performed between levels of cytokines and chemokines and seminal viral load. Resulting regression coefficients were adjusted for the effect of plasma viral load (Table 4.7). In semen, after adjusting for the influence of plasma viral load there were significant associations between viral load and both IL-10 and IFNγ. For every log increase in seminal viral load, IL-10 and IFNγ levels increased by 0.96 and 0.97 pg/ml respectively (Table 4.7).

In the analysis above, a number of cytokines/chemokines in semen were significantly positively correlated with seminal viral load. Thus, it was hypothesised that men shedding virus in semen may have differing levels of cytokines/chemokines compared to non-shedders. To investigate whether men would cluster according to viral loads, participants were divided into three groups, namely HIV-uninfected men, HIV-infected men who had detectable viral loads in semen, and HIV-infected men who have no detectable viral loads in semen. The same analysis was performed in blood. An unsupervised clustering was performed using principle component analysis (PCA). PCA analysis showed no group-specific clustering for any of the analytes measured (Figure 4.7). Secondly, to visualise the levels of cytokines and chemokines in the blood and semen of each participant graphically, hierarchical clustering was used to create heat maps, in order to detect linkage of individuals with detectable viral loads to levels of cytokines and chemokines assayed. None of the groups demonstrated any

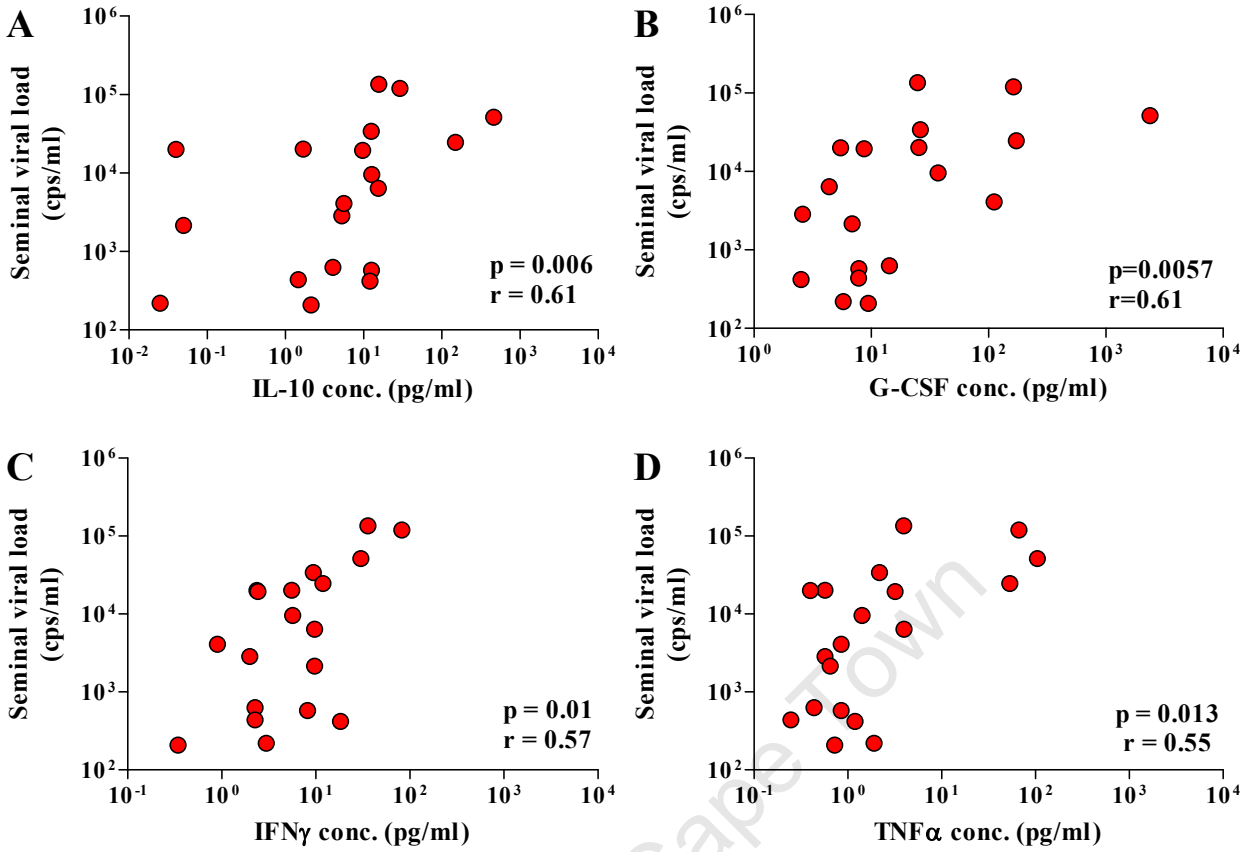


Fig 4.6 Association between seminal viral load and cytokines/chemokines in semen. Shown are correlations for (A) IL-10, (B) G-CSF, (D) IFNγ and TNFα with viral loads of HIV-infected men with detectable seminal viral load (n=19). Each circle represents a viral load (copies/ml) with corresponding cytokine/chemokine concentration (pg/ml). The Spearman Rank test was used and p-values ≤ 0.05 were considered significant. Spearman rho values are indicated.

Table 4.6 Correlations between viral loads and cytokines/chemokines in semen and blood of HIV-infected (ARV-naïve) men.

Cytokines/chemokines		Plasma viral load		Seminal viral load	
		Rho	**p-value**	**Rho**	**p-value**
Pro-inflammatory:	**IL-1α**	**-**	**-**	-0.012	0.96
	IL-1β	0.26	0.2	0.38	0.1
	IL-6	0.14	0.49	0.27	0.26
	IL-8	-0.32	0.11	0.01	0.96
	IL-12p40	-0.068	0.74	0.11	0.63
	IL-12p70	0.17	0.39	0.28	0.23
	TNF-α	**0.53**	**0.0054**	**0.55**	**0.013**
	Eotaxin	0.21	0.31	0.38	0.099
	Fractalkine	0.11	0.58	0.17	0.48
	MCP-1	-0.035	0.86	-0.41	0.079
	MIP-1α	-0.071	0.73	0.32	0.17
	MIP-1β	-0.21	0.29	0.25	0.31
	RANTES	-0.15	0.47	0.037	0.88
	G-CSF	-0.19	0.36	**0.61**	**0.0057**
	GM-CSF	-0.054	0.79	-0.04	0.87
Regulatory:	**IL-10**	0.12	0.56	**0.61**	**0.006**
Adaptive:	**IFN-γ**	0.31	0.13	**0.57**	**0.01**
	IL-2	0.22	0.29	0.065	0.78
	IL-7	0.046	0.82	-0.0035	0.98
	IL-15	-0.079	0.69	0.068	0.78

[a]IL-1α cytokine data in blood not used

Univariate analysis using Spearman Rank test, with p-values considered significant ≤ 0.05 are shown in bold.

Multivariate analysisusing the False discovery rate (FDR) step-down procedure showed no surviving significant p-values.

Table 4.7 Multivariate analysis of association between seminal cytokines/chemokines and seminal viral load adjusted for plasma viral load.

Cytokines/chemokines		β-coefficient (95% CI)	Adj. β-coefficient (95% CI)
Pro-inflammatory:	**IL-1α**	0.07 (-0.57 – 0.72)	0.33 (-0.50 – 1.18)
	IL-1β	-0.01 (-0.66 – 0.63)	0.37 (-0.54 – 1.28)
	IL-6	0.11 (-0.53 – 0.76)	0.59 (-0.34 – 1.53)
	IL-8	0.05 (-0.59 – 0.70)	0.58 (-0.30 – 1.47)
	IL-12p40	0.22 (-0.44 – 0.89)	0.63 (-0.32 – 1.58)
	IL-12p70	-0.45 (-1.12 – 0.21)	-0.02 (-0.88 – 0.83)
	TNF-α	0.30 (-0.37 – 0.97)	0.90 (-0.07 – 1.88)
	Eotaxin	-0.16 (-0.82 – 0.48)	0.47 (-0.40 – 1.35)
	Fractalkine	0.26 (-0.41 – 0.94)	0.50 (-0.41 – 1.42)
	MCP-1	-0.14 (-0.81 – 0.51)	0.44 (-0.44 – 1.34)
	MIP-1α	-0.03 (-0.68 – 0.60)	0.50 (-0.39 – 1.39)
	MIP-1β	0.10 (-0.54 – 0.75)	0.90 (-0.14 – 1.94)
	RANTES	-0.04 (-0.69 – 0.59)	0.24 (-0.54 – 1.04)
	G-CSF	-0.06 (-0.71 – 0.57)	0.80 (-0.15 – 1.76)
	GM-CSF	0.09 (-0.55 – 0.74)	0.33 (-0.56 – 1.22)
Regulatory:	**IL-10**	0.25 (-0.44 – 0.95)	**0.96 (0.01 – 1.91)**
Adaptive:	**IFN-γ**	0.58 (-0.15 – 1.32)	**0.97 (0.001 – 1.94)**
	IL-2	0.17 (-0.47 – 0.83)	0.93 (-0.11 – 1.98)
	IL-7	0.32 (-0.37 – 1.01)	0.70 (-0.14 – 1.56)
	IL-15	0.34 (-0.32 – 1.01)	0.86 (-0.16 – 1.90)

[a]β-coefficient: logistic regression between plasma cytokines/chemokines and seminal viral load

[b]Adj. β-coefficient: logistic regression adjusted for presence of ARVs

[c]Numbers in bold represent statistically significant associations

clustering in either blood (Figure S1) or semen (Figure S2), with HIV-infected men with detectable viral loads in blood or semen spread throughout the uninfected and HIV-infected undetectable viral load groups.

In summary, using multiple methods to investigate the relationship between semen cytokines/chemokines and viral loads, little evidence of any particular cytokine associating with viral shedding in semen was apparent. Although TNFα was significantly positively correlated with plasma viral load, and in semen, TNFα, G-CSF, IL-10 and IFNγ were significantly positively correlated with seminal viral load, these associations were lost after adjusting for multiple comparisons. In semen, controlling for the influence of plasma viral load, IL-10 and IFNγ were significantly positively associated with increases in seminal viral load when regression analysis was applied. Lastly, cluster analyses revealed that cytokines and chemokines did not group into clusters according to the presence or absence of viral replication in semen in HIV-infected men.

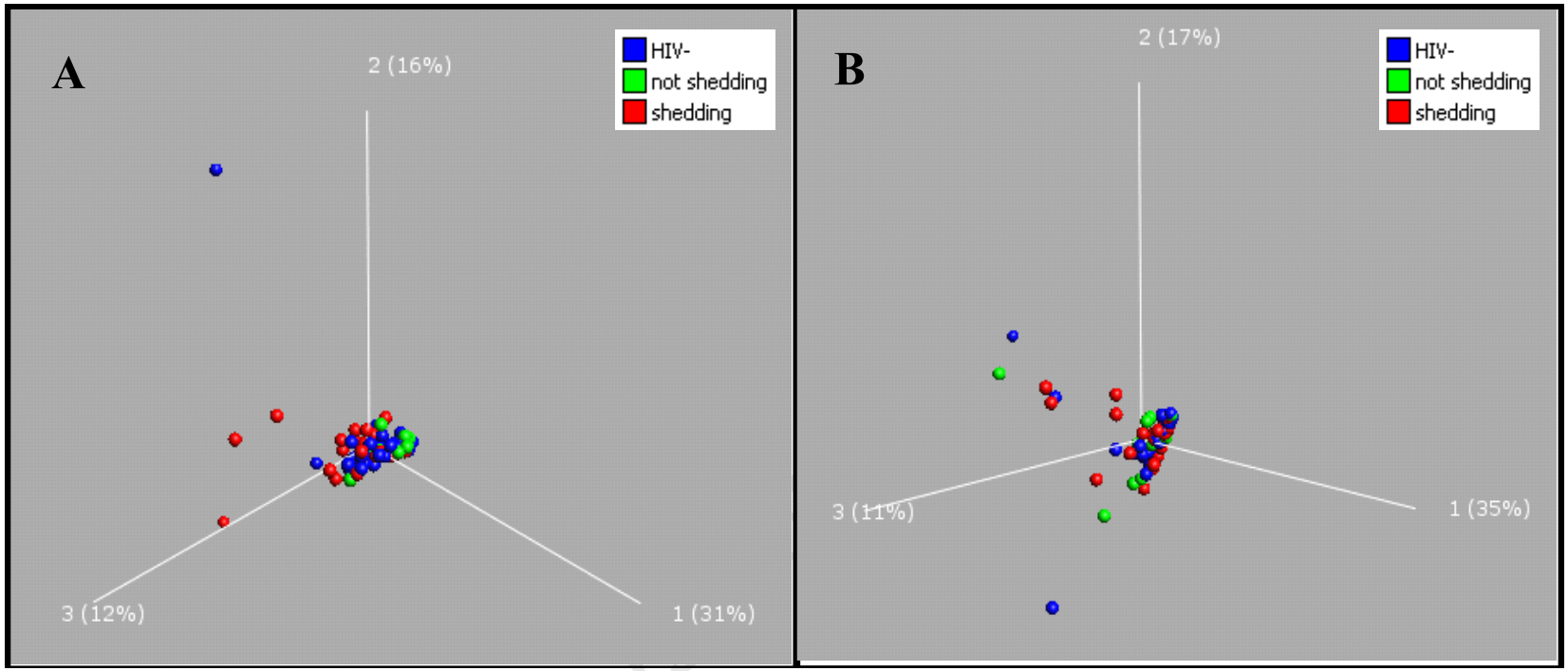


Figure 4.7 Schematic representation of principal component analysis (PCA). Shown are plots of all study participants that are HIV-uninfected (blue), have no detectable viral loads (green) and have detectable viral loads (red) in (A) blood and (B) semen. PCA plots were used to visualize results from unsupervised variance filtration used to test clustering of cytokines and chemokines in abovementioned three groups.

4.3.6 The relationship between cytokines/chemokines and cellular activation

As can be seen from data presented in Chapter 3 and here in Chapter 4, levels of both immune activation and inflammation in the male genital tract were high compared to blood. This was evidenced by higher frequencies of activated T cells and higher concentrations of inflammatory cytokines/chemokines in semen. Although immune activation may be either directly or indirectly linked to viral replication (Appay & Sauce 2008), the contribution and effect of inflammation on immune activation, especially in the genital tract, needs further examination. Thus, the relationship between local genital tract inflammation, T cell activation and viral load was investigated.

A range of analyses were conducted to explore these relationships. To investigate the relationship between inflammatory cytokines and chemokines and levels of activated T cells in semen, univariate correlations were performed. All p-values and Spearman rho values can be found in Table S4 (for semen) and Table S5 (for blood). In the semen of HIV-uninfected men, frequencies of $CD8^+CD38^+$ T cells were significantly positively correlated with both MCP-1 (p=0.0044, r=0.45; Figure 4.8A) and Eotaxin (p=0.0002, r=0.56; Figure 4.8B), whereas in the semen of HIV-infected men (ARV-naïve), although weak, both $CD4^+$ and $CD8^+$ $CCR5^+$ T cell frequencies were significantly inversely correlated with IL-2 (p=0.046, r=-0.49) and IL-7 (p=0.045, r=-0.43), respectively. In the semen of HIV-infected (ARV-treated) men (n=12), frequencies of $CD4^+CD38^+$ T cells were significantly negatively correlated with IL-1α (p=0.024, r=-0.73), IL-1β (p=0.035, r=-0.7), G-CSF (p=0.035, r=-0.7) and MIP-1β (p=0.049, r=-0.67). Also in the semen of HIV-infected (ARV-treated) men, frequencies of $CD8^+CCR5^+$ T cells were significantly positively correlated with IL-1β (p=0.021, r=0.71). In contrast, in blood, the strongest positive correlation was found between IL-10 levels and frequencies of $CD8^+CD38^+$ T cells in HIV-infected (ARV-treated) men (p=0.0027, r=0.78; Figure 4.8D), whereas a strong negative correlation existed between MIP-1β levels and frequencies of $CD8^+CCR5^+$ T cells in HIV-infected (ARV-naïve) men (p=0.0021, r=-0.58; Figure 4.8C). After adjusting for multiple comparisons, only Eotaxin levels in semen of HIV-uninfected men remained positively associated with activated $CD38^+$ CD8 T cells (p=0.044, r=0.56 data not shown). None of the correlations in blood remained significant after adjusting for multiple comparisons.

Immune activation in the genital tract may be as a direct result of viral replication. However, activated immune cells and other local cells produce pro-inflammatory cytokines and chemokines that may also attract more immune cells to sites of infection (Appay & Sauce 2008). To investigate the influence of seminal viral loads on the association between cellular activation and cytokines and chemokines in semen, a univariate regression analysis was performed between levels of cytokines and chemokines and frequencies of activated T cells. Resulting regression coefficients were adjusted for the influence of seminal viral load in a multivariate analysis (Table 4.8). In univariate analysis, high levels of G-CSF were significantly associated with high frequencies of $CD8^+CCR5^+$ T cells. After adjusting for the effect of seminal viral load, this association remained significant (Table 4.8). This indicated that for a log increase in the frequency of $CD8^+CCR5^+$ T cells, the concentration of G-CSF would increase by 0.36 pg/ml after controlling for the influence of seminal viral load. By comparison, the same analysis was performed in the systemic compartment. Here it was found that even after adjusting for the influence of plasma viral load, there was a significant positive association between frequencies of $CD4^+CD38^+$ T cells and levels of RANTES as well as $CD8^+CD38^+$ T cells and levels of IL-2 (Table 4.9). There was a significant positive association between frequencies of $CD8^+CD38^+$ T cells and levels of IL-7, however, after controlling for the influence of plasma viral load, this association was lost.

Thus, in the semen of HIV-infected men, an increase in immune activation was associated with an increase in G-CSF even after accounting for the effect of seminal viral load. In comparison, correlative analysis revealed a significant positive association between activated T cells and Eotaxin in the semen of HIV-uninfected men alone. When comparing compartments, there were no shared associations of any of the analytes investigated. In blood, levels of RANTES and IL-2 remained positively associated with increases in frequencies of activated $CD8^+$ T cells after controlling for the influence of plasma viral load.

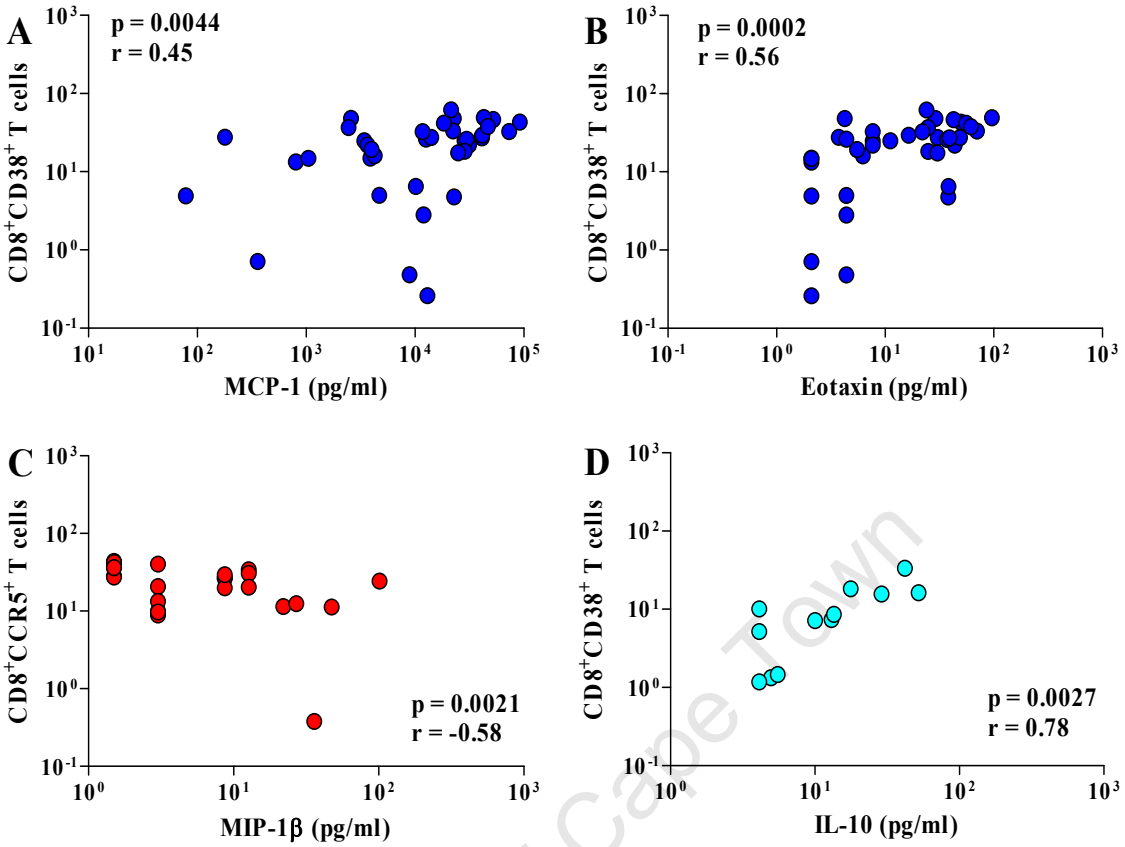


Figure 4.8 Association between activated T cells and cytokines and chemokines. Shown are the two correlations with the highest significant p-values in semen between $CD8^+CD38^+$ T cells and MCP-1 levels (A) and Eotaxin levels (B) in HIV-uninfected men [blue] as well as the two correlations with the highest significant p-values in blood between (C) $CD8^+CCR5^+$ T cells and MIP-1β levels in HIV-infected (ARV naïve) men [red] and between $CD8^+CD38^+$ T cells and (D) IL-10 levels in HIV-infected (ARV-treated) men [cyan]. Each circle represents a cytokine/chemokine concentration (pg/ml) with a corresponding frequency of an activated T cell subset. Spearman Rank test were used and p-values ≤ 0.05 were considered significant. Spearman rho values are indicated.

Table 4.8 Multivariate analysis of association between levels of cytokines/chemokines and T cell activation subsets in semen of HIV-infected men adjusted for seminal viral load.

Cytokines/ chemokines	$CD4^+$ T cells				$CD8^+$ T cells			
	CCR5		CD38		CCR5		CD38	
Pro-inflammatory	**β-coeff. (95% CI)**	**Adj. β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**Adj. β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**Adj. β-coeff. (95% CI)**	**β-coeff. (95% CI)**	**Adj. β-coeff. (95% CI)**
IL-1α	0.21 (-0.2 – 0.62)	0.22 (-0.19 – 0.63)	-0.14 (-0.5 – 0.22)	-0.14 (-0.5 – 0.23)	0.16 (-0.2 – 0.52)	0.15 (-0.21 – 0.52)	0.027 (-0.31 – 0.37)	0.024 (-0.32 – 0.37)
IL-1β	0.18 (-0.21 – 0.57)	0.15 (-0.28 – 0.58)	-0.22 (-0.58 – 0.14)	-0.24 (-0.61 – 0.12)	0.17 (-0.18 – 0.53)	0.16 (-0.2 – 0.52)	0.087 (-0.25 – 0.43)	0.072 (-0.27 – 0.42)
IL-6	0.026 (-0.37 – 0.42)	-0.02 (-0.44 – 0.4)	-0.26 (-0.6 – 0.08)	-0.27 (-0.61 – 0.08)	0.057 (-0.3 – 0.42)	0.035 (-0.34 – 0.41)	-0.2 (-0.53 – 0.13)	-0.22 (-0.56 – 0.12)
IL-8	0.005 (-0.45 – 0.46)	-0.019 (-0.48 – 0.45)	0.2 (-0.19 – 0.6)	0.19 (-0.22 – 0.6)	0.003 (-0.39 – 0.4)	-0.012 (-0.42 – 0.39)	-0.22 (-0.55 – 0.11)	-0.22 (-0.55 – 0.11)
IL-12p40	0.12 (-0.32 – 0.56)	0.088 (-0.37 – 0.55)	-0.099 (-0.45 – 0.25)	-0.13 (-0.5 – 0.24)	0.029 (-0.33 – 0.39)	0.005 (-0.36 – 0.38)	-0.037 (-0.38 – 0.31)	-0.066 (-0.42 – 0.29)
IL-12p70	0.31 (-0.16 – 0.78)	0.29 (-0.2 – 0.78)	-0.22 (-0.6 – 0.15)	-0.21 (-0.59 – 0.18)	0.25 (-0.096 – 0.61)	0.28 (-0.084 – 0.64)	-0.016 (-0.36 – 0.33)	-0.001 (-0.35 – 0.35)
TNF-α	0.14 (-0.25 – 0.53)	0.1 (-0.35 – 0.55)	-0.2 (-0.58 – 0.17)	-0.24 (-0.63 – 0.14)	0.13 (-0.22 – 0.48)	0.1 (-0.27 – 0.48)	-0.024 (-0.36 – 0.32)	-0.07 (-0.43 – 0.29)
Eotaxin	-0.026 (-0.49 – 0.44)	-0.056 (-0.53 – 0.42)	0.12 (-0.24 – 0.48)	0.11 (-0.26 – 0.48)	0.26 (-0.11 – 0.62)	0.25 (-0.13 – 0.62)	0.095 (-0.24 – 0.43)	0.085 (-0.26 – 0.43)
Fractalkine	0.081 (-0.33 – 0.49)	0.048 (-0.39 – 0.48)	0.015 (-0.33 – 0.36)	-0.003 (-0.36 – 0.35)	0.14 (-0.21 – 0.5)	0.12 (-0.25 – 0.5)	-0.033 (-0.37 – 0.3)	-0.055 (-0.4 – 0.29)
MCP-1	-0.2 (-0.62 – 0.21)	-0.18 (-0.61 – 0.24)	-0.1 (-0.45 – 0.24)	-0.084 (-0.44 – 0.27)	-0.17 (-0.53 – 0.2)	-0.16 (-0.53 – 0.21)	-0.13 (-0.47 – 0.2)	-0.12 (-0.46 – 0.22)
MIP-1α	0.09 (-0.35 – 0.53)	0.06 (-0.4 – 0.52)	-0.2 (-0.58 – 0.17)	-0.21 (-0.59 – 0.17)	0.058 (-0.3 – 0.42)	0.043 (-0.33 – 0.41)	-0.056 (-0.4 – 0.29)	-0.068 (-0.42 – 0.28)
MIP-1β	0.069 (-0.32 – 0.46)	0.058 (-0.34 – 0.46)	-0.22 (-0.58 – 0.13)	-0.22 (-0.58 – 0.14)	0.072 (-0.28 – 0.42)	0.063 (-0.29 – 0.42)	-0.12 (-0.46 – 0.22)	-0.13 (-0.47 – 0.21)
RANTES	0.094 (-0.43 – 0.62)	0.06 (-0.49 – 0.61)	-0.15 (-0.51 – 0.21)	-0.15 (-0.51 – 0.21)	-0.026 (-0.4 – 0.34)	-0.033 (-0.41 – 0.34)	0.077 (-0.26 – 0.41)	0.073 (-0.27 – 0.42)
G-CSF	0.23 (-0.14 – 0.62)	0.22 (-0.17 – 0.61)	-0.094 (-0.49 – 0.3)	-0.1 (-0.5 – 0.3)	**0.37 (0.036 – 0.7)**	**0.36 (0.017 – 0.71)**	0.029 (-0.31 – 0.37)	0.0098 (-0.34 – 0.36)
GM-CSF	0.062 (-0.36 – 0.49)	0.028 (-0.42 – 0.47)	-0.059 (-0.4 – 0.29)	-0.066 (-0.42 – 0.28)	0.036(-0.35 – 0.42)	0.024 (-0.36 – 0.41)	0.075 (-0.26 – 0.41)	0.071 (-0.27 – 0.41)
Regulatory								
IL-10	0.27 (-0.099 – 0.64)	0.25 (-0.14 – 0.65)	-0.31 (-0.69 – 0.072)	-0.33 (-0.71 – 0.059)	0.18 (-0.16 – 0.52)	0.16 (-0.19 – 0.52)	-0.24 (-0.57 – 0.089)	-0.27 (-0.61 – 0.061)
Adaptive								
IFN-γ	0.26 (-0.1 – 0.63)	0.27 (-0.19 – 0.74)	-0.27 (-0.6 – 0.066)	-0.34 (-0.69 – 0.014)	0.24 (-0.1 – 0.58)	0.23 (-0.14 – 0.61)	-0.21 (-0.54 – 0.12)	-0.27 (-0.62 – 0.078)
IL-2	0.074 (-0.36 – 0.51)	0.046 (-0.41 – 0.5)	-0.23 (-0.6 – 0.13)	-0.24 (-0.61 – 0.13)	0.014 (-0.35 – 0.37)	0.01 (-0.35 – 0.38)	0.053 (-0.29 – 0.4)	0.049 (-0.3 – 0.39)
IL-7	-0.005 (-0.4 – 0.39)	-0.007 (-0.41 – 0.39)	-0.036 (-0.39 – 0.32)	-0.04 (-0.4 – 0.32)	-0.18 (-0.52 – 0.16)	-0.19 (-0.54 – 0.16)	-0.067 (-0.4 – 0.27)	-0.07 (-0.41 – 0.27)
IL-15	0.075 (-0.38 – 0.53)	0.039 (-0.44 – 0.52)	0.038 (-0.31 – 0.39)	0.017 (-0.34 – 0.38)	-0.027 (-0.41 – 0.36)	-0.066 (-0.47 – 0.34)	-0.16 (-0.5 – 0.18)	-0.19 (-0.53 – 0.15)

[a]β-coefficient (95% confidence interval) linear regression analysis of T cell activation subsets and cytokines/chemokines;
[b]Adjusted β-coefficient (95% confidence interval) for ARV treatment
Red-shaded cells represent significant associations

Table 4.9 Multivariate analysis of association between levels of cytokines/chemokines and T cell activation subsets in blood of HIV-infected men adjusted for plasma viral load.

Cytokines/ chemokines	$CD4^+$ T cells				$CD8^+$ T cells			
	CCR5		CD38		CCR5		CD38	
Pro-inflammatory	[a]β-coeff. (95% CI)	[b]Adj. β-coeff. (95% CI)	[a]β-coeff. (95% CI)	[b]Adj. β-coeff. (95% CI)	[a]β-coeff. (95% CI)	[b]Adj. β-coeff. (95% CI)	[a]β-coeff. (95% CI)	[b]Adj. β-coeff. (95% CI)
IL-1β	0.07 (-0.25 - 0.41)	0.05 (-0.32 - 0 .42)	0.005 (-0.33 - 0.34)	-0.16 (-0.51 - 0.18)	0.022 (-0.31 - 0.36)	-0.07(-0.43 - 0.29)	0.22 (-0.09 - 0.55)	0.10 (-0.24 - 0.45)
IL-6	-0.08 (-0.42 - 0.24)	-0.11 (-0.46 - 0.23)	0.04 (-0.29 - 0.37)	-0.039 (-0.10 - 0.53)	-0.10 (-0.44 - 0.22)	-0.16(-0.50 - 0.17)	0.065 (-0.27 - 0.40)	-0.012 (-0.34 - 0.31)
IL-8	0.034 (-0.30 - 0.37)	0.028 (-0.31 - 0.37)	-0.073 (-0.41 - 0.26)	-0.099 (-0.42 - 0.22)	-0.12 (-0.45 - 0.21)	-0.13(-0.46 - 0.19)	0.13 (-0.19 - 0.47)	0.11 (-0.20 - 0.43)
IL-12p40	0.28 (-0.04 - 0.60)	0.27 (-0.058 - 0.60)	-0.051 (-0.38 - 0.28)	-0.10 (-0.42 - 0.21)	0.086 (-0.25 - 0.42)	0.057(-0.28 - 0.39)	0.10 (-0.23 - 0.43)	0.051 (-0.27 - 0.37)
IL-12p70	0.12 (-0.21 - 0.45)	0.10 (-0.24 - 0.45)	-0.005 (-0.33 - 0.34)	-0.085 (-0.41 - 0.24)	0.029 (-0.30 - 0.36)	-0.021(-0.36 - 0.32)	0.11 (-0.22 - 0.44)	0.026 (-0.30 - 0.35)
TNF-α	-0.14 (-0.48 - 0.18)	-0.23 (-0.61 - 0.13)	0.12 (-0.21 - 0.45)	-0.047 (-0.40 - 0.31)	-0.028 (-0.36 - 0.30)	-0.15 (-0.52 - 0.22)	0.14 (-0.18 - 0.48)	-0.015 (-0.37 - 0.34)
Eotaxin	0.18 (-0.14 - 0.51)	0.17 (-0.18 - 0.52)	0.14 (-0.19 - 0.47)	0.090 (-0.26 - 0.44)	-0.074 (-0.41 - 0.26)	-0.20 (-0.53 - 0.12)	0.14 (-0.19 - 0.47)	0.036 (-0.30 - 0.37)
Fractalkine	-0.067 (-0.40 - 0.26)	-0.10 (-0.46 - 0.25)	0.26 (-0.05 - 0.59)	0.17 (-0.15 - 0.51)	-0.14 (-0.47 - 0.19)	-0.22(-0.57 - 0.11)	0.28 (-0.038 - 0.60)	0.19 (-0.13 - 0.52)
MCP-1	0.071 (-0.26 - 0.40)	0.069 (-0.27 - 0.41)	-0.21 (-0.54 - 0.11)	-0.22 (-0.53 - 0.08)	-0.10 (-0.43 - 0.23)	-0.10(-0.43 - 0.22)	-0.22 (-0.55 - 0.10)	-0.23 (-0.54 - 0.081)
MIP-1α	-0.064 (-0.40 - 0.27)	-0.058 (-0.40 - 0.28)	0.072 (-0.26 - 0.40)	0.098 (-0.22 - 0.41)	-0.17 (-0.50 - 0.15)	-0.16(-0.49 - 0.17)	0.096 (-0.24 - 0.43)	0.12 (-0.19 - 0.44)
MIP-1β	-0.22 (-0.55 - 0.10)	-0.22 (-0.55 - 0.11)	-0.30 (-0.62 - 0.01)	-0.25(-0.56 - 0.05)	-0.30(-0.62 - 0.013)	-0.28(-0.60 - 0.044)	-0.051 (-0.38 - 0.28)	0.006 (-0.32 - 0.33)
RANTES	-0.016 (-0.35 - 0.32)	-0.017 (-0.35 - 0.32)	**0.41 (0.10 - 0.72)**	**0.41 (0.12 - 0.70)**	0.047 (-0.29 - 0.38)	0.043(-0.29 - 0.37)	**0.35 (0.034 - 0.66)**	**0.34 (0.046 - 0.64)**
G-CSF	-0.070 (-0.40 - 0.26)	-0.086 (-0.43 - 0.25)	0.23 (-0.08 - 0.56)	0.18 (-0.13 - 0.50)	-0.098 (-0.43 - 0.23)	-0.13(-0.47 - 0.20)	0.21 (-0.11 - 0.54)	0.16 (-0.15 - 0.48)
GM-CSF	0.11 (-0.22 - 0.44)	0.11 (-0.22 - 0.45)	-0.069 (-0.40 - 0.26)	-0.081 (-0.40 - 0.23)	-0.021 (-0.35 - 0.31)	-0.028(-0.36 - 0.30)	0.10 (-0.23 - 0.44)	0.092 (-0.22 - 0.41)
Regulatory								
IL-10	0.086 (-0.25 - 0.42)	0.068 (-0.28 - 0.42)	-0.051 (-0.38 - 0.28)	-0.15 (-0.48 - 0.17)	0.054 (-0.28 - 0.39)	0.001(-0.34 - 0.35)	0.11 (-0.22 - 0.44)	0.021 (-0.31 - 0.35)
Adaptive								
IFN-γ	0.014 (-0.32 - 0.35)	-0.032 (-0.41 - 0.35)	0.13 (-0.39 - 0.27)	-0.28 (-0.63 - 0.06)	0.001 (-0.33 - 0.33)	-0.11(-0.49 - 0.26)	0.10 (-0.22 - 0.44)	-0.067 (-0.43 - 0.29)
IL-2	-0.077 (-0.41 - 0.25)	-0.12 (-0.49 - 0.23)	0.26 (-0.06 - 0.58)	0.15 (-0.18 - 0.49)	-0.017 (-0.35 - 0.32)	-0.10(-0.46 - 0.25)	**0.46 (0.16 - 0.76)**	**0.38 (0.069 - 0.70)**
IL-7	-0.22 (-0.55 - 0.10)	-0.25 (-0.58 - 0.084)	-0.31 (-0.71 - 0.086)	-0.31 (-0.71 - 0.094)	-0.18 (-0.51 - 0.14)	-0.22(-0.56 - 0.10)	**0.35 (0.041 - 0.67)**	0.30 (-0.007 - 0.61)
IL-15	-0.016 (-0.35 - 0.32)	-0.035 (-0.38 - 0.31)	-0.068 (-0.40 - 0.26)	-0.14 (-0.47 - 0.17)	-0.048 (-0.38 - 0.28)	-0.094(-0.43 - 0.24)	0.18 (-0.14 - 0.51)	0.11 (-0.21 - 0.44)

[a]β-coefficient (95% confidence interval) linear regression analysis of T cell activation subsets and cytokines/chemokines;
[b]Adjusted β-coefficient (95% confidence interval) for ARV treatment
Red-shaded cells represent significant associations

4.4 Discussion

This study investigated the concentrations of cytokines and chemokines in the male genital tract, using semen samples, in order to describe the immune milieu of this compartment in the context of HIV infection. Analyte levels were compared between blood plasma and semen, and between HIV-infected and uninfected men. The main findings of this study were that (i) semen and blood differed substantially with respect to the cytokine milieu, with the majority of cytokines and chemokines found at higher concentrations in semen than blood, both in HIV-infected and uninfected men; (ii) there were no significant differences between any cytokines/chemokines in the semen of HIV-infected vs uninfected men after rigorous statistical analysis; (iii) although specific differences at the individual cytokine level were not evident between HIV-infected and uninfected men, HIV-infection caused a dysregulation of the network of cytokine/chemokine associations in semen; (iv) no significant associations were found between levels of cytokines/chemokines and seminal viral load after rigorous statistical analyses; and (v) investigating relationships between T cell activation and cytokine levels revealed that higher levels of G-CSF in semen of HIV-infected men were associated with higher levels of $CCR5^{+}CD8^{+}$ T cells in semen.

Why would the cytokine milieu differ so substantially between blood and the genital tract? Being a mucosal surface, exposed to the outside environment, the genital tract is likely to differ intrinsically from the systemic compartment. It is a mucosal effector site to which immune cells from the periphery traffic for immune defence, in response to chemokine gradients, and therefore contains a range of immune molecules involved in recruitment, maturation, and signalling of different immune cells (Kutteh *et al*, 2005). The differences may also be due to the reproductive functions associated with the male genital tract. Semen is a reproductive fluid and contains substances involved in immune regulation, such as those inducing immune tolerance to spermatozoa within the male genital tract itself, or dampening inflammatory responses to seminal components after semen deposition in the vagina (Kutteh *et al*, 2005). Particular immune proteins such as TGF-β, present in very high levels, have been shown to have a role in reproduction (Robertson *et al.*, 2002, Robertson 2005).

Of all the cytokines and chemokines assayed in this study, monocyte chemoattractant protein-1 (MCP-1/CCL2) was found in the highest concentrations in semen, regardless of HIV infection status. The concentration of MCP-1 was approximately 60 times higher in semen than in blood plasma. MCP-1 is a chemokine for monocytes, T cells, and dendritic cells, produced by various cell types, including monocytes themselves, as well as non-haematopoeitic cells such as endothelial and epithelial cells, fibroblasts and smooth muscle cells (Cushing *et al.,* 1990, Standiford *et al.,* 1991). MCP-1 regulates the infiltration of monocytes, memory T cells, dendritic cells and NK cells (Deshmane *et al.,* 2009). In HIV-1 infection, plasma MCP-1 levels have previously been correlated with viral load (Weiss *et al.,* 1997). This study did not show any association with viral load. In fact high MCP-1 levels were present in those with viral suppression on ART, and in HIV-uninfected participants. This may reflect the existing high levels of inflammation in the male genital tract, possibly due to continuous exposure of this mucosal surface to sexually-transmitted pathogens. Recruitment of the additional producers of MCP-1, monocytes and macrophages (Cinque *et al.,* 1998), likely only serves to enhance the levels of this chemokine in semen. An alternative explanation may be that this chemokine is found at high concentrations in semen naturally. Highly concentrated levels of MCP-1 were also found in semen in two other studies (Politch *et al.,* 2007, Anderson *et al.,* 2010). In the Politch study, healthy men expressed high levels of MCP-1 in semen; thus, the levels that we have detected in this study may represent constitutively high expression levels of MCP-1, and not necessarily elevation due to concurrent infections or conditions.

What may be the consequences for HIV acquisition? It has been shown that memory $CD4^+$ T cells express CCR2 at high levels and therefore MCP-1 recruitment can facilitate HIV-1 infection of these cells (Matsushima *et al.,* 1989). High MCP-1 levels may therefore recruit potential target cells, thereby facilitating the replication and spread of HIV. If this is the case, this opens up potential avenues for modulating leukocyte infiltration in the genital tract; doxycycline and leukotriene receptor antagonists (LTRAs) can downregulate MCP-1 or block chemotaxis via MCP-1 (Deshmane *et al.,* 2009). However, these approaches must always take into account the potential for pleiotropic effects and the beneficial role of MCP-1 in protective immune responses or normal functioning in the male genital tract. Endogenous MCP-

1 in the female genital tract has been shown to be up-regulated by exposure to seminal plasma in a dose-dependent manner (Sharkey *et al.,* 2007), indicating that MCP-1 may also play a role in recruiting immune cells to the vaginal mucosa in the female genital tract subsequent to ejaculation, which may have a role in HIV transmission.

Although not as dramatically elevated as MCP-1, levels of IL-8, Fractalkine, GM-CSF, IL-7 and IL-15 were more than 5-fold higher in semen than in blood regardless of HIV status. Fractalkine, produced by endothelial venule cells as well as dendritic cells, can recruit monocytes, $CD8^+$ T cells and NK cells to sites of inflammation (Fong *et al.,* 1998). GM-CSF, produced by T cells, macrophages and endothelial cells are mainly responsible for the maturation of progenitors into dendritic cells and monocytes (Abbas & Lichtman 2007). IL-15 is produced mainly by monocytes to stimulate the proliferation of NK cells and also functions as a T cell growth factor (Mueller & Katsikis 2010). These findings confirm those of Politch *et al.* (2009) and Anderson *et al*. (2010), who also found higher levels of IL-7 and IL-8 in semen regardless of HIV infection. IL-7 plays a role in T and B cell development and is secreted by stromal cells in many tissues (Chahroudi & Silvestri 2010). Specifically, IL-7 plays a role in supporting $CD4^+$ and $CD8^+$ T cells, binding to CD127 (the IL-7 receptor) to enhance T cell survival. In semen, high levels of IL-7 are likely to play a similar role. IL-8 is a chemokine that attracts neutrophils to sites of inflammation upon production by mast cells, endothelial cells and monocytes (Sheridan *et al.,*1997, Male 2006). IL-8 has been associated with the enhancement of HIV-1 replication (Lane *et al.,* 2001). In HIV-infected individuals these authors also found high levels of TGFβ, SDF1α, IL-5 and monokine induced by IFNγ (MIG) in semen, which was not tested for in the present study. In the present study higher levels of RANTES were found in blood compared to semen, reflecting the same finding as Anderson *et al.* (2010). Thus, the male genital tract contains high levels of immune mediators involved in the recruitment, maturation and survival of immune cells at this site.

Differences in the cytokine and chemokine levels in the semen of HIV-uninfected, infected and antiretroviral-treated men were investigated. Surprisingly, there were no significant differences between the levels of cytokines and chemokines in semen of the three groups investigated. One explanation is that pre-existing levels of inflammation in the seminal compartment may have masked any significant

differences between cytokine and chemokine levels in HIV-uninfected and infected men. There was, however, evidence of dysregulation of cytokine networks in HIV-infected men compared to uninfected men. Regulation of cytokines and chemokines is complex. The dysregulation of proinflammatory cytokines and chemokines in HIV infection has been reported previously (reviewed in Decrion *et al.,* 2005). In the present study the associations and interactions between cytokines and chemokines in semen of HIV-uninfected men highlights the complexity of their regulation, and how HIV may disrupt this.

Previous studies have described an association between seminal viral loads and levels of IL-1β, IL-6, IL-12, IFNγ and RANTES (Sheth *et al.,* 2005, Storey *et al.,* 1999, Berlier *et al.,* 2006a). In the present study, plasma levels of TNFα and IFNγ levels were positively associated with plasma viral load. In semen, however, there were no significant relationships evident between seminal viral load and the cytokines and chemokines measured. The present study differed from previous smaller studies in several aspects; here a larger number of cytokines were assayed (20); statistical analyses were rigorous, adjusting for multiple comparisons for all tests. An additional explanation could be that other genital tract co-infections (discussed below) may have created a high background inflammatory 'noise' which precluded the ability to link HIV replication with any particular immune mediators.

HIV-induced immune activation can lead to enhanced HIV replication, promoting further cellular activation. This cycle is further exacerbated by pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα working synergistically to elevate levels of activation (Appay & Sauce 2008, Decrion *et al.,* 2005). In the present study, the influence of levels of inflammatory, regulatory and adaptive cytokines and chemokines on cellular activation was assessed. In semen, enhanced levels of granulocyte colony stimulating factor (G-CSF) were found to be associated with higher levels of $CD8^{+}CCR5^{+}$ T cells in HIV-infected men, independent of seminal viral load. G-CSF is a pro-inflammatory cytokine produced by macrophages and endothelial cells at sites of infection and attracts and promotes the survival of neutrophils (Abbas & Lichtman 2007). G-CSF may function in the same way in the male genital tract, where the presence of high levels of neutrophils is important in innate immune protection against pathogenic bacteria. In fact, in this study (Chapter

2), high levels of neutrophils were found in semen. The association of $CD8^+CCR5^+$ with G-CSF may thus be indirect, related to underlying bacterial co-infections in HIV-infected men, rather than with HIV replication.

The present study had a number of limitations. The possible (and likely) presence of sexually-transmitted infections (STIs) that could have contributed to genital tract inflammation was not assessed. A high incidence of HSV-2 seroprevalence (17%), chlamydia (12.4%), syphilis (8.1%) and gonorrhoea (3.3%) infection have been demonstrated among men, many going untreated, leading to some describing a "hidden epidemic of STIs" in South Africa (reviewed in Johnson *et al.*, 2005). Ongoing genital tract inflammation due to concurrent STIs may have masked any effects that HIV replication alone may have had on inflammation and cellular activation in this compartment. A further limitation was that only 12 men among the HIV-infected cohort were on ART, weakening statistical power in this group. Furthermore, we limited the study to detecting the 20 cytokines in the Milliplex MAP kits. The presence of other cytokines and chemokines in seminal plasma, which may be important in the context of HIV-infection and inflammation, needs further investigation. These include MIP-3α, shown to be important in the female genital tract for recruitment of target cells during initial HIV acquisition (Haase 2010), and upregulated by semen in the vagina (Berlier *et al.,* 2006b); and TGF-β, previously described to be at high levels in semen (Politch *et al.,* 2007). TGF-β is known to play a role in reproduction, as well as a central role in T_H17 cell differentiation. These cells produce IL-17, which has an important role in neutrophil mobilisation and the expression of antimicrobial factors (Campillo-Gimenez *et al.,* 2010) and has not been characterised in semen. T_H2 cytokines such as IL-4, IL-5 and IL-13 could provide further insight into the critical balance between pro- and anti-inflammatory cytokines during HIV infection. A future study could include measuring these analytes by high sensitivity ELISA or an extended multiplex panel.

Taken together, the data in this chapter demonstrate that the immune milieu of the genital tract differs substantially from blood, with the majority of cytokines and chemokines tested elevated in semen. However, unexpectedly, there were no striking differences in the levels of cytokines and chemokines in the semen of HIV-infected and uninfected men, or HIV-infected men on suppressive ART. Thus, even in the

absence of HIV infection, the male genital tract appears to maintain a state of inflammation in the participant population that was studied, which may have been the result of undetected and untreated co-infections. This inflammation may have masked any additional effect of HIV replication. However, comparisons of relationships between cytokines in the seminal compartment of HIV-uninfected and infected men, indicated that a disruption of interrelated associations may be due to dysregulation of cytokine and chemokine levels as a result of HIV infection. No associations between any cytokines and HIV replication levels in semen were evident after adjustment for multiple comparisons. When investigating the impact of inflammation in the male genital tract on cellular immune activation, only G-CSF was linked to increases in CCR5-expressing $CD8^+$ T cells. This illustrates that the relationship between inflammation, cellular activation and viral replication is unlikely to be simplistic.

CHAPTER 5

Discussion

The largest HIV-1 epidemic in the world is in sub-Saharan Africa, where the majority of HIV transmission occurs through sexual contact (UNAIDS 2010). A prophylactic vaccine to prevent new HIV infections or effectively halt disease progression and transmission is urgently needed. In Chapter 2, functional T cells and other immune subsets were characterised in semen after developing optimal protocols to isolate these cells from semen. These protocols could be applied to assessing immunological responses in clinical trials of candidate HIV vaccines. Current efforts to develop an HIV vaccine acknowledge that both robust cellular immune responses and strong humoral immune responses may be needed for effective protection. These responses may need to be elicitied in the genital tract, where most infection takes place. Only a handful of studies have characterised HIV-specific cellular immune responses in the male genital tract, and this has been in the context of HIV infection and not vaccine trials. HIV-1 Env-, Gag- and Pol-specific $CD8^+$ T cells have been identified in semen (Quayle *et al.,* 1998). Sheth and colleagues found that $CD8^+$ T cell responses in semen did not correlate with levels of virus in semen, and concluded that these cells were not effectively controlling HIV replication locally (Sheth *et al.,* 2005). This is similar to findings in blood, where the overall frequency of responses may be a poor measure of effective HIV-specific responses, and that it is rather the functional nature or specificity for particular parts of the virus that dictate efficacy of T cell responses (Betts *et al.,* 2006; Ferre *et al.,* 2009; Kiepiela *et al.,* 2007). Although our study established optimised protocols for T and other immune cell isolation from semen, the yield of cells obtained is still too low to perform many or detailed immunological assessments. Establishing additional protocols to supplement these methodologies, such as expanding seminal T cells in culture, are warranted.

With regard to humoral immunity required to protect against HIV infection, this translates into the presence of local broadly neutralising HIV-specific antibodies in the genital tract. In Chapter 2 of the present study, B cells were found in far greater numbers in semen than T cells. Previous studies have shown that the major antibody isotype in seminal plasma was IgG (Moldoveanu *et al.,* 2005), although characterisation of penile washes suggested a preponderance of IgA (Anderson *et al.,* 2011). Very little is known about HIV-specific B-cell responses in the male genital tract and whether HIV-specific neutralising antibodies are found within the male genital tract and its secretions in infected men. Clearly, there are still major gaps in

our knowledge concerning HIV infection and the immunology of the male genital tract that are important areas for future study.

During sexual transmission, semen remains the major carrier of HIV and contains cell-free and cell-associated HIV (Doncel *et al.,* 2011). There is strong evidence indicating that the main factor that influences transmission is the viral load in semen, which peaks during acute and again during late stages of infection in untreated disease (Tachet *et al.,* 1999; Pilcher *et al.,* 2007). During early HIV infection, seminal viral load can reach a peak level of 4.5 $\log_{10}$ copies/ml (Pilcher *et al.,* 2007). In sero-discordant couples where the uninfected partners became infected, 80% of the infecting partners had plasma viral loads of >10 000 HIV RNA copies/ml, whereas no transmissions were found when the infected partner's viral load was <1500 HIV RNA copies/ml (Quinn *et al.,* 2000). In the present study, 13/26 ARV-naïve men (50%) had plasma viral loads of >10 000 HIV RNA copies/ml, whereas 6/26 (23%) men had <1500 HIV RNA copies/ml. In semen, 50% of ARV-naïve men had viral loads >1500 HIV RNA copies/ml. Highly active antiretroviral treatment (HAART) is successful in lowering viral load systemically and in semen (Vernazza *et al.,* 2000), although genital HIV shedding was evident in 2 out of 12 men on ARV treatment in the present study. This illustrates the substantial risk of HIV transmission to unprotected partners posed by genital HIV shedding both without and even with ARV treatment.

An unexpected finding from this study was the large degree of immune activation in semen, even in the absence of HIV infection. Levels of immune activation were highest in semen of untreated HIV-infected men and levels of seminal immune activation were substantially higher in semen than blood regardless of HIV status. Genital tract inflammation was assessed by studying various pro-inflammatory and other immune mediators, and consistent with the T cell activation findings, levels of seminal inflammation were higher than systemic levels regardless of whether men were HIV infected or not. In the genital tract, one of the major factors causing inflammation is sexually transmitted co-infections (Johnson *et al.,* 2009). In South Africa, syndromic management of STIs has reduced STI prevalence among both sexes (Johnson *et al.,* 2011); however, it appears clear from this study that there are either a large amount of untreated STIs, or asymptomatic STIs in this study population, giving

rise to persistent inflammation and T cell activation. This may enhance recruitment of HIV target cells to the genital tract or act locally in activating resident T cells, elevating the risk of HIV acquisition in the male genital tract. In HIV-infected men, this may give rise to greater shedding of HIV. For example, co-infection with HSV-2 increases seminal HIV viral load (Lingappa *et al.,* 2010), and sub-clinical asymptomatic HSV-2 replication may attract HIV target cells to the genital tract (Zhu *et al.,* 2009).

Levels of genital tract immune activation in healthy women in sub-Saharan Africa have been shown to be elevated compared to levels of immune activation in their counterparts in the United States (Cohen *et al.,* 2010), even after taking the effect of common STIs into account. This is an intriguing finding and warrants further study. Although the present study did not control for the effects of common STIs or compare levels of immune activation between local and non-local participants, this observation may account for the unexpectedly high levels of immune activation and CCR5-expressing target cells in the semen of HIV-uninfected men, and may provide a partial explanation for the higher incidence of HIV infection among men in sub-Saharan Africa.

Although the immune cells within semen may be important for HIV infection, there are limitations to studying semen as a representative of the entire male genital tract. Other tissues of the male genital tract may be very important in HIV acquisition, such as the foreskin and penile urethra. Only three studies have investigated HIV infection using *in vitro* models of the male genital tract. Patterson *et al.* (2002) showed that HIV preferentially infects Langerhans cells and CCR5-expressing $CD4^+$ T cells of the inner foreskin. Fischetti *et al*. (2009) introduced HIV to foreskin, glans, meatus and urethral tissue, and found that R5-tropic HIV strains infected all these tissues equally well. Ganor and colleagues (2010) developed *in vitro* models using inner and outer foreskin tissue as well as layered foreskin epithelia and exposed these to free HIV virions or HIV-infected cells. The inner foreskin was preferentially infected by HIV-infected cells through the formation of synapses with keratinocytes, and virions that budded from these synapses were internalised by Langerhans cells, that then migrated to the dermal layer where HIV was transferred to T cells. These studies not only emphasize the need for modeling penile infection in men to elucidate the early events

of HIV infection in the male genital tract, but also highlight the fact that activated $CD4^+$ T cells and other cell types such as Langerhans cells in these 'frontline' tissues may be more important in the susceptibility to infection than activated T cells elsewhere in the male genital tract, that were measured in semen in the present study. On the other hand, amplification of initial foci of HIV infection in the male genital tract may be due to the pool of activated $CD4^+$ T cells and other cell types locally, that then migrate to the rest of the body and establish infection. In addition, although removal of the foreskin may reduce HIV target cells and therefore decrease infection in up to 60% of men, activated $CD4^+$ T cells and other cell types in uncircumcised men in other tissues may then contribute to establishing productive infection. To date, animal models characterising early transmission events have been limited to SIV infection of the female macaque genital tract, and early events in male genital tissue are largely unknown. Recently, two groups described the development of macaque models of penile HIV transmission, and these promise to yield important new insights into early events following HIV infection of the penis (Keele 2011; Yeh 2011).

A controversial question in HIV transmission remains the issue of to what extent cell-associated HIV transmission, in addition to infection by cell-free viral particles, is responsible for establishing a productive infection. HIV-infected cells may be important vectors in transmission of HIV-1 (reviewed in Anderson *et al.,* 2010), and a recent macaque model of cell-associated infection was developed, and demonstrates evidence of cell-associated HIV transmission may indeed occur (Salle *et al.,* 2010). In contrast, another recent study in men who have sex with men (MSM), provided evidence that the cell-free HIV component of semen, and not the cell-associated fraction, was responsible for productive HIV infection (Butler *et al.,* 2010). If indeed cell-associated transmission of HIV occurs, one of the major questions is what are the events that lead to this type of HIV transmission, and how can HIV prevention strategies target these (Anderson *et al.,* 2010). Thus, more research is needed to clear up this controversy.

In conclusion, this study has contributed to research on the male genital tract by optimising methodologies for the isolation of immune cells from semen that may be applied to vaccine trials, and the identification of a range of immune cell types in semen, opening up areas of future research on innate and humoral immunity in the

male genital tract. Furthermore, characterisation of immune activation and levels of CCR5-expressing potential $CD4^+$ target cells in semen provides novel insights into immune activation within the male genital tract of HIV-uninfected and infected men. Finally, a comprehensive, multivariate analysis of levels of inflammatory markers and other immune mediators in semen highlights major differences with the systemic compartment, and illustrates their relationship with HIV replication.

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APPENDIX

Table S1 Assay sensitivities. The minimum detectable concentration for the two assayed used are listed.

Kit	Cytokines/ chemokines	MinDC[a]
High Sensitivity:	**IL-1β**	0.06
	IL-2	0.16
	IL-6	0.10
	IL-7	0.12
	IL-12p70	0.11
	IL-10	0.15
	TNFα	0.05
	IFNγ	0.29
	GM-CSF	0.46
Normal Sensitivity:	**IL-1α**	3.50
	sIL-2Rα	4.40
	IL-8	0.20
	IL-12p40	10.5
	IL-15	0.40
	Eotaxin	1.20
	Fractalkine	6.00
	G-CSF	0.50
	MCP-1	0.90
	MIP-1α	3.50
	MIP-1β	4.50
	RANTES	1.00

[a]Minimum detectable concentration, pg/ml

Table S2 Comparisons between levels of cytokines and chemokines in the semen of HIV-uninfected, HIV-infected (ARV naïve) and HIV-infected (ARV-treated) men.

Cytokines/chemokines		HIV-uninfected[a]	ARV naïve[a]	ARV-treated[a]	p-value[b]	p-value[c]	p-value[d]
Pro-inflammatory:	**IL-1α**	16.3 (5.5 – 32.9)	17.2 (5.3 – 79.3)	17.4 (6.5 – 82)	0.26	0.45	0.98
	IL-1β	0.25 (0.08 – 0.4)	0.17 (0.05 – 0.69)	0.32 (0.044 – 1)	0.60	0.69	0.71
	IL-6	32.5 (6.8 – 118.3)	22 (8.7 – 122)	33.4 (7.3 – 147.2)	0.92	0.92	0.86
	IL-8	1 486 (241 – 2 771)	1 110 (643 – 2 115)	1 141 (540.5 – 2 947)	0.59	0.99	0.77
	IL-12p40	2.8 (1.3 – 3.5)	1.9 (1.4 – 3.5)	3.1 (1.78 – 9.85)	0.75	0.088	0.13
	IL-12p70	0.05 (0.03 – 1.9)	0.036 (0.02 – 0.99)	1.2 (0.036 – 2.57)	0.25	0.33	0.12
	TNF-α	1.82 (0.79 – 4.5)	1.2 (0.57 – 3.95)	1.88 (0.27 – 5.9)	0.67	0.73	0.86
	Eotaxin	23 (4.4 – 38.9)	16.3 (8.6 – 37.9)	28.2 (23.3 – 41.3)	0.76	0.19	0.11
	Fractalkine	802.9 (333 – 1 636)	655.8 (251 – 1 489)	875.6 (250 – 1 019)	0.42	0.55	0.94
	MCP-1	14 110 (3 908 – 31 028)	9 805 (3 211 – 18 029)	17 720 (7 486 – 27 430)	0.097	0.92	0.16
	MIP-1α	3.15 (1.3 – 4.9)	7.5 (2.3 – 36)	15.7 (5.5 – 86.4)	**0.045**	**0.0076**	0.35
	MIP-1β	78.1 (31.9 – 112.2)	53 (35.4 – 171.6)	83.4 (30.2 – 195.8)	0.85	0.72	0.78
	RANTES	370.9 (189.2 – 649.3)	266.5 (98.6 – 818.3)	419.5 (178.6 – 1 239)	0.59	0.67	0.2
	G-CSF	29.5 (12.2 – 60.5)	21.2 (7.5 – 84.3)	20 (12.9 – 58.1)	0.23	0.65	0.73
	GM-CSF	2.4 (0.65 – 7.35)	2.9 (1.6 – 6.2)	5.05 (2.1 – 14.7)	0.51	0.26	0.25
Regulatory:	**IL-10**	27.1 (7.1 – 52.4)	10.9 (2.02 – 17.5)	7.7 (0.8 – 24.5)	**0.022**	0.053	0.85
Adaptive:	**IFN-γ**	6.3 (2.7 – 21.9)	5.6 (1.9 – 10.2)	9.8 (0.78 – 40.5)	0.57	0.73	0.55
	IL-2	0.4 (0.03 – 2.56)	0.2 (0.026 – 1.22)	1.6 (0.022 – 3.52)	0.52	0.88	0.47
	IL-7	786.4 (395.9 – 1 930)	715.8 (347 – 1 865)	524.4 (414 – 2 011)	0.72	0.47	0.86
	IL-15	27.8 (8.8 – 45.4)	27.6 (10.1 – 64.8)	30.7 (27.6 – 39.5)	0.56	0.54	0.84

[a]Median (IQR); Mann-Whitney t test for comparison of unmatched groups, p-values ≤ 0.05 were considered significant and are in bold

[b]Comparison between HIV-uninfected and ARV naïve

[c]Comparison between HIV-uninfected and ARV-treated

[d]Comparaison between ARV naïve and ARV-treated

Table S3 Comparisons between levels of cytokines and chemokines in the blood of HIV-uninfected, HIV-infected (ARV naïve) and HIV-infected (ARV-treated) men.

Cytokines/chemokines		HIV-uninfected[a]	ARV naïve[a]	ARV-treated[a]	p-value[b]	p-value[c]	p-value[d]
Pro-inflammatory:	**IL-1β**	0.15 (0.04 – 0.33)	0.26 (0.012 – 0.59)	0.09 (0.012 – 0.35)	0.15	0.44	0.087
	IL-6	6.5 (4 – 12.3)	6.6 (4 – 14.1)	5.5 (3.2 – 9.2)	0.89	0.43	0.43
	IL-8	2.3 (0.27 – 4.7)	2.5 (1.07 – 3.7)	2.8 (0.04 – 9.2)	0.76	0.84	0.73
	IL-12p40	5.4 (0.47 – 44.7)	0.47 (0.47 – 46.1)	0.47 (0.47 – 3.07)	0.62	0.12	0.49
	IL-12p70	0.01 (0.01 – 0.17)	0.01 (0.01 – 1.16)	0.01 (0.01 – 0.01)	0.37	0.51	0.22
	TNF-α	5.9 (4.6 – 7)	10.1 (7.02 – 12.5)	7.6 (6.4 – 9.8)	**0.0001**	**0.019**	0.18
	Eotaxin	89.1 (37.8 – 182.8)	89.2 (59 – 195.1)	49.8 (44.2 – 68.3)	0.42	0.24	**0.0087**
	Fractalkine	42.6 (11.1 – 102.3)	42.6 (18.8 – 82.4)	0.59 (0.59 – 42.6)	0.66	**0.013**	**0.025**
	MCP-1	216.7 (174.1 – 301.1)	221.3 (168.4 – 364.7)	220.3 (155.2 – 303.8)	0.49	0.82	0.54
	MIP-1α	1.95 (1.95 – 9.52)	1.95 (1.95 – 7.24)	1.95 (1.95 – 5.9)	0.49	0.5	0.79
	MIP-1β	8.7 (1.5 – 12.7)	3.01 (1.5 – 12.7)	5.1 (1.5 – 16.1)	0.86	0.69	0.77
	RANTES	2 809 (1 997 – 3 156)	2 663 (2 080 – 3 207)	2 637 (2 483 – 3 332)	0.99	0.79	0.96
	G-CSF	25.8 (15.5 – 47.8)	40.02 (24.6 – 53.2)	23.3 (15.5 – 30.7)	0.084	0.35	**0.0077**
	GM-CSF	0.01 (0.01 – 0.86)	0.26 (0.01 – 0.69)	0.02 (0.01 – 0.69)	0.48	0.97	0.67
Regulatory:	**IL-10**	1.26 (0.88 – 2.02)	2.02 (1.26 – 2.8)	1.07 (0.88 – 1.5)	**0.017**	0.83	**0.025**
Adaptive:	**IFN-γ**	0.86 (0.37 – 2.9)	2.4 (0.86 – 7.8)	1.21 (0.37 – 1.33)	0.052	0.56	**0.021**
	IL-2	0.1 (0.01 – 0.98)	0.47 (0.05 – 1.39)	0.08 (0.01 – 0.86)	0.20	0.74	0.13
	IL-7	3.12 (1.27 – 7.44)	3.13 (1.5 – 5.26)	1.91 (0.54 – 4.9)	0.81	0.24	0.26
	IL-15	0.37 (0.25 – 1.25)	0.25 (0.25 – 1.25)	0.25 (0.25 – 0.25)	0.62	**0.033**	0.11

[a]Median (IQR); Mann-Whitney t test for comparison of unmatched groups, p-values ≤ 0.05 were considered significant and are in bold

[b]Comparison between HIV-uninfected and ARV naïve

[c]Comparison between HIV-uninfected and ARV-treated

[d]Comparaison between ARV naïve and ARV-treated

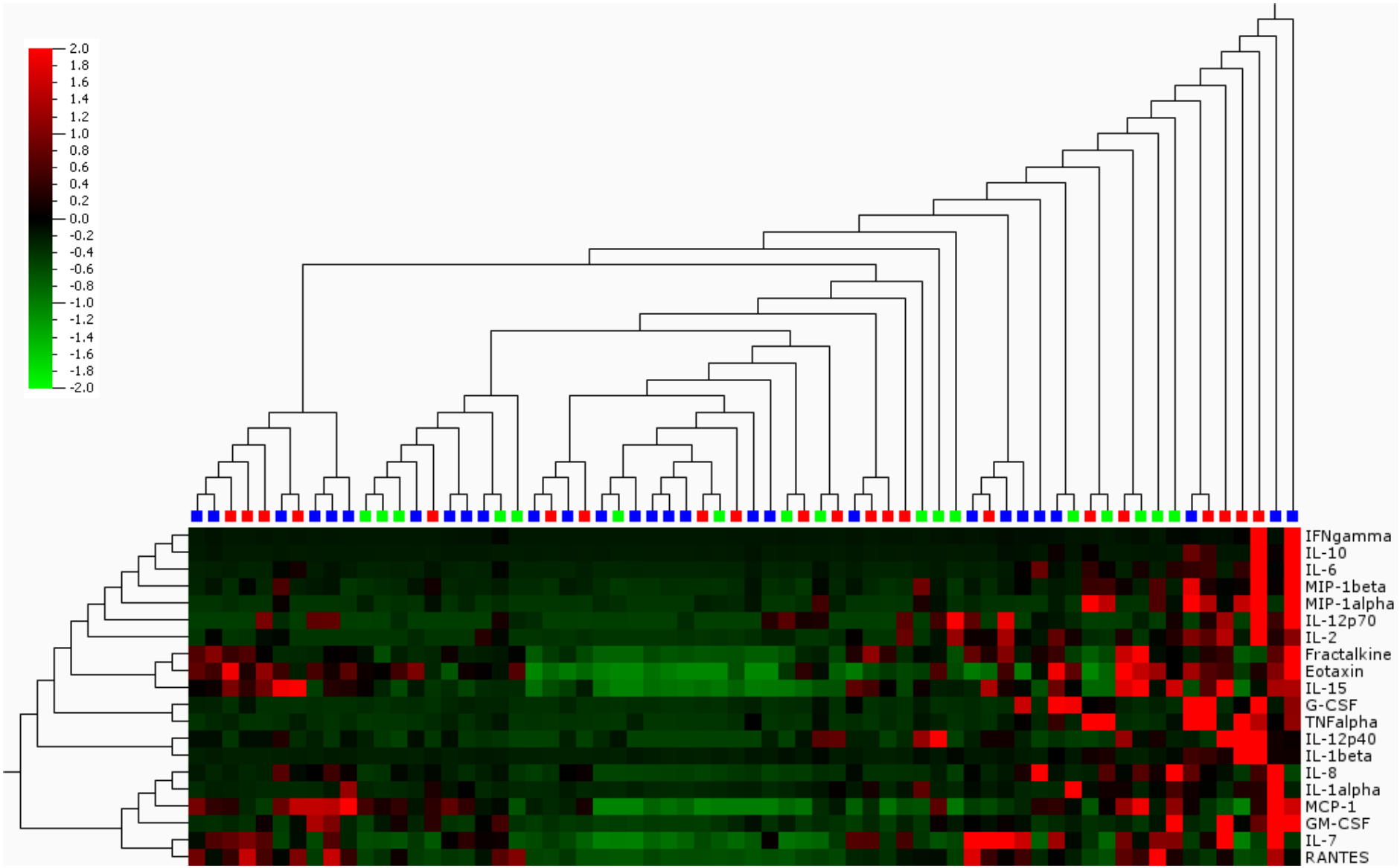

Figure S1 Heat map of cytokine and chemokines assayed in semen. Shown are the cytokine and chemokine responses in the semen of all participants ordered by hierarchical clustering (Spearman correlation with average linkage) creating a condition tree. Coloured squares at the top of each profile indicate HIV-uninfected (blue), HIV-infected men with detectable viral loads (red) and HIV-infected men with undetectable viral loads (green).

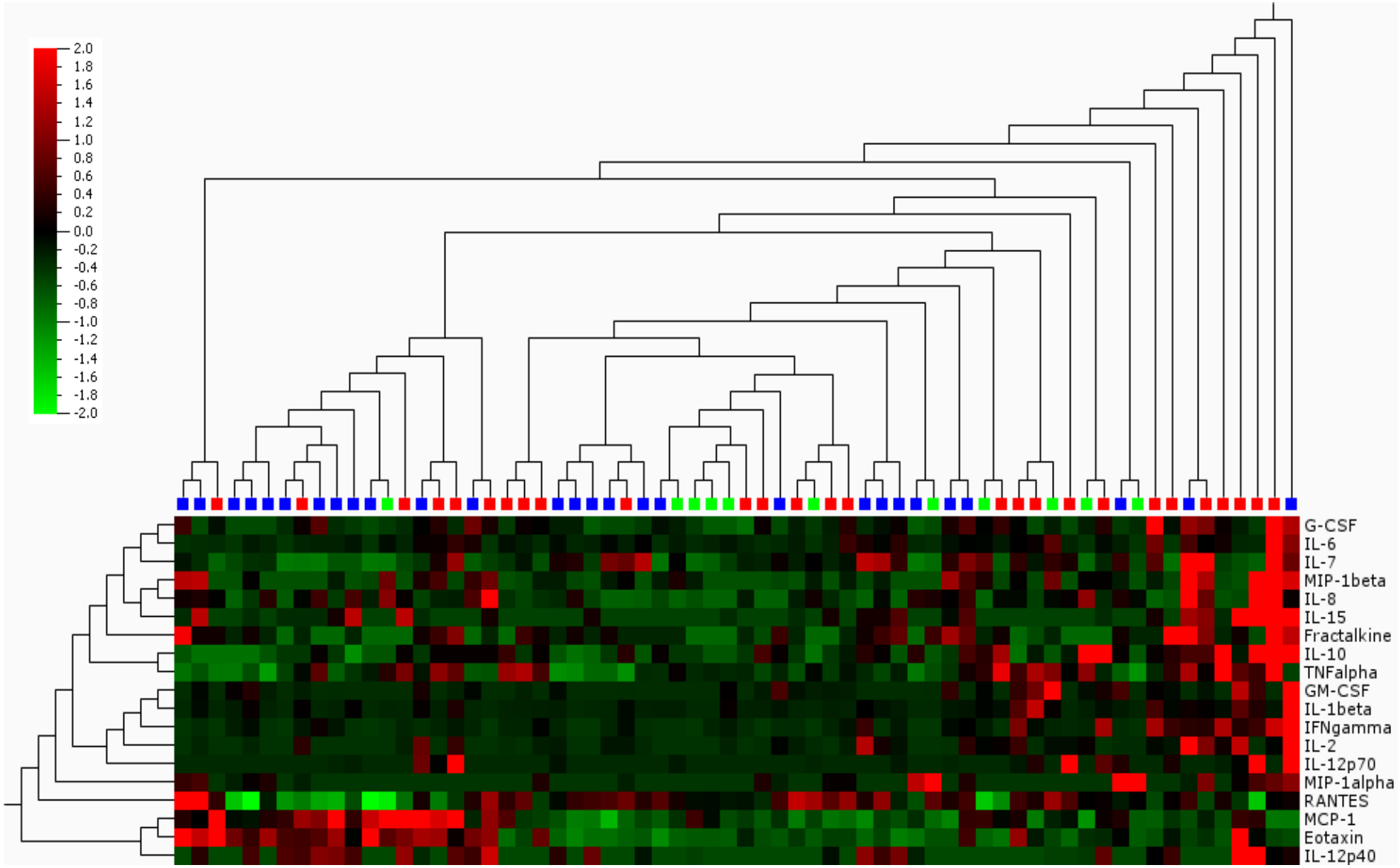

Figure S2 Heat map of cytokine and chemokines assayed in blood. Shown are the cytokine and chemokine responses in the blood of all participants ordered by hierarchical clustering (Spearman correlation with average linkage) creating a condition tree. Coloured squares at the top of each profile indicate HIV-uninfected (blue), HIV-infected men with detectable viral loads (red) and HIV-infected men with undetectable viral loads (green).

Table S4 Correlations between activated T cell subsets and cytokines/chemokines in semen of HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men.

Semen	HIV-uninfected[a]				HIV-infected, ARV-naïve[b]				ARV-treated[c]			
Cytokines/ chemokines	CD4		CD8		CD4		CD8		CD4		CD8	
Pro-inflammatory	CD38 Rho, p-value	CCR5 Rho, p-value	CD38 Rho, p-value	CCR5 Rho, p-value	CD38 Rho, p-value	CCR5 Rho, p-value	CD38 Rho, p-value	CCR5 Rho, p-value	CD38 Rho, p-value	CCR5[d] Rho, p-value	CD38 Rho, p-value	CCR5 Rho, p-value
IL-1α	0.046, 0.79	0.12, 0.57	0.2, 0.21	0.21, 0.27	0.051, 0.82	-0.047, 0.86	0.13, 0.51	-0.047, 0.86	**-0.73, 0.024**	-	-0.41, 0.21	0.35, 0.33
IL-1β	-0.26, 0.22	-0.15, 0.59	-0.079, 0.69	0.027, 0.91	0.014, 0.95	-0.33, 0.19	0.34, 0.093	-0.077, 0.73	**-0.7, 0.035**	-	0.009, 0.98	**0.71, 0.021**
IL-6	0.067, 0.75	0.15, 0.59	0.28, 0.16	0.16, 0.48	-0.088, 0.69	-0.31, 0.23	0.06, 0.77	-0.1, 0.64	-0.53, 0.14	-	-0.38, 0.25	0.15, 0.68
IL-8	-0.026, 0.88	0.042, 0.84	0.29, 0.073	0.094, 0.61	0.28, 0.19	0.14, 0.59	-0.25, 0.22	0.14, 0.59	-0.48, 0.18	-	-0.3, 0.37	0.25, 0.49
IL-12p40	-0.003, 0.99	0.053, 0.79	0.14, 0.4	0.087, 0.64	0.25, 0.25	0.035, 0.89	0.22, 0.28	0.035, 0.89	-0.48, 0.19	-	-0.12, 0.73	0.14, 0.7
IL-12p70	-0.0002, 0.99	0.2, 0.47	0.13, 0.52	-0.067, 0.77	0.17, 0.43	0.11, 0.67	0.36, 0.067	0.039, 0.86	-0.52, 0.15	-	-0.036, 0.92	0.59, 0.074
TNF-α	-0.18, 0.4	-0.047, 0.86	0.01, 0.96	0.23, 0.32	0.047, 0.83	-0.33, 0.2	0.2, 0.32	0.099, 0.66	-0.67, 0.049	-	-0.28, 0.4	0.45, 0.19
Eotaxin	0.28, 0.098	0.29, 0.15	**0.56, 0.0002**	0.39, 0.032	0.28, 0.18	0.078, 0.76	0.32, 0.11	0.078, 0.76	0.017, 0.97	-	0.24, 0.48	0.43, 0.21
Fractalkine	0.058, 0.74	0.1, 0.61	0.21, 0.2	0.21, 0.26	0.12, 0.56	-0.071, 0.79	-0.18, 0.38	-0.071, 0.79	-0.1, 0.8	-	-0.018, 0.96	0.39, 0.26
MCP-1	0.2, 0.24	0.29, 0.14	**0.45, 0.0044**	0.31, 0.087	0.15, 0.5	0.024, 0.93	-0.25, 0.22	0.025, 0.93	0.033, 0.93	-	-0.14, 0.69	0.006, 0.99
MIP-1α	0.05, 0.77	0.09, 0.66	0.26, 0.11	0.27, 0.14	0.24, 0.27	0.33, 0.2	0.039, 0.85	0.33, 0.2	-0.56, 0.13	-	-0.22, 0.51	0.2, 0.58
MIP-1β	0.042, 0.81	0.16, 0.45	0.047, 0.77	0.15, 0.41	0.26, 0.23	0.21, 0.42	-0.037, 0.86	0.21, 0.42	**-0.67, 0.049**	-	-0.064, 0.85	0.3, 0.4
RANTES	-0.014, 0.93	0.33, 0.099	0.14, 0.38	0.28, 0.12	0.14, 0.53	0.15, 0.57	-0.18, 0.39	0.15, 0.57	-0.033, 0.93	-	-0.31, 0.35	0.48, 0.16
G-CSF	0.049, 0.78	0.096, 0.64	0.12, 0.47	0.3, 0.11	0.14, 0.53	0.027, 0.92	-0.15, 0.45	0.027, 0.92	**-0.7, 0.035**	-	-0.054, 0.87	0.39, 0.26
GM-CSF	-0.12, 0.58	0.044, 0.87	0.091, 0.64	-0.09, 0.7	0.071, 0.75	-0.074, 0.78	0.26, 0.2	-0.094, 0.68	-0.18, 0.64	-	-0.25, 0.45	0.24, 0.51
Regulatory										-		
IL-10	-0.27, 0.19	0.1, 0.7	0.028, 0.88	0.33, 0.15	-0.21, 0.33	-0.24, 0.35	0.054, 0.79	0.23, 0.31	-0.53, 0.14	-	-0.25, 0.47	0.27, 0.45
Adaptive										-		
IFN-γ	-0.11, 0.61	0.12, 0.66	0.15, 0.43	0.17, 0.45	-0.21, 0.34	-0.022, 0.93	0.022, 0.91	0.09, 0.69	-0.43, 0.24	-	-0.25, 0.47	0.49, 0.15
IL-2	-0.27, 0.19	0.12, 0.66	-0.21, 0.29	0.25, 0.27	-0.27, 0.21	**-0.49, 0.046**	0.091, 0.66	-0.31, 0.15	-0.57, 0.11	-	-0.045, 0.89	0.55, 0.098
IL-7	-0.26, 0.21	0.24, 0.36	0.25, 0.36	0.079, 0.73	-0.098, 0.66	-0.17, 0.52	-0.13, 0.54	**-0.43, 0.045**	-0.1, 0.8	-	0.054, 0.87	0.39, 0.26
IL-15	0.077, 0.66	0.22, 0.27	0.22, 0.17	0.27, 0.14	0.069, 0.75	0.17, 0.51	-0.039, 0.85	0.17, 0.51	-0.033, 0.93	-	0.09, 0.79	0.38, 0.28

[a]N = 42; [b]N = 26; [c]N = 12; [d]Correlations not performed – only 4/12 ARV-treated men with $CD4^{+}CCR5^{+}$ T cell yields; Spearman Rank test withp-values ≤ 0.05 considered significant; Red shaded cells show significant p-values

Table S5 Correlations between activated T cell subsets and cytokines/chemokines in blood of HIV-uninfected, HIV-infected (ARV-naïve) and HIV-infected (ARV-treated) men.

Blood	HIV-uninfected[a]				HIV-infected, ARV-naïve[b]				ARV-treated[c]			
Cytokines/ chemokines	CD4		CD8		CD4		CD8		CD4		CD8	
Pro-inflammatory	CD38	CCR5	CD38	CCR5	CD38	CCR5	CD38	CCR5	CD38	CCR5	CD38	CCR5
	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value	Rho, p-value
IL-1β	-0.34, 0.079	0.19, 0.32	0.067, 0.73	0.2, 0.3	-0.018, 0.38	-0.052, 0.8	-0.066, 0.75	-0.27, 0.17	-0.021, 0.95	0.039, 0.9	0.35, 0.26	0.23, 0.47
IL-6	-0.15, 0.44	0.14, 0.47	-0.32, 0.098	0.22, 0.27	-0.048, 0.81	-0.087, 0.67	-0.13, 0.53	-0.26, 0.2	-0.12, 0.72	-0.028, 0.93	-0.1, 0.74	-0.07, 0.83
IL-8	-0.27, 0.095	0.16, 0.33	-0.11, 0.51	-0.11, 0.5	-0.049, 0.81	-0.18, 0.37	0.097, 0.64	-0.3, 0.13	-0.066, 0.84	0.5, 0.096	0.021, 0.94	0.021, 0.95
IL-12p40	-0.12, 0.45	0.13, 0.41	-0.29, 0.065	-0.085, 0.6	-0.13, 0.52	**0.43, 0.029**	-0.023, 0.91	0.012, 0.95	-0.083, 0.8	-0.031, 0.92	0.26, 0.41	-0.016, 0.96
IL-12p70	-0.038, 0.85	0.2, 0.31	-0.079, 0.69	0.21, 0.27	-0.29, 0.15	0.09, 0.66	-0.19, 0.35	-0.22, 0.28	**0.64, 0.024**	-0.11, 0.73	**0.65, 0.022**	-0.005, 0.99
TNF-α	0.01, 0.96	0.016, 0.94	-0.0008, 0.99	0.065, 0.74	0.17, 0.39	-0.31, 0.12	-0.042, 0.84	-0.34, 0.088	-0.063, 0.85	-0.13, 0.69	0.31, 0.33	0.14, 0.66
Eotaxin	-0.013, 0.94	0.054, 0.74	0.2, 0.21	-0.002, 0.99	-0.1, 0.62	0.13, 0.52	0.093, 0.65	-0.039, 0.85	-0.56, 0.06	0.34, 0.29	-0.52, 0.08	0.3, 0.34
Fractalkine	-0.11, 0.48	0.077, 0.64	0.057, 0.73	-0.045, 0.78	-0.21, 0.31	-0.095, 0.64	-0.044, 0.83	-0.35, 0.08	**0.64, 0.024**	-	0.23, 0.46	-0.19, 0.55
MCP-1	0.18, 0.24	0.048, 0.77	0.2, 0.23	-0.1, 0.53	-0.24, 0.24	0.33, 0.097	-0.21, 0.29	-0.12, 0.57	-0.33, 0.3	-0.39, 0.21	-0.36, 0.24	-0.48, 0.11
MIP-1α	0.056, 0.73	-0.27, 0.094	**0.35, 0.025**	-0.25, 0.12	-0.007, 0.97	-0.1, 0.62	0.19, 0.36	-0.046, 0.82	0.29, 0.36	0.37, 0.24	-0.064, 0.84	0.046, 0.89
MIP-1β	-0.17, 0.29	0.26, 0.11	0.25, 0.12	0.11, 0.49	0.26, 0.19	-0.12, 0.57	-0.19, 0.36	**-0.58, 0.0021**	-0.14, 0.67	-0.17, 0.6	0.041, 0.89	-0.064, 0.84
RANTES	-0.27, 0.46	0.26, 0.11	0.034, 0.83	0.034, 0.83	0.26, 0.2	0.14, 0.5	0.34, 0.87	0.26, 0.21	0.56, 0.058	0.42, 0.89	0.36, 0.25	0.36, 0.25
G-CSF	0.0054, 0.97	0.23, 0.15	-0.058, 0.72	0.049, 0.77	0.059, 0.77	0.084, 0.68	-0.11, 0.59	-0.28, 0.16	0.3, 0.33	-0.097, 0.77	0.49, 0.1	-0.068, 0.83
GM-CSF	-0.11, 0.59	0.15, 0.43	0.31, 0.11	0.25, 0.2	-0.15, 0.48	0.053, 0.8	-0.074, 0.72	-0.05, 0.81	-0.045, 0.89	0.22, 0.5	0.13, 0.68	0.24, 0.46
Regulatory												
IL-10	0.07, 0.81	-0.028, 0.89	-0.062, 0.75	0.22, 0.35	-0.08, 0.7	-0.15, 0.47	-0.23, 0.27	-0.19, 0.36	0.36, 0.25	-0.15, 0.64	**0.78, 0.0027**	0.12, 0.71
Adaptive												
IFN-γ	-0.077, 0.69	-0.026 0.89	0.021, 0.92	0.029, 0.88	-0.06, 0.77	-0.025, 0.9	-0.022, 0.91	-0.12, 0.54	-0.1, 0.75	0.018, 0.96	0.21, 0.51	0.18, 0.58
IL-2	-0.029, 0.88	0.096, 0.63	-0.023, 0.91	0.25, 0.2	0.15, 0.46	-0.12, 0.56	0.28, 0.17	-0.089, 0.66	0.17, 0.6	-0.022, 0.95	**0.62, 0.031**	0.13, 0.69
IL-7	-0.036, 0.86	0.083, 0.67	-0.044, 0.82	0.22, 0.25	-0.04, 0.85	-0.2, 0.33	-0.01, 0.96	-0.22, 0.29	**0.58, 0.048**	-0.26, 0.41	**0.68, 0.015**	-0.27, 0.39
IL-15	-0.3, 0.058	0.27, 0.097	-0.11, 0.5	0.062, 0.7	-0.047, 0.82	0.014, 0.95	0.13, 0.52	0.093, 0.65	0.21, 0.52	0.39, 0.21	-0.12, 0.7	0.37, 0.24

[a]N = 42; [b]N = 26; [c]N = 12; Spearman Rank test withp-values ≤ 0.05 considered significant; red shaded cells show significant p-values