IMPACT OF HIV INFECTION ON THE FREQUENCY AND PHENOTYPE OF TH17 CELLS IN THE FEMALE GENITAL TRACT

By Amy Leia Salkinder

A dissertation submitted in fulfilment of the requirements for the degree of Master of Science (Med) in the Department of Clinical and Laboratory Sciences, Division of Medical Virology, Faculty of Health Sciences, University of Cape Town

December 2010

Table of Contents

ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisor, Dr. Jo-Ann Passmore, for her patience, mentorship and knowledge throughout my MSc. You have been very approachable and without your enthusiasm and guidance this dissertation would not have been possible. I have learnt a lot from you and I could not ask for a better role model in the science field.

I would also like to sincerely thank all members of the mucosal immunology group: Pam, your advice on the FACS analysis and for your input during my thesis write-up. I would also like to thank you for all the advice you have given me for life in general. I would like to thank Lindi for her help with the Luminex assays. Without your patience, this crucial part of my project would not have been completed. I would like to thank Cobus, Lenine, Hoyam, Nono and Alfred for helping me with my lab work. Working with you all has been a great learning experience.

I would like to thank Nazma from the SATVI lab for LSRII training. I would also like to thank Associate Professor Willem Hanekom for use of the LSRII.

I would like to thank my wonderful family: my mom, thank you for always being there for me and to my dad, I know I would have made you proud. Ilana and Mia, I am proud to call you my sisters.

Lastly I would like to thank my funders: the Wellcome Trust, Investec Trust and the Poliomyelitis Research Foundation. Without their support this project would not have been possible.

LIST OF ABBREVIATIONS

ACD	Acetate Citrate Dextran
AIDS	Acquired Immune Deficiency Syndrome
ARV	Antiretroviral drug
APC	Allophycocyanin
APC-H7	Allophycocyanin-H7
BAL	Broncho-alveolar lavage
BFA	Brefaldin A
CCR4	Chemokine receptor 4
CCR5	Chemokine receptor 5
CCR7	Chemokine receptor 7
CS	Cellulose sulphate
CD	Cluster of differentiation
CEF	Cytomegalovirus, Epstein Barr virus and Influenza virus
CMCs	Cervical mononuclear cells
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
CXCR4	CXC chemokine receptor 4
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EAE	Extrinsic allergic encephalitis
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
EU	Endotoxin units
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGT	Female Genital Tract
FITC	Fluorescein isothyocyanate
FMO	Fluorescence minus one

FSC	Forward scatter
FSC-H	Forward scatter-height
Gag	Group specific antigen
GALT	Gut-associated lymphoid tissue
GATA	GATA-associated trans-acting protein
GIT	Gastrointestinal tract
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAART	Highly active antiretroviral therapy
HCL	Hydrocholoric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human Immunodeficiency Type-1
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IL	Interleukin
iTreg	Induced T regulatory cell
KCL	Potassium chloride
KOH	Potassium hydroxide
LAL	Limulus Amebocyte Lysate
LAP	Latency associated protein
LBP	Lypopolysaccharide binding protein
LN	Lymph node
LPS	Lipopolysacharide
LTNP	Long-term non-progressor
MALT	Mucosa-associated lymphoid tissue
mCD14	Membrane-bound CD14
mg	milligram
MIP-1β	Macrophage inflammatory protein-1 beta
MIP-1α	Macrophage inflammatory protein-1 alpha
ml	millilitre
NAb	Neutralizing Antibody
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Services
NICD	National Institute for Communicable Diseases

NIH	National Institute of Health
NK	Natural killer
nm	nanometer
NOS-2	Nitric oxide synthase-2
PE	Phycoerythrin
PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
pNA	p-nitroaniline
PMA	Phorbol myristate acetate
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
ROR	Retinoic acid receptor-related orphan receptor
SATVI	South African Tuberculosis Vaccine Initiative
sCD14	Soluble CD14
SIV	Simian Immunodeficiency Virus
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STI	Sexually transmitted infection
TCR	T cell receptor
Tc-17	CD8 Th17 effector cells
TER	Transepithelial electrical resistance
TGF-β	Transforming Growth Factor beta
Th	T helper
TNF-α	Tumor necrosis factor-alpha
TLR-4	Toll-like receptor 4
Treg	T regulatory cell
Vivid	Violet-fluorescent reactive dye
WPBTS	Western Province Blood Transfusion Services
µg	Microgram
µl	Microlitre
°C	Degree Celsius

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Background. T helper (Th) 17 cells have recently been implicated in regulating gut mucosal immunity during HIV infection by sustaining gut mucosal barrier integrity, although they do not respond to HIV directly. Depletion of Th17 cells from the gut mucosa during HIV infection has been suggested to contribute to elevated microbial translocation and immune activation. The role of Th17 cells in regulating genital mucosal immunity during HIV infection is less well described. The aims of this study were (1) to compare the frequency and phenotype of Th17 cells in the female genital tract and blood in uninfected compared to HIV-infected women; and (2) to investigate the role of inflammatory/regulatory cytokines and bacterial burden in modulating Th17 cell frequencies in genital secretions and plasma.

Methods. Cervical cytobrush and PBMC-derived T cells were isolated from 40 chronically HIV-infected and 13 uninfected women. Of the 40 HIV-infected women enrolled, 24/40 were on HAART while 16/40 were therapy naïve. The frequency and phenotype of Th17 cells were investigated by differential staining with CD3, CD4, CD8, CD45RA, CCR7, IL-17, IFN-γ and a marker of cell viability Vivid using an LSR2 flow cytometer following stimulation with PMA/ionomycin, HIV Gag subtype C overlapping peptides or CEF peptide pools. Pro-inflammatory and regulatory cytokines in plasma and cervical secretions were measured by ELISA and Luminex Multiplex Flow Cytometry (TGF-β, IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-17 and IL-23). Markers of bacterial and fungal burden [lipopolysaccharides (LPS), soluble CD14 (sCD14) and 1-3-β-δ-glucan] were also measured by ELISA in plasma and cervical secretions.

Results. In blood, the frequency of $CD4^+$ Th17 cells were significantly reduced in HIV-infected compared to uninfected women (p=0.005). In contrast, a significantly higher proportion of $CD4^+$ Th17 cells were detected at the cervix of HIV-infected (irrespective of HAART status) compared to uninfected women (p=0.03 for $HAART^-$ and p=0.04 for $HAART^+$). In both the cervix and in blood, $CD4^+$ Th17 cells were predominantly effector memory T cells ($CD45RA^-CCR7^-$). The inflammatory cytokines IL-12p40, IL-6, IL-8 and IL-1β were more concentrated in HIV^+ compared to uninfected women in both compartments. While only low concentrations of IL-17 were found in genital secretions, IL-17 concentrations correlated positively with inflammatory cytokines IL-1β and IL-12p40 at the cervix. The frequencies of Th17 cells detected at the cervix or in blood were not associated with the concentration of inflammatory cytokines detected in either compartment.

Conclusion. The results indicate an enriched inflammatory environment and enriched Th17 cell frequencies at the cervix during HIV infection. Increased exposure of genital tract immune cells to bacterial and fungal components could be associated with enrichment of Th17 cells in this compartment. The lack of association between inflammation and Th17 cell frequencies argues against selective recruitment of Th17 cells along a pro-inflammatory gradient, but may suggest selective resistance of Th17 cells in this compartment to cell death. Future experiments will need to investigate expression of HIV co-receptors by Th17 cells and include markers of immune activation and apoptosis.

Chapter 1

Literature Review

CHAPTER 1

Table of contents

1.1 Introduction

Human Immunodeficiency Virus Type-1 (HIV-1) causes the deadly Acquired Immunodeficiency Syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983). It is currently estimated that 31 to 36 million people are infected with HIV worldwide (UNAIDS, 2008). The pandemic is mainly driven by sexual mucosal transmission via vaginal and rectal routes (Hladik & Hope, 2009). The epidemic is most severe in Sub-Saharan Africa (20.8-24.1 million) (Figure 1.1), with 67% of the total number of infected people living here and 60% of the infected population being women (UNAIDS, 2008).

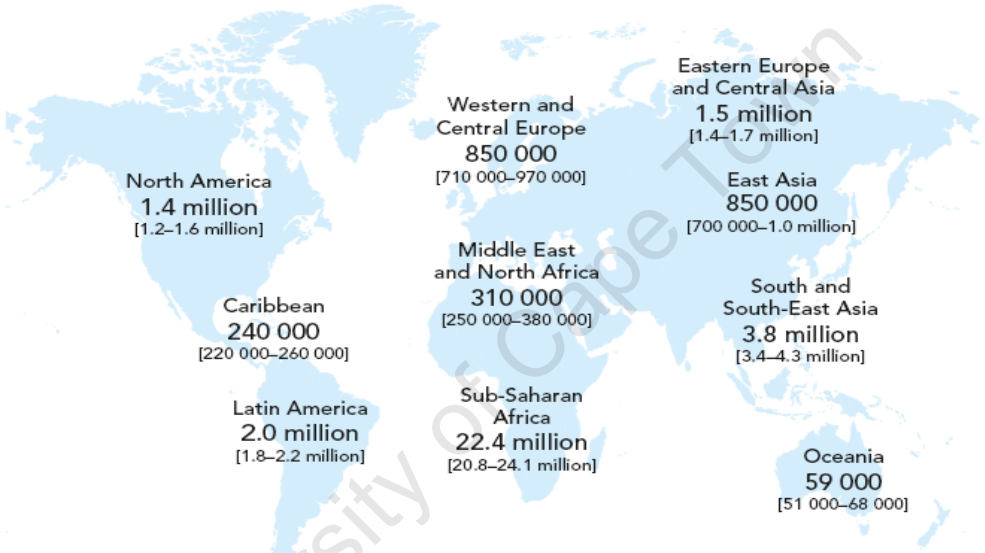


Figure 1.1: Global HIV disease burden. This map shows the prevalence of the population living with HIV globally. The numbers of people infected with HIV are shown for each region. The numbers in brackets shows the range of infected individuals. Sub-Saharan Africa is the most severely affected region with 22.4 million HIV-positive people (WHO, 2008).

Young women represent the fastest growing population who are becoming newly infected with HIV (UNAIDS, 2008). Persistent HIV infection and its associated chronic disease are a major global health concern. The World Health Organization (WHO) in December 2008 reported that approximately 4 million people were receiving highly active anti-retroviral therapy (HAART) in low and middle-income countries, which is 10 fold more than reported in 2002. Despite increased access to HAART, 2 million HIV-infected people are estimated to have died worldwide due to AIDS-related illnesses in 2008.

Transmission of HIV from males to females accounts for approximately 12.6 million cases of HIV worldwide (Hladik & Hope, 2009). The risk of infection via heterosexual contact is estimated to be higher for women than men (Mastro, 1996) and this is attributed in part to the biology of the female genital tract (FGT) (Moore & Shattock, 2003).

1.2 HIV lifecycle

HIV is a retrovirus in the genus *Lentivirus*, as it has to transcribe its RNA into double-stranded DNA for integration into the human genome. HIV is transmitted as a single-stranded enveloped RNA virus. A viral enzyme called reverse transcriptase, contained in the virus particle, converts the RNA genome to double-stranded DNA when entering the target cell (Figure 1.2). Another viral enzyme called integrase then combines viral DNA and its host cellular co-factors into host cellular DNA so that transcription can take place. After infection, the virus can become latent and the cell continues to function or the cell is activated and the virus replicates. The virus buds off from the infected cell and can then infect other cells (Smith *et al.*, 2006).

$CD4^+$ T cells and macrophages are the primary targets for HIV infection (Veldhoen *et al.*, 2008). T cells require activation through the T cell receptor (TCR) or cytokines to become susceptible to HIV infection (Unutmaz *et al.*, 1999). HIV-1 enters target cells through the virion envelope glycoprotein gp120 interacting with CD4 and chemokine co-receptors on target cells (Chan *et al.*, 1997). HIV that uses the β-chemokine receptor CCR5 for entry is termed R5. R5-tropic viruses have been shown to primarily be transmitted during acute phases of infection (Coakley *et al.*, 2005). HIV that enters target cells during the chronic phase can also use the α-chemokine receptor CXCR4 for entry and are termed X4 viruses. Dual-tropic strains of HIV can use both CCR5 and CXCR4 co-receptors for entry (Deng *et al.*, 1996).

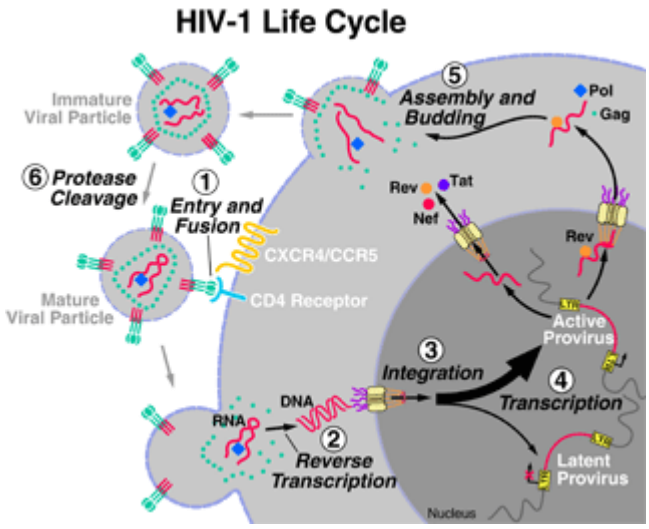


Figure 1.2: The HIV lifecyle. In step 1, a mature HIV viral particle enters and fuses to the target cell via CCR5 or CXCR4 receptors. Reverse transcriptase converts the single-stranded RNA into double-stranded DNA (step 2), allowing intergrase to combine the human genome with the viral DNA (step 3). Transcription into latent or active provirus then occurs (step 4). A new virus is assembled from an active pro-virus, allowing the virus to bud off from the infected cell (step 5). An immature viral particle can then cleave into a mature viral particle (step 6), completing the HIV lifecycle (Gladstone Institute of Virology and Immunology).

1.3 HIV pathogenesis

The devastating effect of HIV on the immune system is due to the virus infecting and killing the immune-regulating helper T lymphocytes. Because CD4 T cells are the main targets for HIV infection and replication, HIV infection is characterized by a dramatic loss of memory $CD4^+$ T cells in circulating blood and at mucosal sites (Veazey *et al.*, 1998). Progression to AIDS is a combination of both direct killing of $CD4^+$ T cells and indirect mechanisms such as apoptosis (Cecchinato e*t al.*, 2010). During HIV infection, enhancing expression of several co-stimulatory molecules may contribute to higher levels of $CD4^+$ T cell apoptosis (Kammerer *et al.*, 1996).

The main site of virus replication and memory $CD4^+$ T cell depletion during early and chronic HIV infection is gut-associated lymphoid tissue (GALT) (Guadalupe *et al.*, 2003). In rhesus macaques, SIV infection has been reported to manipulate resting $CD4^+$ T cells to produce high levels of virus, activating $CD4^+$ T cell targets to sustain virus production (Zhang *et al.*, 2004). This results in direct depletion of $CD4^+$ T cells through lytic infection and indirect depletion through apoptosis (Li *et al.*, 2005). During acute infection, depletion of $CD4^+$ T cells could also be the result of constant antigenic stimulation (Veazey *et al.*, 2003).

During peak SIV infection, 30-60% of gut memory $CD4^+$ T cells are infected and after 4 days post-infection approximately 80% of these cells are depleted (Mattapallil *et al.*, 2005). Chronic HIV infection is characterized by slow depletion of $CD4^+$ T cells, whereas acute infection is characterized by rapid $CD4^+$ T cell depletion (Brenchley *et al.*, 2004). During chronic infection, naive memory $CD4^+$ T cells decrease while memory $CD4^+$ T cells expand, becoming targets of infection (Hazenberg *et al.*, 2000). During this chronic phase of HIV infection, it has been estimated that less than 1% of all memory $CD4^+$ T cells are infected (Douek *et al.*, 2002). This could be due to the virus containing a large proportion of non-infectious particles and because naïve $CD4^+$ T cells are highly resistant to SIV infection (Mattapallil *et al.*, 2005; Nishimura *et al.*, 2004).

In the absence of HAART, $CD4^+$ T cells are unable to reconstitute in mucosal tissues (Mattapallil *et al.*, 2005) and are only partially reconstituted following therapy (Mehandru *et al.*, 2004). Figure 1.3 shows the natural history of $CD4^+$ T cell decline during acute and chronic infection. During the first few weeks of HIV infection, there is massive depletion of $CD4^+$ T cells (blue line) during peak viremia (red line). Once infection has been established after a few years (chronic infection), peak viremia declines with a transient loss of $CD4^+$ T cells (blue line) (Mattapallil & Roederer 2006).

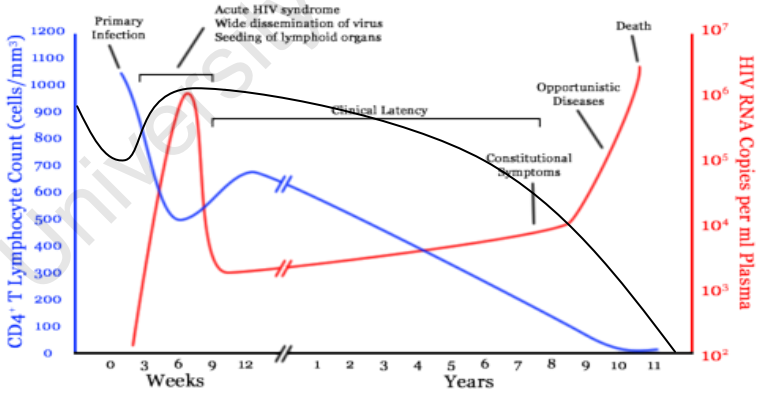


Figure 1.3: Natural history of HIV-1 infection in an untreated individual. Following 2-4 weeks after infection, cytotoxic $CD8^+$ T cell responses are detected in blood, coinciding with a major reduction in peak HIV viremia (red line). This is detected before the appearance of neutralizing antibodies (Nab). Cell-mediated rather than Nab immunity is responsible for initial control of HIV replication (Koup *et al.*, 1994). During acute infection (3–9 weeks), HIV viremia peaks while $CD4^+$ T responses subside due to $CD4^+$ T cell depletion (blue line). As infection establishes, viremia increases with a corresponding decrease in $CD4^+$ T cells, resulting in AIDS and subsequent death (Kahn *et al.*, 1998).

1.4 Correlates of protection against HIV

The main correlates of protection against HIV-1 infection are HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T cells and neutralizing antibodies (Belyakov *et al.,* 2004). These host responses form part of adaptive immunity. Neutralizing antibodies interfere with binding and entry of viral particles into new target cells, while cell-mediated immunity protects against disease progression (Pantaleo *et al.,* 2006). A small number of treatment naïve HIV-1 infected individuals are able to control HIV replication to a lower level and have preserved CD4 T cell counts over time and are called long-term non-progressors (LTNPs). HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses are preserved in this population compared to individuals who progress (Duvall *et al.,* 2006). These individuals provide evidence that HIV-specific cell-mediated immune responses can effectively control virus replication without drug therapy during chronic infection, although the mechanisms are still being investigated.

1.4.1 $CD8^+$ T cell immune responses to HIV infection

Emergence of HIV-specific $CD8^+$ CTL responses that can both directly kill infected target cells and produce anti-viral cytokines correlate with reduction in circulating viral load following peak viremia (Figure 1.3). Polyfunctional HIV-specific $CD8^+$ T cells have been described as the best predictor of viral replication control (Betts *et al.,* 2006). Directly, primates in which $CD8^+$ T cells had been depleted by CD8 monoclonal antibody administration prior to SIV infection showed increased viral loads after infection and more rapid progression to disease than untreated SIV-infected primates (Jin *et al.,* 1999). Studies showing direct mutational viral escape from recognition by $CD8^+$ CTL responses are further evidence for the role of $CD8^+$ T cell control of viral replication (Evans *et al.,* 1999). Furthermore, studies have reported that $CD8^+$ CTL responses are detectable in the genital tracts of women who have high-risk exposure to HIV yet have undetectable antibody responses (Rowland-Jones *et al.* 1995, 1998; Kaul *et al.,* 2000). The presence of genital tract $CD8^+$ T cell responses without a detectable systemic infection in these women implies that $CD8^+$ T cells are controlling the virus at a local level.

1.4.2 CD4$^+$ T cell immune responses to HIV infection

CD4$^+$ T cell ability to become activated, proliferate and function during HIV infection is vital for an effective immune response to occur. During infection, HIV-specific IFN-γ-secreting CD4$^+$ T cells remain detectable during infection, despite a huge loss of HIV-1-specific CD4$^+$ T cells (Pitcher *et al.,* 1999). During untreated HIV-1 infection, CD4$^+$ T cells become hyper-activated and HIV replication co-stimulates CD4$^+$ T cell activation, contributing to chronic immune activation (Haas *et al.,* 2010). Haas and colleagues (2010) suggest that CD4$^+$ T cell activation is dependent on antigen and cytokine-induced inflammation. Although HIV-1 preferentially infects activated CD4$^+$ T cells, the majority of HIV-1 specific CD4$^+$ T cells remain virus-free even in the presence of high-level viremia (Duvall *et al.,* 2006).

The clearance of certain viruses such as Hepatitis C (Grakoui *et al.,* 2003), Epstein-Barr virus (Adhikary *et al.,* 2006) and cytomegalovirus (van Leeuwen *et al.,* 2006) are highly dependent on antigen-specific CD4$^+$ T cell responses. During HIV-1 infection, better control of HIV-1 viral replication has been associated with strong HIV-1–specific CD4$^+$ T cell responses (Rosenberg *et al.,* 1997) and viral escape from CD4$^+$ T cell epitopes has been observed (Rychert *et al.,* 2007). However, it is not yet clear whether the presence of HIV-1–specific CD4$^+$ T cell responses is a consequence of low viremia or if these responses effectively contribute to viral suppression (Streeck *et al.,* 2010).

Escape mutations in CD4$^+$ T cell targeted epitopes can develop, showing that CD4$^+$ T cells exert selection pressure on the virus, especially during acute infection (Rychert *et al.,* 2007). Effectiveness of CD8$^+$ T cell responses in controlling viral replication is affected by the presence or absence of CD4$^+$ T cells (Betts *et al.,* 2006) and has been associated with the presence and preservation of CD4$^+$ T cell responses (Rosenberg *et al.,* 1997). HIV-1–specific CD4$^+$ T cell responses have an effector profile and capacity to proliferate (Harari *et al.,* 2002). They are polyfunctional as they secrete both IFN-γ and IL-2 (Tilton *et al.,* 2007). IL-2 production by CD4$^+$ T cells has been associated with prevention of HIV-associated disease (Pantaleo *et al.,* 2006). CD4$^+$ T cells can become exhausted by continuous antigenic encounter (Petrovas *et al.,* 2006). Since a large proportion of CD4$^+$ T cells are depleted during HIV infection, of which the majority exist at mucosal surfaces associated with GALT, it is likely that mucosal dysfunction occurs during HIV infection, due to a lack of CD4$^+$ T cell help at the mucosa (Mehandru *et al.,* 2004).

1.5 Classification and differentiation of CD4 T helper cell subsets

Four main CD4 T helper cell subsets have been identified according to the cytokines they produce, including T helper 1 (Th1), Th2, Th17 and T regulatory (Treg) cells (Figure 1.4; Zhou *et al.,* 2009). Each of these subsets arise from naïve CD4 T cells (Th0 cells) and differentiate into these functionally distinct CD4 T helper cell subsets under the influence of distinct cytokines. Th1 cells are $CD4^+$ IFN-γ and IL-2-producing T cells. They are important in eradicating intracellular bacterial and viral infections by activating macrophages. Naïve Th0 cells are stimulated to become Th1 cells under the influence of IFN-γ and IL-12. This activates the transcription factor T-bet which allows differentiation through STAT-1. Th2 cells secrete IL-4, IL-5 and IL-13 and protect against extracellular parasites and metazoan and are important in humoral immunity. During differentiation of Th0 cells to Th2 cells, the transcription factor GATA-3 is activated under the influence of IL-4, allowing differentiation to occur through STAT-6 (Zhu *et al.*, 2001).

Th17 cells have the ability to produce IL-17, IL-22 and IL-26. During differentiation of Th17 cells from Th0 cells, TGF-β, IL-1β, IL-6 and IL-21 activates the transcription factor RORC-2 which in turn activates STAT-3. This induces production of IL-23, initializing full differentiation into Th17 cells (Liang *et al.,* 2006). Tregs suppress immune system activation and inflammation, tolerate self-antigens and sustain immune system equilibrium (Crome *et al.,* 2010) through production of IL-10 and TGF-β (Weaver *et al.,* 2007). The transcription factor Foxp3 is activated by TGF-β, IL-10 and IL-2, initializing differentiation of these cells (Di Cesare *et al.,* 2009).

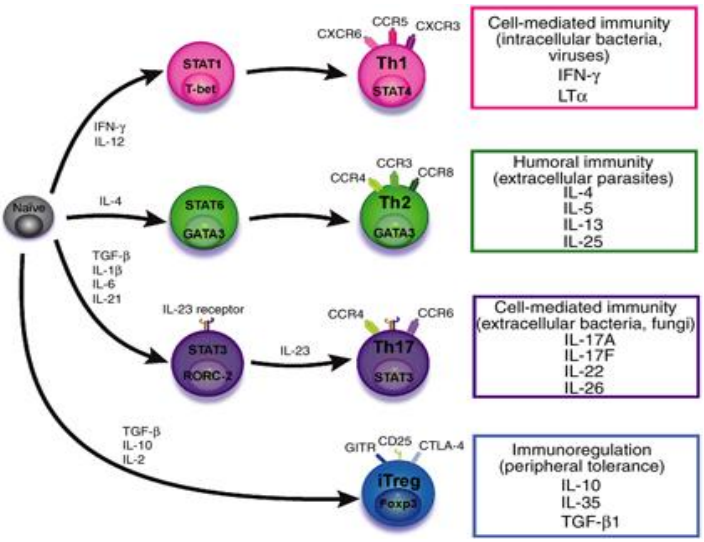


Figure 1.4: Differentiation of Th1, Th2, Th17 and regulatory T cells from Th0 cells. Th1 cells differentiate in the presence of IL-12 and secrete IFN-γ. Th2 cells depend on IL-4, STAT-6 and GATA-3 to differentiate and mainly secrete IL-4. Th17 cells require a combination of TGF-β and IL-1β, IL-6, and/or IL-21 to differentiate. RORC-2 is the transcriptional regulator needed for differentiation. IL-23 stabilizes differentiation (described in section 1.6). Th17 cells mainly secrete IL-17A, IL-17F and IL-22. *In vitro* differentiation of Th17 cells leads to cells with single expression of IL-17 or IL-22 or co-expression of IL-17 and IL-22, also observed *in vivo* (Liang *et al.,* 2006). Naïve $CD4^+$ T cells also differentiate into induced Treg (iTreg) cells in the presence of IL-2 and TGF-β or IL-10 and have an important immunoregulatory function (Di Cesare *et al.,* 2009).

1.6 Th17: a new member of the T cell subset family

The pro-inflammatory cytokine IL-17 was first described in 1993 by Rouvier and colleagues and $CD4^+$ T helper cells capable of secreting IL-17 were defined as Th17 cells by Oppmann and colleagues (2000) as a new member of the T helper family. In addition to IL-17, Th17 cells have also been shown to produce IL-22 and IL-26 (Korn *et al.,* 2009). Th17 cells are considered to be the bridge between innate and adaptive immunity because they regulate both the adaptive and innate immune responses (Khader *et al.,* 2009). In addition, Th17 differentiation and production of IL-17 occur earlier during infection than development of Th1 and Th2 cell responses (Harrington *et al.,* 2005). Th17 cells are found in non-lymphoid tissues (intestine) and secondary lymphoid tissue (mesenteric lymph nodes, spleen and Peyer's patches) (Campillo-Gimenez *et al.,* 2010).

When they were first described, Th17 cells were strongly associated with the destructive effects of autoimmunity in murine extrinsic allergic encephalitis model (EAE), an autoimmune disease inducible in mice (Chen *et al.,* 2007). Mice over-expressing TGF-β generated more Th17 cells and showed increased EAE pathology than wild type mice. In humans, Th17 cells were shown to produce IL-17A and IL-17F and have been found to be important in host protection against extracellular pathogens (such as bacterial and fungal infections) and in autoimmune diseases (rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis) but not in viral defence (Brenchley *et al.,* 2008; Ghilardi *et al*, 2007; Hashimoto *et al.,* 2005; Scriba *et al,*. 2008).

Both IL-17A and IL-17F bind to a type I cell surface receptor called IL-17R (McAllister *et al.,* 2005), although IL-17F is only 50% homologous to IL-17A (Stockinger *et al.,* 2007). Scriba *et al.* (2008) suggested that Th17 cells may play a role in acute inflammation associated with mycobacterial infections. In addition to $CD4^+$ Th17 cells, IL-17 is also produced by a wide number of immune cell types including γδ T cells (Deknuydt *et al.,* 2009), neutrophils (Fenoglio *et al.,* 2009), $CD8^+$ T cells (Kader *et al.,* 2009), natural killer (NK) cells (Langrish *et al.,* 2005), and NK T cells (Yoshiga *et al.,* 2008). Mast cells (Mrabet-Dahbi *et al.,* 2009) and alveolar macrophages (Song *et al.,* 2008) might also be induced to produce IL-17 under certain conditions. During SIV infection in rhesus macaques, Kader *et al.* (2009) showed that Tc-17 cell responses increased in peripheral blood and remained elevated during therapy, whereas Th17 cells were not reconstituted. In healthy uninfected primates, Th17 cells outnumber Tc-17 cells by 3 to 1, suggesting a homeostatic balance between Th17 and Tc-17 cells in maintaining mucosal integrity and function.

1.6.1 Characterization of Th17 cells

The Th17 cell lineage is characterized by the expression of the transcription factor RORγt and surface markers CCR6, CCR4 and IL-23R (Figure 1.5; Acosta- Rodriguez *et al.,* 2007). When the transcription factor RORγt becomes activated, the transcription factor Foxp3 in iTregs becomes inhibited. The differentiation of naïve T cells into Th17 cells depends on exposure to TGF-β and IL-6 (Ouyang *et al.,* 2008). Although development of Th17 phenotypes requires the presence of TGF-β, the additional contribution of IL-6 skews the response towards a Th17 phenotype (Afzali *et al.,* 2007).

In addition, IL-23 is required to maintain and stabilize commitment to the Th17 lineage (Korn *et al.,* 2009). Polarizing cytokines TGF-β and IL-6 induce expression of the transcription factors RORγt and RORα in newly activated T cells and allow Th17 differentiation to occur (Ivanov *et al.,* 2006; Yang *et al.,* 2008). Cytokines such as IL-1β, TNF-α, IL-21 and IL-6 have also been implicated in the differentiation of Th17 cells (van Beelen *et al.,* 2007; Nurieva *et al.,* 2007; Yue *et al.,* 2008).

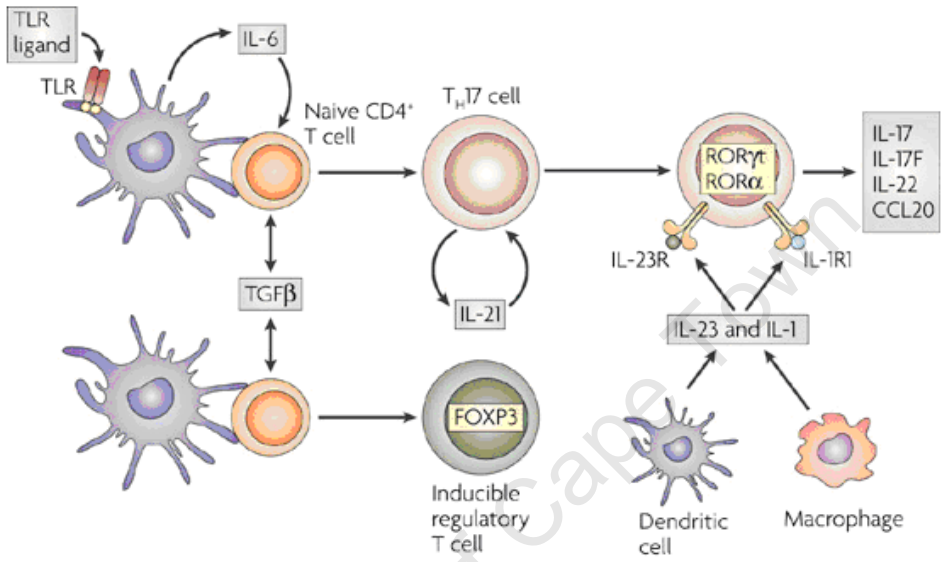


Figure 1.5: Differentiation of naïve CD4$^+$ T helper cells into Th17 cells. Differentiation of naïve Th0 CD4$^+$ T cells into CD4$^+$ Th17 cells occurs in the presence of TGF-β and IL-6, leading IL-21 secretion, reinforcing the differentiation process. This establishes IL-23R and IL-1R1 expression. IL-23 and IL-1 finalize the differentiation cycle and maintain differentiated Th17 cells (Sun *et al.,* 2010). IL-17A receptors are present on antigen presenting cells (APCs) (Acosta- Rodriguez *et al.,* 2007).

RORγt induces the expression of IL-23Rs on newly primed Th17 cells, allowing them to respond to IL-23 to sustain Th17 lineage-specific responses. IL-23 is produced by APCs in response to infection with certain bacteria and viruses (McKenzie *et al.,* 2006). Aggarwal *et al.* (2003) found that IL-23 is involved in recruitment of neutrophils to the site of infection. The IL-23/IL-17 axis is an important mediator of inflammation (Wiekowski *et al.,* 2001). Blocking IL-23 or its downstream factors IL-17 and IL-6 has been shown to significantly suppress disease development in animal models of inflammatory bowel disease and multiple sclerosis and Iwakura & Ishigamr (2006) have suggested that the IL-23/IL-17 axis may be the new therapeutic target for chronic inflammatory diseases.

1.6.2 Role of Th17 cells during inflammation

While FoxP3$^+$ Tregs prevent autoimmunity, Th17 cells have been shown to promote autoimmune tissue inflammation (DiPaolo *et al.*, 2007; Korn *et al.*, 2007). Production of IL-17 enhances T cell priming and stimulates fibroblasts, endothelial cells, macrophages and epithelial cells to produce multiple pro-inflammatory mediators such as IL-1, IL-6, TNF-α, NOS-2, metalloproteases and chemokines, which result in the induction of inflammation (Kolls *et al.*, 2004). Studies have shown that autoimmune pathology was not observed in the absence of Th17 cells (Afzali e*t al.*, 2007). Aggarwal *et al.* (2003) reported that secretion of IL-17A and IL-17F resulted in chemokine production and release of pro-inflammatory cytokines IL-6, TNF-α and IL-1β, resulting in neutrophil recruitment and activation of acute inflammatory responses leading to abscess formation and tissue destruction (Kolls, 2004; Bettelli *et al.*, 2007). IL-27, IFNγ, IL-4 and IL-2 negatively regulate the induction of Th17 cells, thus maintaining a homeostatic equilibrium (Batten *et al.*, 2006; Harrington *et al.*, 2005; Laurence *et al.*, 2007).

The intricate balance between Th17 and Tregs is important in controlling inflammation because these Th lineages share an intermediate stage in their development (Bettelli *et al.*, 2006). While Th17 cells induce inflammation, however, Tregs suppress inflammation. Early during microbial infection, while pathogens are entering through the mucosal barrier, Tregs are important in suppressing inflammation by secreting IL-10, which dampens Th1 and Th2 responses (Figure 1.6).

It has further been proposed that γδ T cells secrete IL-17 which recruits neutrophils to the site of infection and stimulates APCs to secrete IL-12, inducing recruitment of αβ T cells (Cua *et al.*, 2010). During this early phase of inflammation, Th17 cells are inhibited by the Treg response. When pathogens enter the host, IL-23 secretion by APCs induces the final phase of Th17 cell differentiation and IL-17 and IL-22 secretion. Studies have shown that these cytokines induce expression of antimicrobial peptides and neutrophil chemoattractants at mucosal sites and thus play an important role in controlling mucosal infections (Blaschitz *et al.*, 2010). IL-22 results in proliferation of keratinocytes (Zheng *et al.*, 2007) and increases epithelial proliferation and transepithelial resistance to injury in the lung (Aujla *et al.*, 2008).

Th1 responses are activated at later stages of inflammation. Once pathogens are eliminated, Tregs becomes induced, suppressing Th17 responses, preventing inflammatory-induced tissue damage. An imbalance between Th17 and Treg cells results in chronic tissue damage through inflammation (Korn *et al.*, 2009). $CD4^+$ T cells activated by gut antigens in Peyer's patches migrate to the lamina propria, where they die by apoptosis in healthy individuals. Pro-inflammatory cytokines released in response to antigens further increase epithelial permeability, setting up a severe cycle of chronic inflammation (Brandtzaeg *et al.*, 1997).

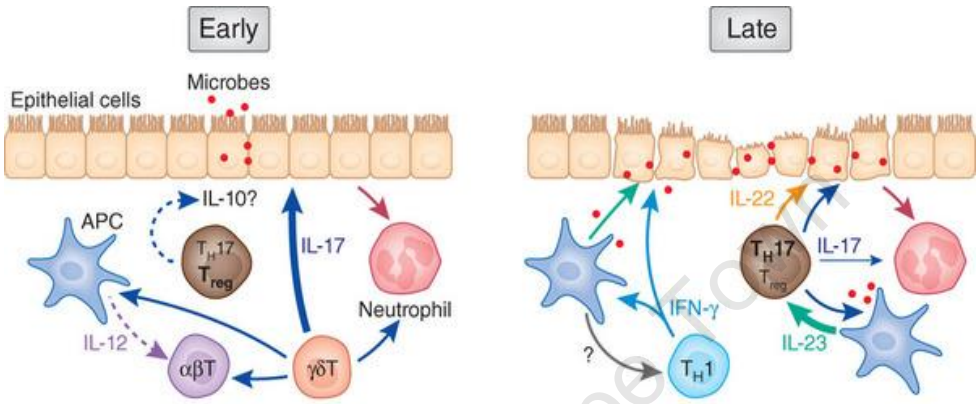


Figure 1.6: A simplified model for Th17 and T regulatory cell cytokines in co-ordinating tissue inflammation (O' Connor *et al.*, 2010). Early during inflammation (on the left), IL-17 produced by γδ T cells in the lamina propria of the gastrointestinal tract (GIT) [a tissue enriched with Th17 cells (Ivanov *et al.*, 2007)], secretes chemoattractants for neutrophils from the stroma to the site of infection and regulates γδ $CD4^+$ T cell responses by acting on APCs, dampening Th1 and Th2 cytokine secretion. Resident Th17 and Treg cells contribute to tissue protection through IL-10, until activated by IL-23 toward a pro-inflammatory phenotype (on the right). IL-17 and IL-22 contribute to the inflammatory response (Haak *et al.*, 2009). IFN-γ production is coupled with IL-23 from APCs and IL-17, increasing neutrophil recruitment in later stages of inflammation (Iwakura & Ishigame, 2006).

IL-17 and IL-22 are believed to contribute to barrier function of mucosal surfaces by formation of tight junctions and mucin (Blaschitz *et al.*, 2010). At mucosal surfaces, tight junction assembly involves protein-protein interactions between epithelial cells, forming a barrier between the lumen and lamina propria (Renu *et al.*, 2010). Tight junctions represent the major barrier between intestinal epithelial cells (Hossain *et al.*, 2008). Pathogens can attack tight junction intercellular proteins (Liu *et al.*, 2009). Mucosal barriers of the GALT become compromised during HIV infection (Nazli *et al.*, 2009), and this is likely to be partially due to mucosal depletion of Th17 cells during HIV infection (Boasso *et al.*, 2009).

Mesquita *et al.* (2009) investigated changes in epithelial barrier tight junctions by measuring transepithelial electrical resistance (TER) after exposure of human epithelial cells to microbicides in a dual-chamber system. They infected cultures with HIV and measured the ability of the virus to cross the epithelium and infect target T cells in the lower chamber. They exposed U1 cells with nonoxynol-9 (N-9) or cellulose sulfate (CS). They found that N-9 and CS resulted in rapid and sustained reduction in transepithelial electrical resistance and increased HIV infection of T cells in the lower chamber. Enhanced HIV transmission resulted from a decrease in TER due to a change in epithelial permeability that was adequate for HIV migration. They also showed persistent disruption in the epithelial barrier of the genital tract exposed to human seminal fluid and down-modulation of junctional proteins triggered by CS.

Inflammation can be beneficial to a pathogen by promoting colonization and host-to-host transmission (Liu *et al.,* 2009). Intestinal pathogens exploit the mucosal host inflammatory response to suppress growth of commensal organisms to colonize the inflamed gut (Macpherson *et al.,* 2005). The gut mucosal surface is colonized by a barrier of millions of protective commensal bacteria that are severely compromised during HIV infection by associated inflammatory responses (Pedron *et al.,* 2008). IL-17 and IL-22 stimulate production of mucins during inflammation, an important nutritional source for pathogens; hence these cytokines decrease host defence against these pathogens (Sugimoto *et al.,* 2008). Alternatively, mucins also protect the mucosal barrier by coating it and preventing pathogenic organisms from contacting cells of the mucosal lining (McAuley *et al.,* 2007).

It is believed that increased local tissue damage favours bacterial translocation and systemic immune activation, leading to the progressive loss of $CD4^+$ T-cell subtypes and development of AIDS (Brenchley *et al.,* 2006; Cechinato *et al.,* 2008) (described in more detail in Section 1.8). Another hypothesis is that increased recruitment of Th1 cells to sites of viral replication (GALT) is associated with increased production of TNF-α, a Th17 differentiation inhibitor (Harrington *et al.,* 2005). Th17 cells, by maintaining epithelial integrity and epithelial tight junctions, may reduce microbial translocation and subsequent immune activation (Cecchinato *et al.,* 2008; Macal *et al.,* 2008).

Studies have shown that activation of mucosal immune responses is involved in containing infections at mucosal surfaces, thus preventing dissemination of pathogens to systemic sites by forming a barrier (Blaschitz *et al.,* 2010). Because Th17 cells are concentrated at mucosal sites, they contribute to mucosal barrier integrity and control of infection (Srinivasan *et al.,* 2010). IL-17A and IL-17F are chemoattractants and elicit inflammatory responses as many innate and adaptive immune cells express the IL-17R (Aggarwal *et al.,* 2003). IL-17, produced by Th17 cells, trigger production of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), chemokines CXCL1-8 and metalloproteinases in a wide range of cell types (Kolls *et al.,* 2004). IL-17 and IL-22 stimulate granulopoiesis by inducing expression of G-CSF (Ye *et al*., 2001). These cytokines contribute to localizing infection to the mucosa, impeding dissemination of pathogens beyond the initial site of infection (Liu *et al.,* 2009).

1.7 The role of Th17 cells in gut mucosal immunity

At mucosal surfaces of the gut, studies have shown that mucosal $CD4^+$ Th17 cells are preferentially depleted during pathogenic HIV and SIV infections but are preserved in non-pathogenic SIV infections in sooty mangabeys and African green monkeys (Khader *et al.,* 2008). Comparison of pathogenic and non-pathogenic models of SIV infection suggest that Th17 cells contribute to the pathogenesis of AIDS (Brenchley *et al.,* 2008). It has been suggested that perturbation of Th17 cells at mucosal surfaces could compromise mucosal defences, resulting in immune activation (Brenchley *et al.,* 2006). Most Th17 cells are lost within two weeks after infection at mucosal sites of SIV-infected macaques and are not replenished over time (Cecchinato *et al.,* 2010).

GALT primarily guard against invading pathogens introduced into the GIT (Pabst & Rothkotter, 2006). GALT alone contains more than 40% of all the body's lymphocytes (Mowat & Viney, 1997; Croitoru *et al.,* 1994) and is an early target for HIV infection, replication and viral persistence (Veazey *et al.,* 1998). The majority of activated $CD4^+$ HIV target cells reside in mucosal tissues (Paiardini *et al.,* 2008). Studies in SIV-infected macaques revealed that up to 60% of intestinal $CD4^+$ T cells are infected during the acute stage of infection and up to 80% of these infected cells are depleted during the first 4 days before loss of blood $CD4^+$ T cells is detectable (Mattapallil *et al.,* 2005, Li *et al.,* 2005).

Similarly, in HIV-infected humans, memory $CD4^+$ T cells are preferentially depleted in the early stages of infection even when HAART is initiated (Mehandru *et al.,* 2004; Brenchley *et al.,* 2004; Guadalupe *et al.,* 2003), and this is likely to contribute to mucosal immune dysfunction observed in chronically HIV-infected individuals (Gordon *et al.,* 2007).

Studies have shown that Th17 cells are mainly localized in mucosal compartments of the gut and broncho-alveolar lavage (BAL) fluid of the lung (Cecchinato *et al.,* 2008; Brenchley *et al.,* 2008). The GALT is continually exposed to food and bacterial antigens, therefore the correct balance between Tregs and effector T-cell responses are important in maintaining its integrity. Th17 cells are highly activated in the gut because of continuous exposure to bacterial antigens, allowing Th17 cells to become a target for HIV infection (Cecchinato *et al.,* 2008). The reduction in Th17 cell number might be due to overall depletion of $CD4^+$ T cells in the mucosa, because the total number of $CD4^+$ T cells in the GALT is severely reduced during infection.

El Hed *et al.* (2010) showed that a large portion of Th17 cells express the HIV co-receptor CCR5 and produce very low levels of MIP-1α and MIP-1β. They further showed that $CCR5^+$ Th17 cells are infected with HIV *in vivo* explaining preferential depletion in the gut of HIV-infected patients (Brenchley *et al.,* 2008). Brenchley *et al.* (2008) also showed that Th17 cells isolated from the human gut express CCR5 and therefore are a target for HIV infection. Both groups showed that Th17 cells represent ideal targets for HIV because of high CCR5 expression and lower secretion of MIP-1α and MIP-1β, whose function is to inhibit HIV entry. They also showed that $CCR5^+$ Th17 cells are infected by CCR5 viruses and depleted during viral replication *in vitro*.

El Hed *et al.* (2010) showed that HIV-infected individuals on HAART had significantly reduced Th17 cell counts compared to uninfected individuals (regardless of viral load or CD4 count), whereas HIV-infected individuals naïve to therapy had normal levels and also suggested that CCR5-tropic viruses preferentially targeted $CCR6^-CCR5^+$ Th17 cells in both treated and untreated individuals. They also showed that Th17 cells were inversely correlated with activated $CD4^+$ T cells in HIV^+HAART^+ individuals. Overall, their results suggest a complex perturbation of Th17 cells during the course of HIV infection through both direct and indirect mechanisms of immune activation.

1.8 Markers of bacterial translocation and immune activation

Breaches in gut mucosal barrier integrity have been implicated in bacterial translocation at gut mucosal surfaces into systemic circulation (MacFie *et al.,* 2004). Brenchley *et al.* (2008) reported that Th17 cell loss during HIV infection was associated with microbial translocation as result of breaches in gut mucosal barrier integrity. This breach in gut mucosal integrity is believed to sustain immune activation during infection. Recently, Yue *et al*. (2008) showed that translocation of bacterial products induced secretion of IL-17 by Th17 cells.

Bacterial translocation can be measured by lipopolysaccharide (LPS) and soluble CD14 (sCD14) levels (Seabra *et al.,* 2009). LPS is found on the outer membrane of gram-negative bacteria and elicit robust immune responses (Kimbrell *et al.,* 2008). LPS contribute greatly to the structural integrity of bacteria and protects their membranes from chemical attack (Stewart *et al.,* 2006). LPS binds to membrane bound CD14 (mCD14) on monocytes or sCD14 (Scherberich *et al.,* 2000).

Binding of LPS to sCD14 is important for initiating an inflammatory response and promoting secretion of pro-inflammatory cytokines in many cell types, especially macrophages (Figure 1.7; Hershberger *et al.,* 1968). When gram-negative bacteria are detected, the liver secretes LPS-binding protein (LBP), allowing LPS and LBP to form a complex. This stimulates toll-like receptor 4 (TLR-4) on macrophages to form mCD14 (through intracellular cell signaling) or sCD14 (Wang *et al.,* 2002). This receptor is specific for LPS. The LBP/LPS complex binds to sCD14 and triggers an inflammatory response, involving the pro-inflammatory Th17 response described earlier, in order to combat invading gram-negative bacteria.

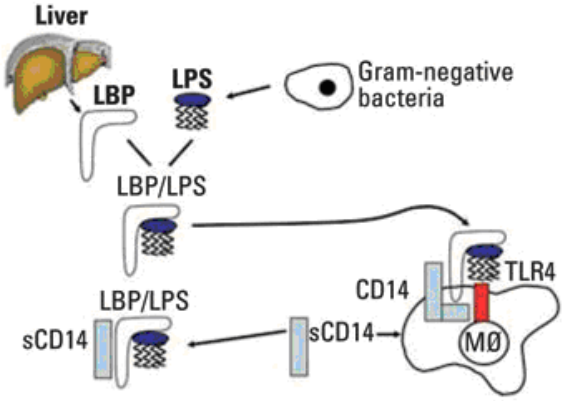


Figure 1.7: The complex LPS recognition system. LBP secreted by the liver acts as a carrier of LPS and is essential for binding of LPS to the sCD14 receptor. This complex assembles with sCD14 molecules, allowing for recognition of LPS by TLR-4 on the surface of immune cells such as macrophages. This leads to induction of a specific inflammatory response towards LPS (Zeldin *et al.,* 2006).

Brenchley *et al.* (2006) showed that HIV-infected individuals had higher concentrations of LPS in the blood than uninfected individuals, indicating that gram-negative commensals entered the blood stream from the gut. Liang *et al.* (2006) found that IL-22 and IL-17A together induced the expression of defensins and other antibacterial products. IL-22 has also been shown to induce LBP by hepatocytes (Wolk *et al.,* 2007) and prevents systemic inflammation provoked by LPS in the blood of HIV-infected individuals (Brenchley *et al.,* 2006). LPS can also induce production of IL-23, IL-6 and IL-1β in human monocyte-derived dendritic cells and result in differentiation of Th17 cells (Lombardi *et al.,* 2009).

Baroncelli *et al.* (2009) evaluated the effects of HIV viral replication on LPS levels in the plasma of $HIV^{+}HAART^{+}$ individuals compared to uninfected individuals. They found that there was reduced microbial translocation in $HIV^{+}HAART^{+}$ individuals when viral replication was suppressed, leading to reduced immune activation. Another recent paper by Epple *et al.* (2009) showed that HAART is able to repair the HIV-induced defect of the gastrointestinal barrier that is the cause of microbial translocation.

Jiang *et al.* (2009) measured plasma levels of bacterial DNA (16S rDNA) as a marker of microbial translocation and found a strong association between T cell activation (and lower CD4 T cell restoration) and higher levels of 16S rDNA in $HIV^{+}HAART^{-}$ individuals. They reported that microbial translocation decreased during HAART and resulted in restoration of $CD4^{+}$ T lymphocyte numbers, although $CD4^{+}$ T cell numbers were not reconstituted to pre-infection levels.

Bacterial 16S rDNA levels in plasma were correlated with bacterial LPS concentrations and inversely correlated with $CD4^+$ T cell restoration after HAART. These studies suggest that the degree of microbial translocation during HAART is strongly associated with the degree of immune restoration through the damaged gut epithelium and plays an important role in the pathogenesis of HIV-induced immunodeficiency.

Nazli *et al.*, (2010) investigated microbial translocation across the mucosal epithelial surfaces during HIV infection. Purified epithelial cells were isolated from the female genital tract (FGT) and exposed to HIV. R5 tropic laboratory strains reduced transepithelial electrical resistance by 30–60% without affecting cell viability. The decrease in transepithelial electrical resistance (TER) correlated with disturbance of tight junction proteins and increased permeability. Significant upregulation of inflammatory cytokines were seen in genital epithelial cells following exposure to HIV. Mucosal epithelial cells responded directly to HIV by significantly upregulating inflammatory cytokines that lead to impairment of barrier functions associated with viral and bacterial translocation across the epithelial monolayers. The increased permeability could be responsible for significant crossing of mucosal epithelium by virus and bacteria present in the lumen of mucosa in the genital tract and can be relevant to mucosal transmission of HIV and immune activation.

1.9 Mucosal immunity at the female genital tract

Depletion of activated memory $CD4^+$ T cells has also been reported in other mucosal sites including the vaginal and lung mucosa (Vajdy *et al.*, 2001; Veazey *et al.*, 2003). The genital tract mucosa is primarily an effector site that guards against sexually transmitted pathogens (Johansson & Lycke, 2003). The genital mucosa is part of the mucosal associated lymphoid tissue (MALT) (Johansson *et al.*, 1998). The FGT does not contain organised structures like the Peyer's patches of the GALT and is therefore considered a tertiary lymphoid site (Nandi & Allison, 1993). Cellular targets that are vulnerable to HIV infection are found in the ectocervical, endocervical and vaginal epithelium as well as in the uterus (Hladik *et al.*, 2008).

1.9.1 Mechanisms for HIV infection in the female genital tract

The structure of the FGT forms the first line of defence against HIV because of physical barriers and innate and adaptive immunological defences (Shacklett *et al.,* 2008). If the intrinsic barriers of the FGT are overcome, HIV-1 is capable of crossing the genital epithelium and establishing an infection. For the virus to do so, a number of intrinsic mechanical, chemical and biological barriers must be avoided. Epithelial cells lining the FGT play a key role in forming a primary barrier to HIV entry. These cells express TLRs that recognize and respond directly to HIV. Tight junctions between the columnar cells of the endocervix and endometrium form a mechanical barrier, preventing pathogens from breaching the epithelium (Kaushic *et al.,* 2010). Physical defences include multi-layered squamous epithelium in the vagina and ectocervix (Shacklett *et al.,* 2009). In addition, innate biological factors produced by epithelial cells such as cytokines, chemokines and antimicrobial peptides, as well as mucous production in the FGT help prevent HIV entry (Fahey *et al.,* 2005). However, it has been proposed that HIV gains access into FGT tissues by a number of mechanisms such as direct infection of epithelial cells, transcytosis of viral particles across the epithelium, entry of virus and/or infected cells through epithelial breaches and binding to and/or direct infection of mucosal dendritic and $CD4^+$ T cells via receptors (Shacklett *et al.,* 2009).

1.9.2 Anatomy of the female genital tract

The FGT is composed of the lower reproductive tract (vagina and ectocervix) which is lined by stratified squamous epithelium and the upper reproductive tract (endocervix, endometrium and fallopian tubes) which is lined by a single layer of columnar epithelium (Kaushic *et al.,* 2010). The lower FGT has three regions relevant to HIV transmission (Figure 1.8): (1) the vaginal mucosa composed of multi-layered squamous epithelium, (2) the ectocervix composed of multi-layered squamous epithelim intersecting with single layered columnar epithelium in the transformation zone and (3) the endocervix composed of only single layered columnar epithelium (Pudney *et al.,* 2005). $CD4^+$ T cells and APCs are enriched in the transformation zone (where the ectocervix transitions into the endocervix) and the transformation zone may be more susceptible to HIV entry as a result (Pudney *et al.,* 2005).

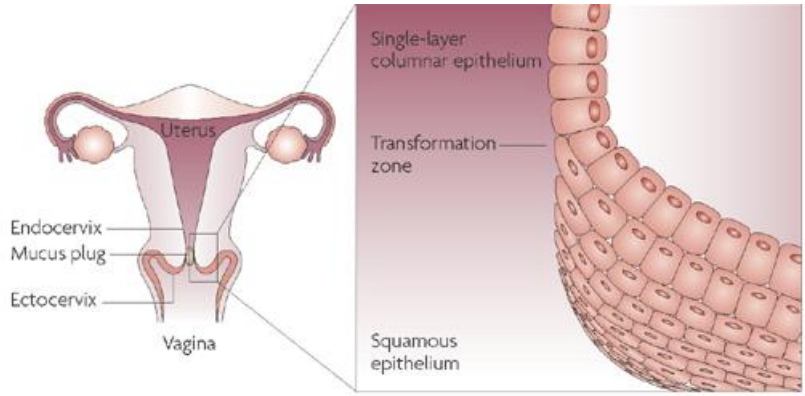


Figure 1.8: Anatomy of the female genital tract. Viral invasion occurs through the non-keratinized squamous epithelium of the vagina and ectocervix and the single-layer columnar epithelium of the endocervix. The endocervical canal is filled with mucus, providing a barrier against pathogens (Hladik & McElrath, 2008; Fahey *et al.*, 2005) and is an inductive and effector site for cell-mediated immunity (Shacklett *et al.*, 2009).

The role of Th17 cells in the FGT has not been extensively studied. Wang *et al.* (2009) reported that the prevalence of Tregs decreased in blood, in uterine lining and the endometrium obtained from women with unexplained recurrent miscarriage, whereas Th17 cell frequencies increased. Proportions of IL-17$^+$ CD4$^+$ T cells were higher in women with unexplained recurrent miscarriage compared to healthy women. Tregs inhibited IL-17 expression through TGF-β and IL-10 *in vitro*, suggesting that more Th17 cells were inhibited in the control group of women compared to women with unexplained recurrent miscarriages. These results suggest an important role for a Th17/Treg balance in the FGT in fertility.

Feinen *et al.* (2010) evaluated Th17 responses in a murine model of *Neisseria gonorrhoeae* in the FGT of mice. They showed that *N. gonorrhoeae* infection elicited an innate immune response which in turn triggered neutrophil recruitment and production of antimicrobial peptides. They showed that IL-17 secretion was induced in human antigen presenting cells *in vitro.* Furthermore, they showed that IL-17 was induced in the iliac lymph nodes during murine genital tract gonococcal infection. When IL-17 secretion was blocked or when the IL-17R was deleted, infection was prolonged and neutrophil recruitment was reduced. In IL-17R knockout mice, neutrophil-attractant chemokines were reduced in response to *N. gonorrhoeae*. These results show a crucial role for IL-17 and Th17 cells in the immune response towards this infection in the FGT of mice and may implicate an important role for Th17 cells in the FGT of humans by protecting against this sexually transmitted infection.

A recent study by Chen *et al.* (2010) evaluated regulation of MyD88, an adaptor molecule required for innate immunity, during *Chlymadia trachomatis* infection in the upper urogenital tract of mice. They showed that MyD88 knock-out mice failed to produce inflammatory cytokines in the FGT during the first week of infection and developed a Th2-dominant response, whereas wild-type mice developed a Th1/Th17-dominant response after infection, demonstrating the importance of the latter response in the FGT in controlling this infection. MyD88 knock-out mice had significantly higher numbers of Chlamydial organisms in the upper genital tract tissues and developed more severe pathology compared to wild-type mice. These results show the critical role in the development of Th1/Th17 adaptive immunity, which may be essential for limiting infection and reducing pathology of the upper genital tract. Taken together, these two studies show the importance of Th17 cells in the genital tract for controlling sexually transmitted infections.

In lungs, Th17 cells produce mucous by the direct effect of IL-17 on epithelial cells by mobilizing mucins. IL-17 increases mRNA encoding mucin genes MUC5AC, MUC5B and the mucin protein in human airway epithelial cells (Ivanov *et al.,* 2007). Gram-negative bacteria in the lungs of mice stimulate IL-23 secretion by APCs, allowing Th17 cells to differentiate, contributing to mucin secretion by recruitment of epithelial cells (Hashimoto *et al.,* 2005). In the GALT, Th17 cells facilitate mucous production by formation of tight junctions and epithelial regeneration (Renu *et al*., 2010).

In the human FGT, it can be postulated that Th17 cells may play a role in mucous production in the same way as in the lung by mobilizing mucins and in the FGT of mice by inducing neutrophil recruitment. Also, in the same way as in the murine respiratory tract, IL-17-producing Th17 cells may produce mucous through IL-23 regulation at the human FGT. Other possible roles for this subset in the human FGT could be maintenance of epithelial cell tight junctions, which could contribute towards mucosal barrier integrity and mucous production, as well as antimicrobial protein production as described in the gut. Investigating Th17 cells in the human FGT during HIV infection is crucial because Th17 cells have an anti-fungal and bacterial function, which could help combat and prevent sexually transmitted infections.

1.10 Study aim and objectives

Overall aim of dissertation

To investigate the impact of HIV infection and inflammation on the frequency and phenotype of Th17 cells in the FGT and the blood of uninfected and HIV-infected women.

Rationale for the study

Th17 cells have shown to be important in regulating gut immunity, particularly during HIV infection because they play a role in sustaining gut mucosal barrier integrity. Although the FGT similarly has a comprehensive ecosystem of bacterial and fungal commensals and pathogens that become dysregulated during HIV infection, the role of Th17 cells in regulating vaginal immunity in humans has not been investigated. The FGT represents a mucosal milieu rich in inflammatory innate mediators and the intersection between Th17 inflammatory cells, genital inflammation, and vaginal bacterial or fungal burden may give important insight into the role of these cells in genital tract mucosal immunity.

Specific Aim 1: To compare the frequency and phenotype of Th17 cells detected in the FGT and blood in uninfected women compared to HIV-infected women naïve to anti-retroviral therapy or receiving therapy.

Hypothesis: As in the gut-associated mucosal tissue, the frequencies of Th17 cells detected in the FGT are significantly reduced during HIV-infection but reconstituted in women receiving HAART.

Specific Aim 2: To investigate the influence of pro-inflammatory and regulatory cytokines on the frequency and phenotype of Th17 cells in the FGT.

Hypothesis: Pro-inflammatory cytokines in the FGT have previously been implicated in local HIV replication and shedding (Gumbi *et al*., 2008; Anderson *et al.,* 2008). Th17 cells may be recruited to the FGT during HIV infection along an inflammatory gradient. Pro-inflammatory cytokines are also needed for Th17 cell differentiation (Di Cesare *et al.*, 2010). Thus, if Th17 cells are recruited to the FGT along an inflammatory gradient, then pro-inflammatory cytokines should be enriched at the cervix.

Specific aim 3: To investigate the association between Th17 cell frequencies in the female genital tract and markers of bacterial (LPS, sCD14) and fungal [(1-3)-β-δ-glucan] burden during HIV infection.

Hypothesis: Increased exposure of genital tract immune cells to bacterial and fungal components and their host by-products [LPS, sCD14 and (1-3)-β-δ-glucan] will be associated with enrichment of Th17 cells in this compartment.

Chapter 2

Materials and Methodology

CHAPTER 2

Table of Contents

2.1 Study Participants

A total of 40 chronically HIV-infected and 13 uninfected women were enrolled in this study from the Empilisweni Clinic and Nyanga Day Hospitals in Cape Town, South Africa (in collaboration with Prof. Lynette Denny of the Dept. Obstetrics and Gynaecology, Dr. David Coetzee of the School of Public Health; and Prof. Anna-Lise Williamson of the Division of Medical Virology, Dept. Clinical and Laboratory Sciences, University of Cape Town). Women who were pregnant, menstruating, post-menopausal, had undergone a hysterectomy, had cervical cancer, vaginal discharge or visible or reported sexually transmitted infections were excluded from this study. Approval for this study was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Cape Town (Nyanga REC 106/2002 and Empilisweni REC 208/2006). All participants gave informed written consent before initiation of the study.

2.2 Sample collection and cervical cell processing

Cervical T lymphocytes were collected from the endocervical canal of the female genital tract using a Digene cervical sampler as described by Passmore *et al.* (2002) and Gumbi *et al.* (2008). The Digene cervical sampler was rotated 360˚ within the endocervical os under speculum examination and then transferred immediately to 3ml R10 transport medium [RPMI 1640 (Gibco™)], with 50mg/ml glutamine (Gibco™), 0.8mg/ml Fungin™ (InvivoGen), 50U penicillin, 50mg/ml streptomycin and 10% fetal calf serum (FCS) (Invitrogen, USA). After collection of cervical T lymphocytes, the cytobrush was placed in a 15ml conical tube containing 3ml of R10 transport media immediately after sampling. The specimens were transported to the laboratory and processed within 3 to 6 hours of collection to minimize cell loss. Cytobrush samples with visible blood contamination were discarded. At the laboratory, the cytobrush was flushed ~30 times using a sterile, plastic disposable pasteur pipette to dislodge cells from the cytobrush. The pipette was then removed and discarded. The cervical cells were washed once at 250g for 10 minutes to pellet cells.

Cervical supernatant (containing the transport medium used to flush the cytobrush with the cells removed) was stored at -80°C for analysis of genital inflammatory cytokine levels and HIV shedding. The cervical lymphocyte pellet was resuspended in 2.2ml R10 media and incubated overnight (approximately 12-18 hours) in a humidified incubator (37°C, 5% CO_2) prior to stimulation and intracellular cytokine staining. Cells were rested overnight so that any apoptotic cells could die off and to reduce background. Intracellular cytokine production by cervical cytobrush-derived T cells was evaluated *ex vivo* since the low yields precluded cryo-preservation.

2.3 Blood collection and peripheral blood mononuclear cell (PBMC) isolation

Forty millilitres of anti-coagulated blood was collected by venipunture into sterile Acetate Citrate Dextran (ACD) BD vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient (Ficoll-Hypaque) centrifugation and counted using a Guava cell counter. Leucosep® tubes (Greiner Bio-one Ltd, UK) was used to isolate PBMCs from anti-coagulated whole blood. Processing was done within 5 hours of venipuncture to maximise T cell recovery (Bull *et al.,* 2007). Leucosep® tubes contain a porous separation disc to stabilize the interface between the Ficoll cushion and PBMC "buffy" layer, thereby allowing for the separation of high density erythrocytes from lower density PBMCs.

Briefly, a volume of 3ml Ficoll Histopaque (Sigma-Aldrich, MO, USA), equilibrated to room temperature, was added to each 15ml Leucosep® tube and centrifuged for 1 minute at 1000g using a Heraeus 1.0R Megafuge at room temperature to force the Ficoll below the separation disc. A volume of 5ml of whole anti-coagulated blood was then added to each 15ml Leucosep® tube and centrifuged for 15 minutes at 1000g using a Heraeus 1.0R Megafuge at room temperature to separate the blood components. A volume of 2ml of plasma was carefully removed from the top of each gradient with a plastic disposable Pasteur pipette. Plasma was stored at -80°C for HIV viral load determination and evaluation of plasma cytokines levels. Plasma was stored in 1ml aliquots at -80°C for assessment of cytokine concentrations.

From each gradient, the white "buffy' layer (enriched with PBMCs) which sediments immediately above the separation disc, was carefully removed using a Pasteur pipette and transferred to a new 15ml tube to be washed twice with 15ml of 1% FCS (Invitrogen, USA) in phosphate buffered saline (PBS; Gibco™). The PBMC pellet was then re-suspended in 6ml of R10 media prior to counting. Isolated PBMCs were cryopreserved in liquid nitrogen until analysis.

2.4 PBMC counting and viability assessment

PBMC counts and viability were performed using an automated Guava PCA cell counter system (Guava Technologies Inc, CA, USA) which records the frequency of nucleated viable cells following staining with Guava ViaCount reagent (Guava Technologies Inc, CA, USA). Guava ViaCount reagent contains two DNA binding dyes that distinguish between live and apoptotic cells. Dead cells and debris are excluded based on negative staining with the viability dye. Analysis was done using CytoSoft™ software (Guava Technologies Inc, CA, USA). Briefly, PBMCs were washed once with 10ml of R10 media. The supernatant was discarded and the pellet was re-suspended in 5ml of R10 media. A volume of 10µl of the PBMC suspension was added to 190µl Guava ViaCount to make a 1:20 dilution. The cells were incubated for 8 minutes at room temperature and immediately acquired on the automated Guava cell counter. All freshly isolated PBMCs had a viability >95%. PBMCs were adjusted to 2 x 10^6 viable cells/ml and rested overnight (approximately 12-18 hours) in R10 media in a humidified incubator (37°C, 5 % CO_2) to reduce background to allow apoptotic cells to die off.

2.5 Cryo-preservation of PBMCs

PBMCs that were not used immediately for intracellular cytokine staining (ICS) and flow cytometry experiments were cryo-preserved within 6 hours of collection. Briefly, PBMCs were suspended in 500µl of 100% FCS (Invitrogen, USA) on ice, then diluted drop wise to 1ml by the addition of 500µl of 20% freezing medium [20% Dimethyl Sulphoxide (DMSO; Sigma-Aldrich, UK) in FCS (Invitrogen, USA)].

Freezing media containing cells were transferred by pipette to pre-cooled 1ml cryovials which were then immediately transferred into pre-cooled (4°C for 1 hour) "Mr Frosty" Cryo freezing containers (Nalgene® Labware, Thermo-scientific Inc). "Mr Frosty" containers have isopropanol which functions to control the freezing rate of cells to -1°C/minute to limit cell death (Weinberg *et al*., 2000). "Mr Frosty" containers were then placed at -80°C overnight. Frozen PBMCs were subsequently transferred to liquid nitrogen for long term storage.

2.6 Thawing of frozen PBMCs

Vials containing frozen PBMCs were transferred from liquid nitrogen to a 37°C water bath until almost completely thawed. Thawed cells were re-suspended by adding 1ml of pre-warmed (37°C) R1 media [RPMI 1640 (Gibco™), 1% FCS (Invitrogen, USA) containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine (Gibco™) and 0.8mg/ml Fungin™ (InvivoGen) drop wise. The solution was made up to 10ml (R1) in a 50ml tube and centrifuged at 320g for 10 minutes. The supernatant was discarded and the pellet re-suspended in 500µl of R10 media with 0.002% DNAse (Roche Diagnostics) for 2 minutes. The addition of DNAse prevented cell clumping. After 2 minutes incubation in DNAse, the samples were washed in 1% FCS in PBS. The supernatant was discarded and the pellet re-suspended in 5ml R10. Cell viability after thawing was assessed as described in section 2.5. Only PBMC samples with >95% viable cells were used in intracellular cytokine staining experiments.

2.7 Intracellular cytokine staining and flow cytometry for evaluation of Th17 and Th1 cell phenotype and function

Flow cytometry allows both the counting and evaluation of phenotypic properties of cells suspended in fluid and passed by an electronic detection apparatus at high speeds (Givan, 2001). Intracellular cytokine staining (ICS) is a technique for identifying single cells that produce cytokines in response to a specific stimulus which can be measured by flow cytometry (Herr *et al*., 1996; Scheibenbogen *et al*., 1997; Suni *et al.,* 1998). This is a useful measure of T cell function (Maecker, 2004). ICS was carried out to analyse the frequency of IL-17 producing Th17 cells and IFN-γ producing Th1 cells.

2.7.1 Antibody staining panels used for flow cytometry

To characterize the phenotype and functionality of IL-17 producing Th17 and IFN-γ producing Th1 cells, PBMCs and cervical cytobrush-derived mononuclear cells (CMCs) were stained with commercially available antibodies against known surface and intracellular functional markers of T cell subsets.

An 8-colour antibody staining panel to simultaneously evaluate IL-17 and IFN-γ production by viable memory T cells was designed by Dr. Tom Scriba [South African Tuberculosis Vaccine Initiative (SATVI), University of Cape Town; Scriba *et al*., 2008]. The panel was composed of antibodies against T cell phenotypic markers (CD3, CD8, and CD4), memory markers (CD45RA and CCR7), intracellular cytokines (IL-17 and IFN-γ) and a viability marker Vivid. Table 2.1 provides details of the fluorochrome combinations used, the antibody clone and the final volume of antibodies used in all experiments.

TABLE 2.1| Eight-colour antibody panel used on the LSRII flow cytometer

Antibody	Fluorochrome	Clone	Volume (µl)*	Supplier
CD3	Allophycocyanin H7 (APC-H7)	SK7	2.5	Becton Dickinson
CD4	Fluorescein isothiocyanate (FITC)	Ber-ACT8	5	Becton Dickinson
CD8	Peridininchlorophyll Cy 5.5 (PerCP-Cy 5.5)	SK7	5	Becton Dickinson
CCR7	Allophycocyanin (APC)	150503	5	R&D Systems
CD45RA	Phycoerythrin Cy 7 (PE-Cy 7)	L48	1.5	Becton Dickinson
IFN- γ	Alexa Flour 700	RPA-T8	1	Becton Dickinson
IL-17	Phycoerythrin (PE)	BL 168	5	BioLegend
Violet Flourescent Reactive dye (Vivid)	PacBlue	N/A	0.5	Invitrogen

*Optimal antibody volumes differed depending on the antibody lot number. The range of volumes used is reported above.

2.7.2 Antigen stimulation of PBMCs and cervical cells

The reagents used for stimulation of T cells and ICS are summarised in Table 2.2. Cervical cytobrush-derived cells (rested at 37^{o}C overnight; as described by Gumbi *et al.*, 2008) and PBMCs were transferred into 3 (cervix) or 4 (PBMC) BD Falcon tubes at ~2 x 10^5/500µl/tube for cervical cells and 1 x 10^6 PBMC/500µl/tube.

Cervical cells and PBMCs were either stimulated with HIV Gag Du422 overlapping peptides (each at a final concentration of 1µg/ml, kindly provided by the NIH AIDS Reagent Repository), PMA/ionomycin [positive control, 1µg/ml PMA and 50µg/ml ionomycin (Sigma-Aldrich, UK)], or left unstimulated (negative control). For PBMCs only (where cell numbers were not limiting), an additional tube was included in each experiment containing CEF peptides (Cytomegalovirus, Epstein Barr virus and Influenza virus immunodominant peptides; table 2.3; Lünemann *et al.,* 2010). Cells were incubated for a total of 6 hours in a humidified incubator (37°C, 5% CO_2). Brefaldin A (BFA; 12.5µg/ml; Sigma-Aldrich, UK) was added to each tube after the first hour of incubation to block Golgi transport of IFN-γ and IL-17 and retain newly produced cytokines inside the cell.

The negative control consisting of unstimulated cells allowed measurement of spontaneous cytokine production. Stimulation with HIV-1 Gag peptides allowed measurement of the magnitude of HIV Gag responsiveness in each compartment by Th1 and Th17 cells. Stimulation with CEF peptides allowed measurement of T cell responses against other common viral antigens (Doisne *et al.,* 2004). The positive control consisting of PMA/ionomycin stimulation allowed assessment of the maximal ability of cells to produce IFN-γ and IL-17. PMA is a phorbol ester which engages the T cell receptor while simultaneous addition of ionomycin opens calcium channels that result in an influx of intra-cellular Ca^{2+} ions (Touraine *et al*., 1977). The Ca^{2+} ions activate the cell to produce large quantities of cytokine (Kawanishi & Joseph, 1992).

Table 2.2| Reagents used in intracellular cytokine staining (ICS)			
Reagent	Composition and mode of action	Quantity	Supplier
CEF peptides	A peptide pool consisting of Cytomegalovirus, Epstein Barr virus and Influenza virus peptides. Epstein Barr virus is found in almost the whole human population (Lünemann *et al.,* 2010). CEF peptides stimulate the release of IFN-γ from $CD8^+$ memory T cells and serves as a peptide-specific positive control (PANATecs International).	Each peptide present at a final conc. of 1µg/ml	NIH AIDS Reagent Repository
HIV Gag superpool	A single pool of 121 15-mer HIV Gag peptides overlapping by 10 amino acids; based on HIV-1 subtype C Du422 sequence.	Each peptide present at a final conc. of 1µg/ml	NIH AIDS Reagent Repository
PMA /Ionomycin	A potent mitogen that stimulates the T cell receptor. When used in conjunction with ionomycin results in massive cytokine production. An ionophore that stimulates Calcium ion release which activates cytokine production (Majewski *et al.,* 2007).	1µg/ml PMA 0.5µg/ml ionomycin	Sigma Aldrich
BFA	Inhibits Golgi transport of newly synthesized proteins thereby accumulating newly synthesized cytokines inside the cell; it is a lactone antibiotic produced by fungal organisms (Loesch *et al.,* 2007).	0.5µg/ml	Sigma Aldrich
Cytofix/Cytoperm (1X)	Formaldehyde and Saponin	500µl per sample	Becton Dickinson
Perm Wash (1X)	FCS and Saponin	2ml per sample	Becton Dickinson
CellFIX (1X)	Formaldehyde and sodium azide. A fixing agent that immobilizes bound antibodies on stained cells.	500µl per sample	Becton Dickinson

2.7.3 Intracellular cytokine staining of PBMCs and cervical cells

Following stimulation, PBMCs and cervical cells were washed twice with 2ml PBS. The supernatant was discarded and the cell pellets were resuspended in the dead volume. Fifty microlitres of a 1:50 dilution of the viability dye Vivid was added. Vivid is a fluorescent reactive dye that reacts with cellular amines. It permeates membranes of dying cells and reacts with amines intracellularly and on the cell surface. This causes intense fluorescent staining. Faint staining is a result of the dye reacting with cell-surface amines of viable cells (Molecular probes®, Invotrogen™, USA). The cells were incubated at room temperature for twenty minutes in the dark, after which they were washed once with FACS wash buffer.

Surface antibodies for the 8-colour panel (Table 2.1; CD4, CD8, CD45RA and CCR7) were prepared in a master mix according to their optimally titrated volumes in FACS wash buffer. A staining volume of 50µl of the mastermix was added to each tube on ice. The samples were incubated at 4°C for 1 hour. Samples were washed once with FACS wash buffer to remove excess unbound antibody.

To identify cytokine producing cells following stimulation, samples were labelled with CD3 (directed against surface antigens) and with IFN-γ and IL-17 (intracellular antigens) using the antibodies described in Table 2.1. Briefly, all tubes were washed once in PermWash™ buffer (Table 2.2; BD Biosciences, San Diego, CA) and the above-mentioned antibodies were added. The tubes were incubated at 4˚C for one hour and then washed once with permwash buffer. The supernatant was discarded and the cell pellets were re-suspended in the dead volume. One hundred microlitres of 1 X BD CytoFix-CytoPerm™ solution (Table 2.2; BD Biosciences, San Diego, CA) (containing 1% formaldehyde to fix stained cells prior to analysis) was added to each tube and stored at 4°C until samples were acquired. Stained cells were captured on a Becton Dickenson LSRII flow cytometer and events analyzed using FlowJo software version 8.5.2 (TreeStar Inc, OR, USA). A minimum of 1 x 10^6 total events were captured per analysis for PBMCs and 100% of the sample was captured per analysis for cervical cells. Cervical samples with ≤100 $CD3^+$ events were excluded from phenotyping analysis to reduce positive bias associated with low cell numbers (Passmore *et al*., 2002; Gumbi *et al*., 2008).

2.7.4 Colour compensation settings and correcting for spectral overlap

Spectral overlap in flow cytometry is a phenomenon associated with fluorochromes that have overlapping emission spectra. Signal from one fluorochrome will "spill over" or register on a photodetector that is specific for another overlapping fluorochrome. Spectral compensation allows "subtraction" of this signal spill-over from neighbouring channels and analysing fluorescently labelled cell populations requires compensation for spectral overlap (Roederer *et al.,* 2004). To compensate for spectral overlap in each experiment, a control panel of tubes containing each antibody in the staining panel (Table 2.3) alone was conjugated onto commercially available microparticles (CompBeads; BD Biosciences, San Diego, CA).

CompBeads are made up of one positive control population of beads that binds the antibody κ chains, and one negative population that does not bind antibody. The beads stained with each antibody individually were acquired on a flow cytometer along with the PBMC and cervical cells stained with all the antibodies in combination, using the same voltages and settings.

During analysis using FlowJo software version 8.5.2 (TreeStar Inc, OR, USA), colour compensation for each panel was determined automatically by using the individually stained CompBeads to determine spectral overlap between the combinations of antibodies used in the panel. In all experiments, the automated colour compensation settings were applied and no manual adjustments were made. Briefly, volumes of each antibody in the panel (Table 2.3) were added individually to 100µl of PBS in 5ml Falcon tubes (BD Biosciences, San Diego, CA). CompBeads were vortexed thoroughly for 1 minute to avoid aggregation and a drop of negative and positive beads was added to each antibody tube. The beads were incubated at 4°C for 15 minutes, and 100µl of 1 x CellFIX (BD Biosciences, San Diego, CA) was added. The beads were acquired on a LSRII flow cytometer (Becton Dickinson, Nenelux NV, Belgium) for the 8-colour panel (Table 2.1).

2.7.5 Event capture, gating and use of fluorescence minus one (FMOs)

In flow cytometry, gating refers to the selection of cells (according to their fluorescence and/or scatter characteristics) that are carried forward for further analysis. It is important that gating is as objective as possible (Givan, 2001). To limit subjectivity in gating cell populations, fluorescence minus one (FMO) gating was used to establish cut-offs for each antibody used in this study. FMOs for the 8-parameter flow cytometry panel in this study included 8 tubes containing PBMCs from healthy individuals labelled with all the fluorochromes in the panel with the exception of one (Table 2.3; Roederer *et al.,* 2004). FMOs allow for gate settings that accurately distinguish positive and negative cell populations during analysis (Roederer *et al.,* 2004). In addition, a positive control was set up containing all fluorochromes in the panel applied to PBMCs from a healthy uninfected individual. The gates set in the positive control are then based on the location of cell populations in FMO panels and these settings are applied to the experimental samples.

TABLE 2.3| Example of FMO gating control set-up for 8-colour antibody panel									
		Antibody volumes for FMOs							
Panel	FMO*	CD3	CD4	CD8	CCR7	CD45RA	IFN-γ	IL-17	Vivid
Negative controls	-CD3	-	2.5	2.5	2.5	2.5	2.5	2.5	2.5
	-CD4	5	-	5	5	5	5	5	5
	-CD8	5	5	-	5	5	5	5	5
	-CCR7	5	5	5	-	5	5	5	5
	-CD45RA	1.5	1.5	1.5	1.5	-	1.5	1.5	1.5
	-IFN-γ	1	1	1	1	1	-	1	1
	IL-17	5	5	5	5	5	5	-	5
	Vivid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
Positive control	None	2.5	5	5	5	1.5	1	5	0.5
*FMO (Fluroescence minus one)									

2.8 Measurement of IL-17 in cervical secretions and plasma by enzyme-linked immunosorbent assay (ELISA)

ELISA is an immunoassay technique which employs a sandwich antibody capture method on a planar surface (usually a 96-well plate). The plate is coated with an antibody that specifically recognises the analyte. Once the analyte is added, a biotin labelled detection antibody binds the analyte that has been immobilised by the capture antibody. An enzyme (e.g. Strepavidine-Horseradish Peroxidase) is added and forms a complex with the biotin-labelled detection antibody. A substrate (e.g. peroxidase) is then added that reacts with the Strepavidine-Horseradish Peroxidase enzyme to form a colour product (Figure 2.1). The intensity of the colour product is measured on an instrument and it directly correlates with the amount of analyte present in the sample.

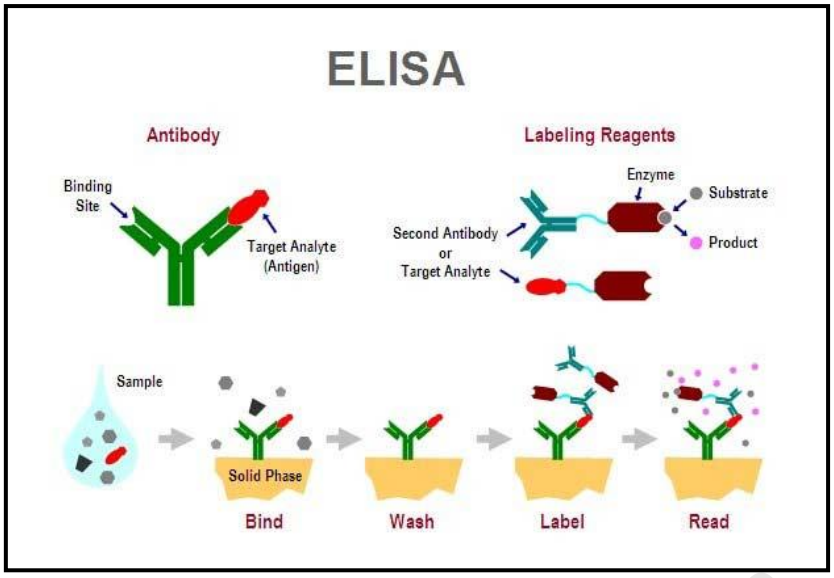


Figure 2.1: Diagram representing the traditional method for an enzyme-linked immunosorbent assay (Abcom®, 2010). ELISA is based on the principle involving an antibody specific for an antigen. These antibodies capture antigens in a sample either specifically or non-specifically and are immobilized on a solid support (a "sandwich" ELISA). A detection antibody (which is either covalently linked to an enzyme or is bioconjugated to a secondary antibody linked to an enzyme) is then added and this forms a complex with the antigen. Between each step the plate is washed with detergent to remove non-specific proteins or antibodies. An enzymatic substrate is added after the last wash step. This produces a visible signal which indicates the amount of antigen present in the sample (Adler *et al.*, 2009).

The Quantikine® Human IL-17 ELISA detection kit (R&D Systems, MN, USA) was used in this study to measure the concentration of IL-17 in plasma and cervical supernatants. The Quantikine® kit contained a 96-well microplate pre-coated with anti-IL-17 capture antibody, a horseradish peroxidase conjugated detection antibody, recombinant human IL-17 as a standard, assay diluents and wash reagents, colour reagents containing hydrogen peroxide substrate and a stop reagent containing HCL. Details of the reagents are provided in Table 2.4. The reported limit of detection for the kit was 15pg/ml of IL-17.

TABLE 2.4| Reagents used in Human IL-17 Quantikine® ELISA kit

Reagent	Composition	Purpose
IL-17 Microplate	96 well polystyrene microplate coated with a mouse monoclonal antibody against IL-17	Wells of plate are the planar surface in which the capture antibody is immobilized
IL-17 Conjugate	Polyclonal antibody against IL-17 conjugated to horseradish peroxidase	Binds plate-bound IL-17. Horseradish peroxidase is the enzyme for the colour product reaction that quantifies the amount of IL-17.
IL-17 Standard	Lyophilized recombinant human IL-17 in a buffered protein base	Known concentration of IL-17 used to generate standard curve of the assay
Calibrator diluent RD5R	Buffered protein solution	Diluent for cell culture supernatants (used to assay cervical supernatants in the study)
Calibrator diluent RD6-21	Buffered protein solution	Diluent for plasma samples
Assay diluent RD1-36	4-fold concentrated buffered protein solution	Diluent used in dissolving lyophilized standard
Wash Buffer concentrate	25-fold concentrated solution of buffered surfactant	Wash solution for automated plate washer
Colour reagent A	Stabilized hydrogen peroxide	Part of the substrate reacts with horseradish peroxidase complex (enzyme)
Colour reagent B	Stabilized chromogen (tetramethylbenzidine)	Substrate that reacts with horseradish peroxidase complex (enzyme) to form colour product
Stop Solution	Diluted HCl solution	Stops colour ELISA reaction
Plate covers	Adhesive strips	Protect assay samples

To generate a standard curve, the lyophilised recombinant human IL-17 standard provided with the kit was reconstituted in 1ml of distilled water to form a 20 000pg/ml stock solution. Two-fold serial dilutions of the stock solution were made in calibrator diluent RD5R (for cervical supernatants) or RD6-21 (for plasma samples) (range 31.25pg/ml-2000pg/ml) according to the manufacturer's instructions. The approporiate calibrator diluent was used as the zero standard (0pg/ml). Cryo-preserved plasma and cervical supernatants were thawed overnight at 4°C. Plasma samples were centrifuged at 2000 rpm (4°C) for 15 minutes and the supernatant carefully removed prior to assaying. All samples and standards were assayed in duplicate.

To measure IL-17 concentrations, a volume of 100µl of assay diluent RD1-36 was added to each well, followed by 100µl of standard, control or sample in duplicate. Plates were then incubated for 3 hours in the dark at room temperature. Each well was then aspirated and washed three times in 1 x wash buffer (wash buffer concentrate diluted in distilled water) using an automated plate washer (ELx50 Auto Strip Washer; Bio-Tek Instruments Inc.). A volume of 200µl of IL-17 conjugate (detection antibody conjugated to horseradish peroxidase) was added to each well and incubated for 1 hour at room temperature. Each well was washed three times in 1 x wash buffer using an automated plate washer. A volume of 200µl of substrate solution (Colour reagent A and Colour reagent B at 1:1 concentration) was added to each well and left to incubate for 30 minutes at room temperature in the dark. Finally, 50µl of Stop Solution containing HCL was added to end the reaction.

Optical density was measured on a VersaMax® microplate reader (Molecular Devices) within 30 minutes of stopping the reaction at wavelength 450nm. Optical imperfections on the plates were corrected by subtracting the reading at 540nm from the reading at 450nm as recommended by the manufacturer. A standard curve from the standards (four parameter logistic curve-fit) was generated using SOFTmax PRO (version 4.3.1, Molecular Devices). SOFTmax PRO software tools were used to calculate the concentration of IL-17 in each plasma and cervical sample using the equation generated from the standard curve.

2.9 Measurement of inflammatory cytokine concentrations in cervical secretions and plasma using the Multiplex Luminex Assay

A Multiplex Luminex Assay was used to measure the concentrations of inflammatory and regulatory cytokines in plasma and cervical supernatant samples from HIV-infected and uninfected women in this study. This assay is similar in prinicipal to the ELISA, in that it is sandwhich immunoassay method. However, the Multiplex Luminex method allows multiple types of analytes to be assayed simultaneously in the same sample (Probst *et al.,* 2003). The principal of this technique is summarised in Figure 2.2. In the Luminex method, the capture antibody is pre-conjugated to polysterene microbeads. The microbeads are dyed with two different flurophores (red and infra-red). Each bead contains each of the two dyes in different ratios, allowing one bead to be distinguished from the other. Beads with different capture antibodies are often mixed in the same assay. Cytokines from the samples are allowed to bind to the conjugated capture antibodies on the beads which are suspended in solution. Once the detection antibody and enzyme are added, beads can be read using the Luminex® 100™ or 200™ detection system to identify and quantify each cytokine (Probst *et al.*, 2003).

MILLIPLEX™ (Millipore Corporation, MA, USA) normal sensitivity kits were used to determine the concentration of 5 human cytokines (IL-1β, IL-6, IL-8, IL-10 and IL-12p40) in plasma and cervical secretions. The reported lower level of detection for the MILLIPLEX™ kits ranged from 0.1 - 9.5pg/ml for each cytokine.

Cervical supernatants and plasma samples were stored at -80°C as described in section 2.3 and 2.4, respectively. Samples were thawed slowly for use in the multiplex assay by placing them on ice and storing overnight at 4°C. The 96-well microtitre filter plate was pre-wet by aliquoting 200µl of Assay Buffer (supplied with the kit) into each well, shaking on a plate shaker for 10 minutes at room temperature and removing the Wash Buffer by vacuum. A volume of 25µl Assay Buffer was added to each of the duplicate background wells and to each of the sample wells.

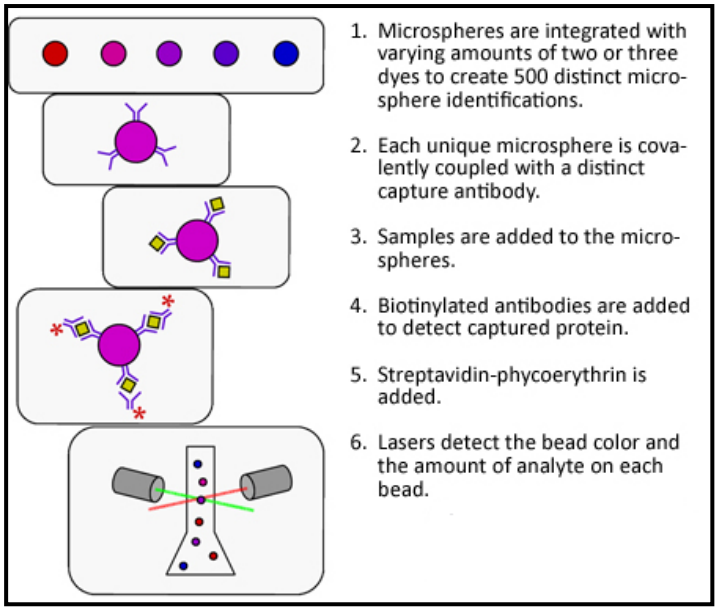


Figure 2.2: Principal of Multiplex Luminex methodology (BioLegend®, 2010). Microspheres are internally colour-coded with two fluorescent dyes. One hundred distinctly coloured bead sets each coated with a specific antibody can formed. After an analyte is captured by the bead, a biotinylated detection antibody is added. The reaction mixture is incubated with the reporter molecule Streptavidin-PE conjugate. This completes the reaction on the surface of each microsphere. The microspheres pass quickly through a laser which excites the internal dyes on the microspheres. A second laser excites the fluorescent dye, PE, on the reporter molecule. A high-speed digital-signal processor identifies each microsphere. The results are quantified based on the fluorescent reporter signals. Multiple results from a single sample can be obtained by adding multiple conjugated beads to each sample (BioLegend®, 2010).

Lyophilised human cytokine standard cocktail (supplied with kit) was diluted to 3.2, 16, 80, 400, 2000 and 10 000pg/ml in Assay Buffer and a volume of 25µl of each standard were aliquoted to the appropriate standard wells. Quality Control vials I and II, each containing a lyophilised mixed cytokine cocktail, were reconstituted in 250µl deionised water and 25µl was aliquoted into each appropriate control well. A volume of 25µl of RPMI was added as a control for background cytokine production in RPMI media. Filtered cervical supernatant samples (diluted 1:2 in PBS) and plasma samples (plated neat), were added to the appropriate sample wells at a volume of 25µl. Each of the 5 antibody-immobilised beads was vortexed, pooled and 25µl of the mix was aliquoted into each well of the microtitre filtration plate. The plate was sealed with an adhesive plate sealer, covered with aluminium foil and incubated on a plate shaker for 1 hour at room temperature.

Following the appropriate incubation periods, fluid was gently removed from the microtitre plates by vacuum. Plates were washed twice by aliquoting 200µl per well of wash buffer, removing fluid by vacuum and blotting the plates on absorbent paper towels to remove excess fluid. A volume of 25µl of biotinylated detection antibody cocktail was added to each well and plates were incubated for 30 minutes on a plate shaker at room temperature. Streptavidin-Phycoerythrin was added at a volume of 25µl to each well containing the detection antibody and the plate was sealed, covered and kept agitated on a plate shaker for 30 minutes at room temperature.

All fluid was removed by vacuum and the plate was washed twice with 200µl per well of wash buffer (as described above). Sheath fluid (Luminex Corporation, USA) was added to all wells at a volume of 150µl per well. The plate was sealed, covered and agitated on a plate shaker for 10 minutes to re-suspend the beads. The cytokine and chemokine levels in plasma and cervical supernatant were quantified in a BioRad Bio-Plex Instrument and analysed using Bio-Plex Manager Systems software (Luminex xMAP^{TM} Technology). Data was collected using a Bio-Plex^{TM} Suspension Array Reader (Bio-Rad Laboratories Inc$^{®}$) and 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 levels that were below the lower limit of detection of the assay were reported as the mid-point between the lowest concentrations measured for each cytokine and zero.

2.10 Measurement of lipopolysaccharide (LPS) concentrations in cervical secretions using the Chromogenic Limulus Amebocyte Lysate (LAL) Assay

This assay has previously been used as an *in vitro* end-product endotoxin test for human biological products (Solum *et al.*, 1973) and was used in this study to measure LPS concentrations in genital secretions. The LAL test measures the amount of gram-negative bacterial endotoxins present in a sample (Young *et al.,* 1972). The LAL assay uses the LAL endotoxin reaction to activate an enzyme which results in p-nitroaniline (pNA) release from a synthetic substrate. This produces a yellow colour (Figure 2.3).

Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL. The rate of activation is determined by the concentration of endotoxin present in the sample. The pNA released is measured photometrically at 405-410nm after the reaction is stopped with stop reagent. The concentration of endotoxin is calculated from the absorbance values of solutions containing known amounts of endotoxin standard. Reagents used in this assay are shown in Table 2.5.

TABLE 2.5| Reagents used in the LAL assay (Cambrex Bio, Walkersville, MD)

Reagent	Composition	Purpose
Pyroplates	96-well endotoxin-free uncoated plates, with lids	Wells of plate are flat-bottomed to assay for LAL
Limulus Amebocyte Lysate	Contains lyophilized lysate prepared from the circulating amebocytes of the horseshoe crab *Limulus polyphemus* reconstituted with LAL reagent water	Used in the endotoxin reaction to activate an enzyme which in turn releases p-nitroaniline (pNA) from a synthetic substrate
E. coli endotoxin	Contains 15-40 EU lyophilized endotoxin reconstituted with LAL reagent water	Known concentration of endotoxin used to generate standard curve of the assay
Chromogenic substrate	Contains 7mg lyophilized substrate reconstituted with LAL reagent water	Used as a substrate in the LAL reaction to form a chromophore which absorbs at 405nm
LAL reagent water	Deionized, endotoxin-free water	Used for reconstituting reagents and for negative controls.

Standards were prepared by diluting endotoxin stock solution with LAL reagent water to give a concentration of 1.0 EU/ml and a two-fold dilution series was used. The microplate method was used for this assay. The microplate was heated on a 37˚C heating block and 50µl of cervical, plasma and standard was added to the appropriate wells. The cervical supernatants were plated in neat and in 10-fold dilutions with R10 media. The blanks contained 50µl of LAL reagent water. At the beginning of the experiment, 50µl of LAL was added to the first column of microplate wells and was left to incubate at 37˚C. After 10 minutes, 100µl of pre-warmed (37˚C) substrate solution was added. After 16 minutes of incubation, 100µl of stop reagent was added.

Optical density was measured on a VersaMax® microplate reader (Molecular Devices). A standard curve from the standards (four parameter logistic curve-fit) was generated using SOFTmax PRO (version 4.3.1, Molecular Devices). SOFTmax PRO software tools were used to calculate the concentration of LPS in each cervical sample using the equation generated from the standard curve. The absorbance was read at 405nm.

2.11 Detection of β-δ-Glucan (fungal antigen) in plasma and cervical secretions

The Fungitell® (Associates of Cape Cod Inc.) assay is a protease zymogen-based colorimetric assay for qualitative detection of (1→3)-β-δ-Glucan in serum and culture supernatants of patients with fungal infections. The concentration of (1→3)-β-δ-glucan, a major cell-wall component of fungi, is used in the diagnosis of mycoses and fungemias (Obayashi *et al.*, 1995). The assay is based upon modification of the LAL pathway (Figure 2.3). For this study, it was used to test cervical samples for fungal (1→3)-β-δ-Glucan. Reagents used in this assay are shown in Table 2.6.

The Fungitell® assay was used to detect (1→3)-β-δ-Glucan produced by *Candida albicans* in cervical samples. Standards were prepared using lyophilised glucan and reagent grade water to make a 100pg/ml solution. A two-fold dilution series was used and 25µl of the standard was used per well. A volume of 5µl of sample was added to each well and each sample was assayed in duplicate, after which 20µl of serum pre-treatment reagent was added. The plate was agitated to mix the contents and incubated at 37˚C for ten minutes in the incubating plate reader. The standards and negative controls (reagent grade water) were then added to the wells, after which 100µl of Fungitell reagent was added to each well.

TABLE 2.6| Reagents used in the (1→3)-β-δ-Glucan (Fungitell®) assay		
Reagent	Composition	Purpose
Pyroplates	96-well glucan-free uncoated plates, with lids	Wells of plate are flat-bottomed to assay for (1→3)-β-δ-Glucan
Fungitell® Reagent	One vial of Fungitell reagent add to 2.7ml of reagent grade water and 2.8ml Pyrosol Reconstitution buffer	A lyphilized (1→3)-β-δ-Glucan specific LAL
Pyrosol reconstitution Buffer	Tris HCL 0.2*M* pH 7.4	For constitution of the Fungitell® Reagent
Glucan Standard	Lyophilized pachyman and inert filler with (1→3)-β-δ-Glucan content shown on the label	Known concentration of glucans used to generate standard curve of the assay
Reagent Grade Water	Deionised, glucan-free water	For glucan-free dilutions of assay reagents and standards
KCI	1.2*M*	For serum pre-treatment reagent reconstitution
KOH	0.25*M*	For serum pre-treatment reagent reconstitution
Serum pre-treatment reagent	900µl each of 0.25*M* KOH and 1.2*M* KCL	Converts triple-helix glucans into single-stranded glucans which are more reactive in the assay

The plate was inserted into the microplate reader (equilibrated to 37˚C) with the lid on and agitated for 5-10 seconds. The reading at 405nm was subtracted from the reading at 490nm, for 40 minutes at 15-20 second intervals at 37˚C. The data was collected and analyzed by calculating the mean rate of optical density change (milli-absorbance units per minute) for all points between 0 and 40 minutes. Optical density was measured on a VersaMax® microplate reader (Molecular Devices). A standard curve from the standard concentrations (four parameter logistic curve-fit) was generated using SOFTmax PRO (version 4.3.1, Molecular Devices). SOFTmax PRO software tools were used to calculate the concentration of (1→3)-β-δ-Glucan in each cervical sample using the equation generated from the standard curve.

Samples <60pg/ml were interpreted as β-δ-Glucan negative while samples with values >80pg/ml were interpreted as β-δ-Glucan positive.

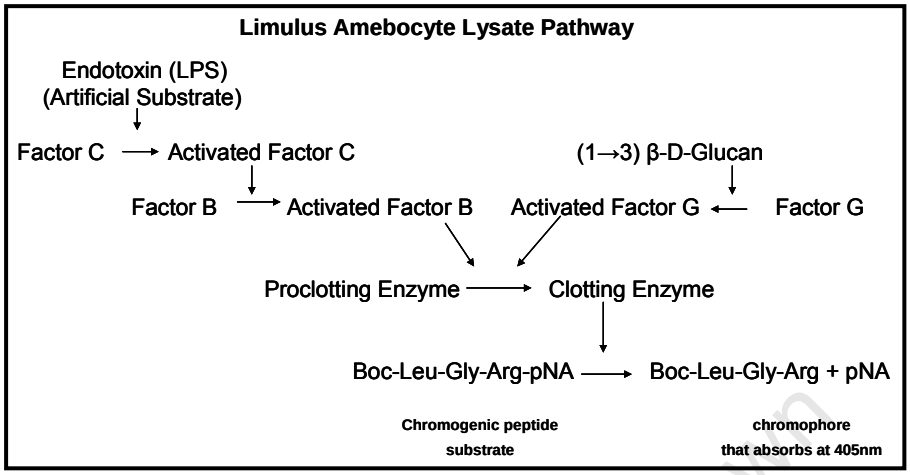


Figure 2.3: Flow diagram representing a modification of the LAL pathway for LPS and (1→3)-β-δ-Glucan detection (described in 2.11 and 2.12). (1→3)-β-δ-Glucan activates Factor G, a serine protease zymogen, while LPS activates Factor C. This in turn activates Factor B. Both the activated Factor G and Factor B converts the inactive proclotting enzyme to the active clotting enzyme, which in turn cleaves pNA from the chromogenic peptide substrate, Boc-Leu-Gly-Arg-pNA, creating a chromophore that absorbs at 405nm. The Fungitell kinetic assay is based upon the rate of optical density increase produced by a sample.

2.12 Detection of soluble CD14 in cervical secretions by ELISA

The human soluble CD14 (sCD14) solid phase sandwich ELISA kit has been developed for quantitative measurement of natural and recombinant human CD14 in serum, plasma and culture medium (Biometic, Enzo Life Sciences®, Austria). Table 2.7 lists the reagents included in the kit, composition and purpose. A mixture of two monoclonal antibodies specific for sCD14 was pre-coated to the wells. CD14 exists in two forms: anchored to the membrane of antigen presenting cells and the other is the soluble form. LPS binds to sCD14 only in the presence of LPS-binding protein (LBP) (Kitchens *et al.*, 2000).

sCD14 is sufficient in low concentrations to confer a response to LPS and therefore has robust responses and gives more accurate measurements of LPS concentrations (Tapping *et al.*, 2000). LPS is an endotoxin and therefore elicits unstable immune responses (Rozhkova *et al.*, 2010) and previous studies have shown that measuring LPS concentrations has proven to be difficult (Brenchley *et al.*, 2006). This ELISA was used to detect sCD14 in cervical supernatants as a surrogate measure of bacterial burden in the FGT.

TABLE 2.7| Reagents used in the sCD14 ELISA assay (Alexis Biochemicals, Biometec)

Reagent	Composition	Purpose
sCD14 Microplate	96 well polystyrene microplate precoated with a monoclonal antibody against sCD14	Wells of plate are the planar surface in which the capture antibody is immobilized
Wash buffer	PBS/Tween 0.05% PBS dissolved in 200ml distilled water and 100µl 0.05% Tween 20	Used to wash unbound protein from wells
PBS	1 tablet dissolved in 200ml distilled water	Used to reconstitute the wash buffer
Dilution buffer	Dilution buffer dissolved with 50ml PBS and 50µl Tween 20	Buffer used to dilute the standards
Reference serum	Reconstituted lyophilized reference serum in 10µl distilled water and 990µl dilution buffer	Serves as a positive control
CD14 standard	30µl distilled water to human-CD14-standard with 970µl dilution buffer.	Known concentration of human CD14 used to generate standard curve of the assay

All reagents were used at room temperature. To generate a standard curve, the lyophilised recombinant human CD14 standard provided with the kit was reconstituted in 30µl of distilled water, after which this content was diluted with 970µl dilution buffer. Fifty microlitres of this was added to 450µl dilution buffer to form a 50ng/ml stock solution. The standards were used in a two-fold dilution series. A volume of 100µl of standards and samples was added to each well and each standard and sample was assayed in duplicate. Plates were incubated for one hour at room temperature while shaking.

The plates were then washed three times with wash buffer. A volume of 100µl of detection antibody was added to each well and incubated at room temperature for one hour and shaking. The plates were washed again three times with wash buffer. A volume of 100µl of substrate solution was added to each well and incubated for 14 minutes at room temperature without shaking, after which 100µl of stop solution was added to each well. Optical density was measured on a VersaMax® microplate reader (Molecular Devices) within 30 minutes of stopping the reaction at wavelength 450nm. A standard curve from the standard concentrations was generated using SOFTmax PRO (version 4.3.1, Molecular Devices). SOFTmax PRO software tools were used to calculate the concentration of sCD14 in each cervical sample using the equation generated from the standard curve.

2.13 Measurement of TGF-β1 in cervical secretions and plasma by ELISA

The Quantikine® Human TGF-β1 ELISA detection kit (R&D Systems, MN, USA) was used in this study to measure the concentration of TGF-β1 in plasma and in cervical secretions. The Quantikine® sandwich ELISA kit contained a 96-well microplate pre-coated with anti-TGF-β1 capture antibody, a horseradish peroxidase conjugated detection antibody, recombinant human TGF-β1 as a standard, assay diluents and wash reagents, colour reagents containing hydrogen peroxide substrate and a stop reagent containing HCL (Table 2.8).

To generate a standard curve, lyophilised recombinant human TGF-β1 provided with the kit as a standard was reconstituted in 2ml of 1 x Calibrator Diluent RD-56 to form a 2000pg/ml stock solution. Two-fold serial dilutions of the stock solution were made in calibrator diluent RD5-26 (ranging from 31.25pg/ml - 2000pg/ml) according to the manufacturer's instructions. Calibrator diluent RD5-26 was used as the zero standard (0pg/ml). Each concentration in the dilution series was assayed in duplicate.

The majority of human TGF-β exists in a latent form bound to Latency Associated Protein (LAP; Lawrence *et al*., 1984) and its biological activity requires release from LAP (Gentry *et al.*, 1988). This is achieved by disrupting LAP by acidification (Pircher *et al.*, 1986; Wakefield, 1987). All samples were acid activated and assessed in duplicate (described below).

TABLE 2.8| Reagents used in Human TGF-β1 Quantikine® ELISA kit		
Reagent	Composition	Purpose
TGF-β1 Microplate	96 well polystyrene microplate coated with a monoclonal antibody specific for TGF-β1	Wells of plate are the planar surface in which the capture antibody is immobilized
TGF-β1 Conjugate	Polyclonal antibody against TGF-β1 conjugated to horseradish peroxidase	Binds plate-bound TGF-β1. Horseradish peroxidase is the enzyme for the colour product reaction that quantifies the amount TGF-β
TGF-β1 Standard	Lyophilized recombinant human TGF-β1 in a buffered protein base	Known concentration of TGF-β1 used to generate standard curve of the assay
Assay diluent RD1-21	Buffered protein solution	Diluent for cell culture supernatants (used to assay cervical supernatants in the study)
Assay diluent RD1-73	Buffered protein solution	Diluent for plasma samples
Calibrator diluent RD5-26	4-fold concentrated buffered protein solution	Diluent used in dissolving lyophilized standard
Wash Buffer concentrate	25-fold concentrated solution of buffered surfactant	Wash solution for automated plate washer
Colour reagent A	Stabilized hydrogen peroxide	Part of the substrate reacts with horseradish peroxidase complex (enzyme)
Colour reagent B	Stabilized chromogen (tetramethylbenzidine)	Substrate that reacts with horseradish peroxidase complex (enzyme) to form colour product
Stop Solution	Diluted HCL solution	Stops colour ELISA reaction
Plate covers	Adhesive strips	Protect assay samples

All samples were acid activated before measurement of TGF-β according to the manufacturer's instructions. Cryo-preserved plasma and cervical supernatants were thawed overnight at 4°C. Plasma samples were centrifuged at 2000g (4°C) for 15 minutes and the supernatant carefully removed to pellet any contaminating platelets containing latent TGF-β (Pircher *et al.,* 1986; Miyazono *et al*., 1988). For acid activation, 20µl of 1N HCL was added to 100µl of cervical supernatant or 40µl plasma. Samples were mixed well by pipette and incubated for 10 minutes at room temperature. The acidification reaction was stopped by neutralizing samples with 20µl of a 1.2N NaOH/0.5M HEPES. Plasma levels required a 1:2 or 1:4 dilution to fall within the standard curve; cervical samples were not diluted.

To assay for TGF-β concentrations, a volume of 50µl of assay diluents RD1-73 (for plasma samples) and RD1-21 (for cervical supernatants) was added to each well, followed by 50µl of acid activated samples in duplicate. Samples were incubated in the plate for 2 hours at room temperature, allowing the anti-TGF-β capture antibody to bind TGF-β. Each well was then washed four times in 1 x wash buffer (supplied with the kit) using an automated plate washer (EL_x50 Auto Strip Washer; Bio-Tek Instruments, Inc.). A volume of 100µl of TGF-β conjugate (detection antibody conjugated to horseradish peroxidase) was added to each well and incubated for 2 hours at room temperature. Each well was washed four times in 1 x wash buffer using an automated plate washer. A volume of 100µl of substrate solution (Colour reagent A and Colour reagent B at 1:1 concentration) was added to each well and left to incubate for 30 minutes at room temperature in the dark. Finally, 100µl of Stop Solution containing HCL was added to end the reaction.

Optical density was measured on a VersaMax® microplate reader (Molecular Devices) within 30 minutes of stopping the reaction at 450nm. Optical imperfections on the plates were corrected for by subtracting the reading at 540nm from the reading at 450nm as recommended by the manufacturer. A standard curve from the standard concentrations (four parameter logistic curve-fit) and the concentration of TGF-β in each plasma and cervical sample using the equation generated from the standard curve was calculated using SOFTmax PRO (version 4.3.1, Molecular Devices).

2.14 Detection of IL-23 in cervical secretions and plasma by ELISA

The Quantikine® Human IL-23 Immunoassay is a solid-phase quantitative sandwich ELISA designed to measure human IL-23 (R&D Systems, MN, USA). Details of the reagents provided by the manufacturer are provided in Table 2.9. All reagents were used at room temperature. To generate a standard curve, lyophilised recombinant human IL-23 provided as a standard with the kit was reconstituted in 1ml of distilled water to form a 10 000pg/ml stock solution. Four-fold serial dilutions of the stock solution were made in calibrator diluent RD5-16 for cervical supernatants (range: 39pg/ml - 10 000pg/ml) according to the manufacturer's instructions. The calibrator diluent was used as the zero standard (0pg/ml). Cryo-preserved cervical supernatants were thawed overnight at 4°C. All samples and standards were assayed in duplicate.

TABLE 2.9| Reagents used in Human IL-23 Quantikine® ELISA kit		
Reagent	Composition	Purpose
IL-23 Microplate	96 well polystyrene microplate coated with a goat polyclonal antibody against the human IL-23 p19 subunit.	Wells of plate are the planar surface in which the capture antibody is immobilized
IL-23 Conjugate	Goat polyclonal antibody against the human Il-23 p40 subunit conjugated to horseradish peroxidase with preservatives	Binds plate-bound Il-23. Horseradish peroxidase is the enzyme for the colour product reaction that quantifies the amount IL-23
IL-23 Standard	Recombinant human IL-23 in a buffered protein base with preservatives, lyophilized	Known concentration of IL-23 used to generate standard curve of the assay
Assay diluent RD1-22	Buffered protein solution	Diluent for cell culture supernatants (used to assay cervical supernatants in the study)
Calibrator diluent RD5-16	Buffered protein solution	Diluent used in dissolving lyophilized standard
Wash Buffer concentrate	25-fold concentrated solution of buffered surfactant	Wash solution for automated plate washer
Colour reagent A	Stabilized hydrogen peroxide	Part of the substrate reacts with horseradish peroxidase complex (enzyme)
Colour reagent B	Stabilized chromogen (tetramethylbenzidine)	Substrate that reacts with horseradish peroxidase complex (enzyme) to form colour product
Stop Solution	2N sulphuric acid solution	Stops colour ELISA reaction
Plate covers	Adhesive strips	Protect assay samples

To assay for IL-23 concentrations, a volume of 100µl of assay diluent RD1-22 was added to each well, followed by 100µl of standard, control or sample in duplicate. Plates were then incubated for 2 hours while shaking at 500 rpm. Each well was then aspirated and washed 4 times in 1 x wash buffer (wash buffer concentrate diluted in distilled water) using an automated plate washer (EL_x50 Auto Strip Washer; Bio-Tek Instruments, Inc.). A volume of 200µl of IL-23 conjugate (detection antibody conjugated to horseradish peroxidase) was added to each well and incubated for 2 hours at room temperature while shaking at 500 rpm. Each well was washed 4 times in 1 x wash buffer using an automated plate washer. A volume of 200µl substrate solution (Colour reagent A and Colour reagent B at 1:1 concentration) was added to each well and left to incubate for 30 minutes at room temperature in the dark. Finally, 50µl of Stop Solution containing sulphuric acid was added to end the reaction.

Optical density was measured on a VersaMax® microplate reader (Molecular Devices) within 30 minutes of stopping the reaction at wavelength 450nm. Optical imperfections on the plates were corrected for by subtracting the reading at 540nm from the reading at 450nm as recommended by the manufacturer. A standard curve from the standards (four parameter logistic curve-fit) was generated using SOFTmax PRO (version 4.3.1, Molecular Devices). SOFTmax PRO software tools were used to calculate the concentration of IL-23 in each cervical sample using the equation generated from the standard curve.

2.15 Determination of plasma and cervical HIV load

Frozen cervical supernatants and plasma samples were thawed overnight at 4˚C. The viral load in thawed plasma and cervical supernatants were measured using Nuclisens Easyq HIV 1 Version1.2 by the NHLS Laboratory at Groote Schuur Hospital. The lower level of detection was 70 copies of HIV RNA per ml. Detection of HIV copies in cervical supernatant was used as an indicator of HIV shedding.

2.16 Statistical analysis

Data analysis was performed using Microsoft® Excel Analysis ToolPack and GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA). For normally distributed data (such as the frequency of phenotypic markers), the student unpaired t-test was applied to compare unmatched data while the paired t-test was used to compare matched data. Pearson correlation was used to investigate the association between variables. For non-parametric data (such as the frequency of functional markers), non-paired medians were compared using the nonparametric Mann-Whitney test while paired data was compared using the Wilcoxon Ranks test. Correlations between nonparametric variables were determined using the Spearman rank correlation test. P-values of less than 0.05 were considered significant. Individual tests applied for each analysis are indicated in the results section.

Chapter 3

Results

CHAPTER 3

Table of contents

3.1 Clinical description of individuals included in this study

Forty women chronically infected with HIV and 13 uninfected women were included in this study (Table 3.1) to investigate the impact of HIV infection on Th17 cell frequencies and function in the female genital tract. Of the forty HIV-infected women, 24/40 (60%) were on HAART (HIV^+HAART^+) while 16/40 (40%) were naïve to HAART (HIV^+HAART^-). All the women were pre-menopausal with a median age of 36 years (IQR 22-52 years). HIV^+HAART^- women had a median blood CD4 count of 378 cells/µl (IQR 243-1215) while HIV^+HAART^+ women had a median CD4 count of 555 cells/µl (IQR 170-1187). Absolute CD4 counts in the blood of HIV^+HAART^+ women was significantly higher than CD4 counts in HIV^+HAART^- women ($p<0.0001$). These results indicate that CD4 T cells are reconstituted in the blood of HIV^+HAART^+ women compared to HIV^+HAART^- women.

The median plasma viral load amongst the HIV^+HAART^- women was 21 200 RNA copies/ml (IQR 2800-134 750). None of the HIV^+HAART^+ women had detectable plasma HIV viral loads. Of the 16 therapy naïve HIV-infected women included in this study, 5/16 (31%) were considered to be shedding HIV into their genital secretions as they had >300 copies of HIV RNA/ml of cervical supernatant. In contrast, none of the $HAART^+$ HIV-infected women were shedding HIV in their genital secretions. In $HAART^-$ HIV-infected women, a significant positive relationship was found between cervical and blood viral loads (Rho=0.3; p=0.6), suggesting that viral load in the blood was a reliable predictor of viral load at the cervix. These results also indicate that viral load is effectively suppressed in both blood and at the cervix of HIV-infected women on HAART.

TABLE 3.1| Clinical descriptions of chronically HIV-infected women included in this study

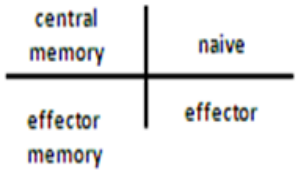
HAART[a] status	Patient ID[b]	Age (years)	CD4 count[c] (mm^3/ml)	Plasma viral load (copies/ml)	Cervical viral load (copies/ml)	HAART regimen[d]	Months on HAART
HAART-	730608	37	ND[e]	280000	1700	-	-
	751129	35	ND	15000	2100	-	-
	770203	33	ND	89000	2000	-	-
	881014	22	ND	2400	<70	-	-
	279-1	42	1215	180000	<70	-	-
	346-1	43	681	3400	<70	-	-
	656-1	34	243	11000	<70	-	-
	656-1	40	264	2600	<70	-	-
	661-1	45	269	190000	<70	-	-
	696-1	29	412	150000	<70	-	-
	704-1	34	589	28000	<70	-	-
	NY065	38	455	3800	<70	-	-
	NY079	38	266	430	<70	-	-
	NY163	33	365	30000	9300	-	-
	NY219	34	349	1300	1900	-	-
	NY248	37	555	78000	<70	-	-
Median (IQR)		37 22-45	388.5 243-681	21 200 2800-134750	2000 (1700-9300)	-	-
HAART+	760104	34	ND	<70	<70	Lamivudine, Stavudine, Nevirapine	ND
	598-1	26	275	<70	<70	Lamivudine, Stavudine, Efivarenz	ND
	602-1	24	379	<70	<70	Lamivudine, Stavudine, Nevirapine	ND
	665-1	43	753	<70	<70	Data unavailable	ND
	676-1	55	ND	<70	<70	Data unavailable	ND
	677-1	38	769	<70	<70	Data unavailable	ND
	692-1	ND	945	<70	<70	Data unavailable	ND
	721-1	52	589	<70	<70	Lamivudine, Stavudine, Efivarenz	ND
	NY061	37	524	<70	<70	Lamivudine, Stavudine, Efivarenz	74
	NY072	36	1187	<70	<70	Combivir, Efivarenz	75
	NY098	39	604	<70	<70	Lamivudine, Stavudine, Efivarenz	65
	NY110	39	170	<70	<70	Lamivudine, Stavudine, Efivarenz	7
	NY125	35	375	<70	<70	Lamivudine, Stavudine, Nevirapine	25
	NY149	34	798	<70	<70	Zidovudin, Efivarenz	73
	NY154	32	764	<70	<70	Lamivudine, Stavudine, Nevirapine	66
	NY159	46	613	<70	<70	Zidovudin, Efivarenz	88
	NY162	36	301	<70	<70	Stocrin	62
	NY164	38	475	<70	<70	Lamivudine, Stavudine, Nevirapine	44
	NY165	29	543	<70	<70	Lamivudine, Stavudine, Nevirapine	16
	NY166	36	349	<70	<70	Lamivudine, Nevirapine, Tenefovir	7
	NY186	36	482	<70	<70	Lamivudine, Nevirapine	36
	NY191	38	279	<70	<70	Combivir, Nevirapine	74
	NY233	50	234	<70	<70	Lamivudine, Stavudine, Nevirapine	5
	NY234	37	516	<70	<70	Data unavailable	ND
Median (IQR)		37 24-55	478.5 170-945	-	-		47.8 29.324

[a] Antireteroviral status; [b] Patient identity; [c] CD4 cell count; [d]HAART regimen; Combivir= Single dose combination of Lamivudine+Zidovudine; IQR (Interquartile range); [e] ND - Not determined

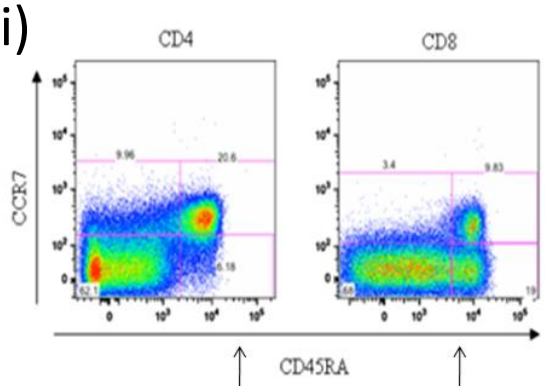
3.2 Flow cytometry to measure Th17 and Th1 frequencies at the cervix and in blood of HIV-infected and uninfected women

An eight colour flow cytometry panel was used to investigate the frequency of Th17 and Th1 memory CD4 and CD8 T cells in the blood (Scriba *et al*., 2008) and at the cervix. Figure 3.1 shows the gating strategy used to define (i) the frequencies of $CD4^+$ and $CD8^+$ T cells, (ii) the proportion of each of these subsets that were naïve or memory, and (iii) the proportion of these that produced IL-17 (defined as Th17 cells) or IFN-γ (defined as Th1 cells) following stimulation with antigen (Gag or CEF peptides) or PMA/ionomycin. Differential staining with maturational markers CCR7 and CD45RA expression by CD4 and CD8 T cells was used to define distinct memory T cell subsets in the blood and cervix of HIV-uninfected, HIV^+HAART^+ and HIV^+HAART^-women. These markers were used to define four distinct T cell subsets: naïve T cells ($CD45RA^+CCR7^+$), effector T cells ($CD45RA^+CCR7^-$), central memory T cells ($CD45RA^-CCR7^+$) and effector memory T cells ($CD45RA^-CCR7^-$) (Sallusto *et al.*, 1999).

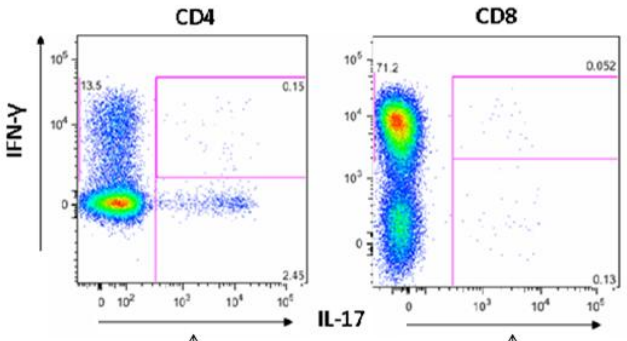
In both HIV-infected and uninfected women, $CD8^+$ T cells in blood and at the cervix produced IFN-γ in response to PMA/ionomycin stimulation but did not produce IL-17 (Figure 3.1). $CD4^+$ T cells produced either IL-17 or IFN-γ in response to PMA/ionomycin stimulation and $CD4^+$ T cells producing both cytokines were not detected. Those that produced IL-17 in response to PMA/ionomycin were considered to be Th17 cells while cells that produced IFN-γ were considered to be Th1 cells.



(ii)

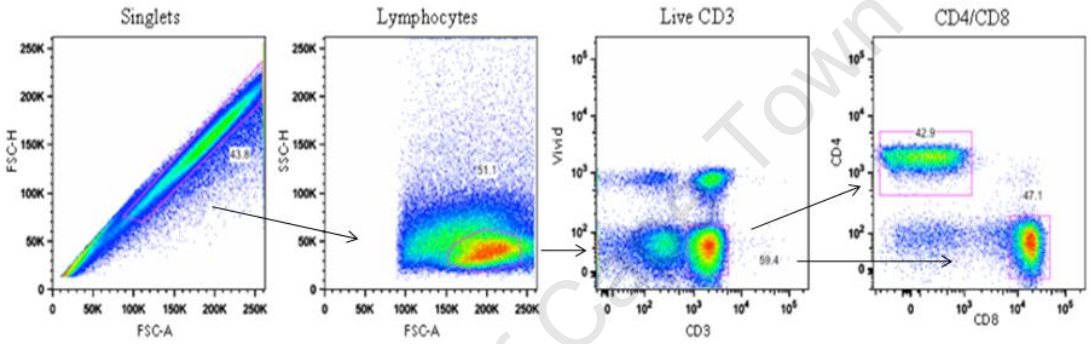


(iii)

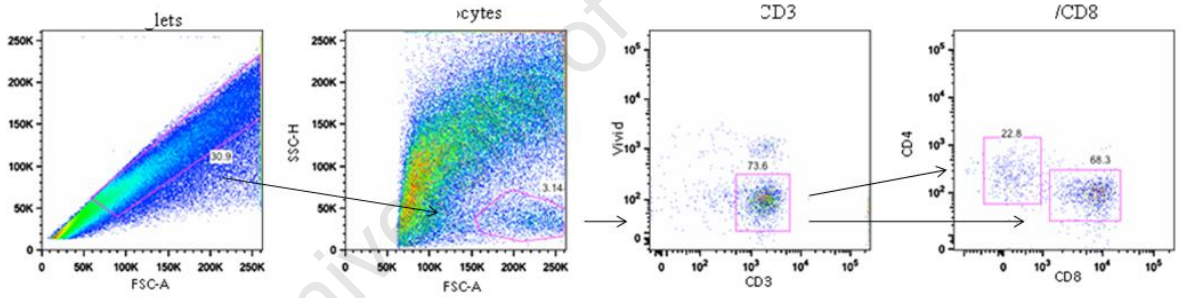


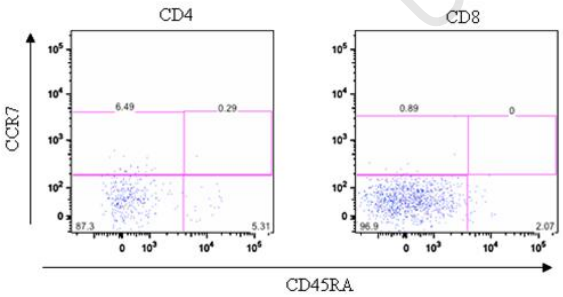
Blood

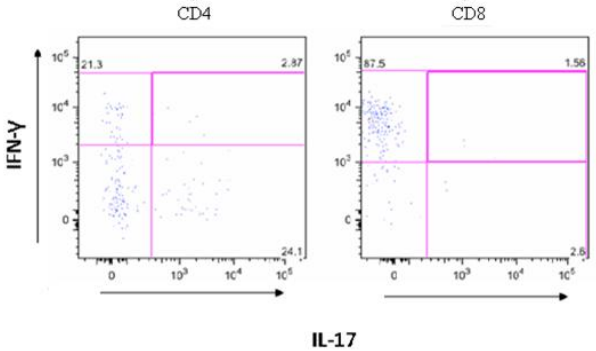
(i)



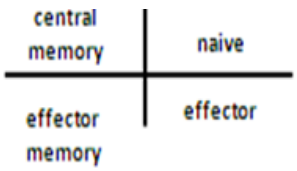
Cervix







(ii)



(iii)

Figure 3.1: Representative plots showing the gating strategy used to define (i) CD4 and CD8 T cells in blood (top) and at the cervix (bottom) of (ii) distinct memory phenotypes that (iii) produced IFN-γ and IL-17 in response to stimulation with PMA/ionomycin stimulation. (i) The singlet gate was based on forward vs. side scatter properties; the lymphocyte gate was based on side scatter (SSC) vs. forward scatter (FSC) properties, CD3 positive events were gated according to Vivid versus CD3 and CD4 and CD8 positive events were visualized on CD4 vs. CD8. (ii) Gating strategy used to characterize the phenotypic markers expressed by $CD4^+$ and $CD8^+$ naïve ($CD45RA^+$) and memory ($CD45RA^-$) T cell subsets. This phenotype panel included an antibody panel consisting of CD4, CD8, CD45RA and CCR7 using eight-colour flow cytometry. (iii) Gating strategy used to characterize IFN-γ and IL-17 production by $CD4^+$ and $CD8^+$ T cell subsets in response to PMA/ionomycin. IFN-γ and IL-17 events were gated on CD4 and CD8 T cell properties. Fluorescence minus one (FMO) was used to set gates. Numbers within gates indicate the percentage of positive gated events.

3.3 Impact of HIV infection on CD4 T cell frequencies at the cervix and in blood

The frequency of $CD4^+$ T cells in the blood and at the cervix of uninfected and HIV-infected women was evaluated (Figure 3.2). In both the cervix and blood, the frequencies of $CD4^+$ T cells were significantly higher in uninfected compared to HIV-infected women not on therapy (Figure 3.2A; p=0.02 at the cervix; p=0.0001 in blood). In addition, the frequency of $CD4^+$ T cells in blood was also significantly higher in uninfected women compared to HIV^+HAART^+ women (Figure 3.2B; p=0.0007). These results suggest that $CD4^+$ T cells may not only be depleted in the blood of HIV-infected women compared to uninfected women but also at the genital mucosa of the cervix. Although HAART resulted in partial restoration of $CD4^+$ T cell proportions in both compartments compared to therapy naive HIV-infected women, $CD4^+$ T cell frequencies in the blood of $HAART^+$ women was still significantly lower than in uninfected individuals.

The association between $CD4^+$ T cell frequencies in blood and at the cervix was investigated to assess whether depletion of blood $CD4^+$ T cells predicted cervical $CD4^+$ T cell depletion during HIV infection (Figure 3.2C). There was a significant positive association between $CD4^+$ T cell frequencies in matched blood and cervical cytobrush samples (Figure 3.2C; Rho=0.4, p=0.02; Spearman Correlation Test). These results suggest that depletion of $CD4^+$ T cells in blood is mirrored at the cervix.

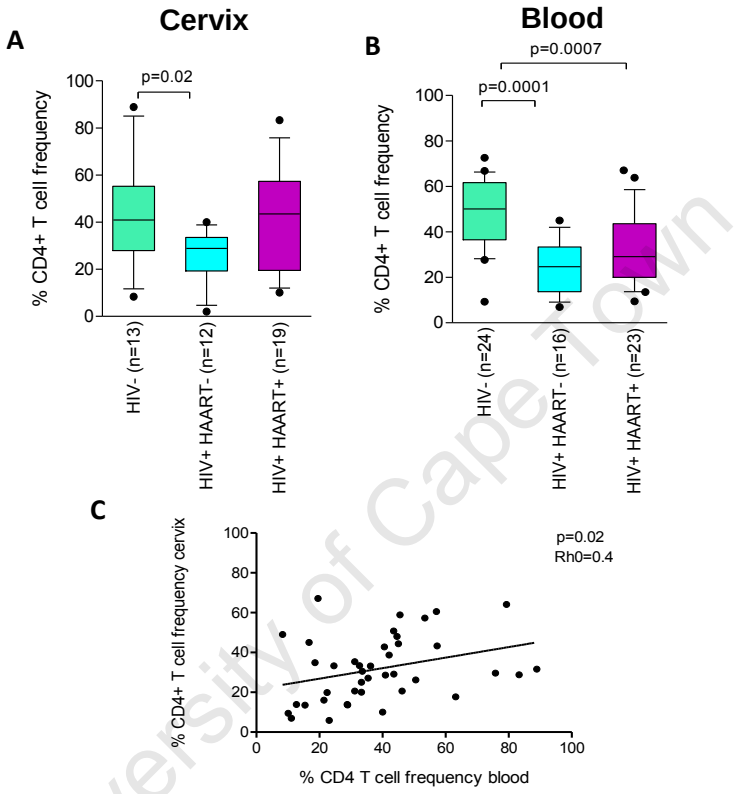


Figure 3.2 Comparison of CD4 T cell frequencies at the cervix (A) and in blood (B) of uninfected (HIV^-), therapy naïve HIV-infected (HIV^+HAART^-) and HIV-infected women on HAART (HIV^+HAART^+). The proportion of $CD3^+$ T cells that were also $CD4^+$ was evaluated in cervical cytobrush samples (A) and blood (B) from uninfected women (green boxes), HIV^+HAART^- women (blue boxes) and HIV^+HAART^+ women (purple boxes). n = sample size. Horizontal lines indicate median values while upper and lower limits of the box indicate the 75^{th} and 25^{th} percentile. Whiskers indicate the 10^{th} and 90^{th} percentile and dots indicate outliers. P-values ≤ 0.05 were considered significant and a non-parametric t-test was used to calculate significance. All women (n=40) were used to evaluate the association of $CD4^+$ T cell frequencies in (C). Spearman Rho values indicate the strength of correlation of $CD4^+$ and $CD8^+$ T cells between blood and cervical compartments.

3.4 Impact of HIV infection on Th17 cell frequencies at the cervix and in blood

The impact of HIV infection on $CD4^+$ Th17 cell proportions was assessed by comparing Th17 cell proportions in the cervix and blood from uninfected, HIV^+HAART^- and HIV^+HAART^+ women following stimulation with PMA/ionomycin (Figure 3.3). Previous studies have shown that Th17 cells are depleted from blood and gut-associated lymphoid tissue during HIV-infection and that this mirrors total CD4 T cell depletion during HIV disease progression (Brenchley *et al.*, 2006; 2008; Dandekar *et al.*, 2010). In blood, this study found that $CD4^+$ Th17 cells were present at significantly reduced frequencies in HIV-infected women compared to uninfected women (Figure 3.3B; p=0.005). Despite showing improved total CD4 T cell proportions in blood (Figure 3.2), HIV-infected women on HAART also had significantly lower frequencies of blood $CD4^+$ Th17 cells compared to uninfected women (p=0.005). In contrast to blood, a significantly higher proportion of $CD4^+$ Th17 cells were detected at the cervix of HIV-infected women compared to uninfected women (Figure 3.3A; $HAART^- HIV^+$, p=0.03; $HAART^+HIV^+$, p=0.04). In contrast to blood, these results suggest that Th17 cells were enriched or selectively preserved at the cervix of HIV-infected women compared to uninfected women. Furthermore, no association was found between the frequency of $CD4^+$ Th17 cells in the cervix and the blood (R=-0.3, p=0.07; Spearman Test; data not shown).

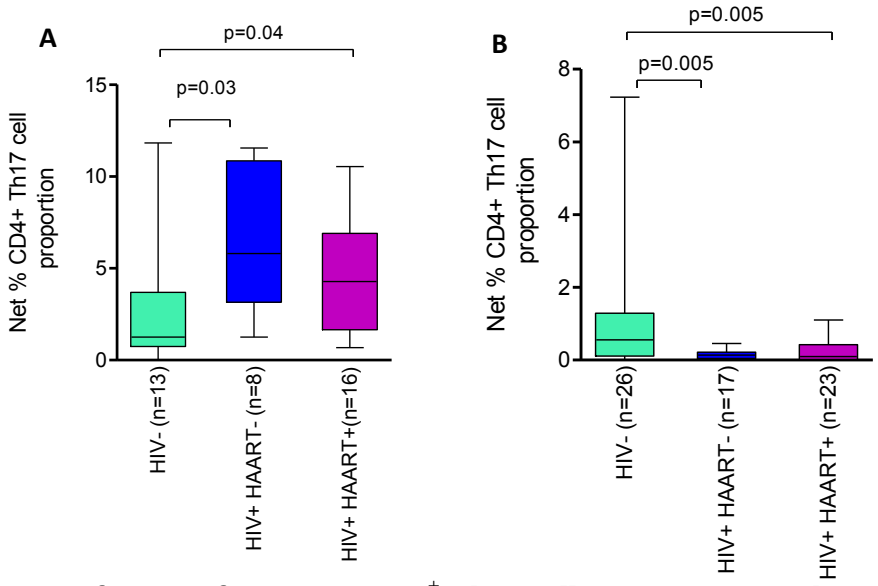


Figure 3.3: Impact of HIV infection on CD4$^+$ Th17 cell proportions at the cervix (A) and blood (B) of HIV uninfected, HIV$^+$HAART$^-$ and HIV$^+$HAART$^+$ women. CD4$^+$ Th17 cell proportions were evaluated in HIV uninfected women (green bars), HIV$^+$HAART$^-$ women (blue bars) and HIV$^+$HAART$^+$ women (purple bars). n = sample size. Horizontal lines indicate median value and $p \leq 0.05$ was considered significant and was calculated using a non-parametric t test.

3.5 Memory status of CD4$^+$ and CD8$^+$ T cells at the cervix and in blood

The maturational status of T cells at the cervix or in blood was further compared in all women in this study based on expression of CD45RA and CCR7 (Figure 3.4). Both CD4$^+$ and CD8$^+$ T cells in the cervix and the blood were predominantly effector memory (CD45RA$^-$CCR7$^-$; Figure 3.4). Significantly higher frequencies of CD4$^+$ T cells from the cervix of uninfected and HAART$^+$ HIV-infected women were effector memory compared with matching blood samples (p=0.03 and p=0.005, respectively). In contrast, naïve T cells and terminally-differentiated effectors were present at significantly lower frequencies in the cervix compared to the blood of HIV uninfected (p=0.004; p=0.01) and HAART$^+$ HIV-infected women (p=0.0001 and p=0.02, respectively). Terminally differentiated effector frequencies were also significantly lower in the cervix compared to blood in HAART$^-$HIV-infected women (p=0.02) (figure 3.4). Similarly, CD8$^+$ T cells at the cervix and in blood were predominantly effector memory, with significantly higher frequencies of cervical CD8$^+$ T cells being effector memory than in blood for HIV-infected women on and off HAART (Figure 3.4B; p=0.0002 and p=0.02, respectively). Furthermore, significantly lower frequencies of CD8$^+$ T cells at the cervix were naive compared to blood in uninfected and HAART$^-$ HIV-infected women (p=0.01 and p=0.002, respectively).

Comparisons of the memory status of $CD4^+$ and $CD8^+$ T cells in HIV uninfected women could not be made as there were no significant differences.

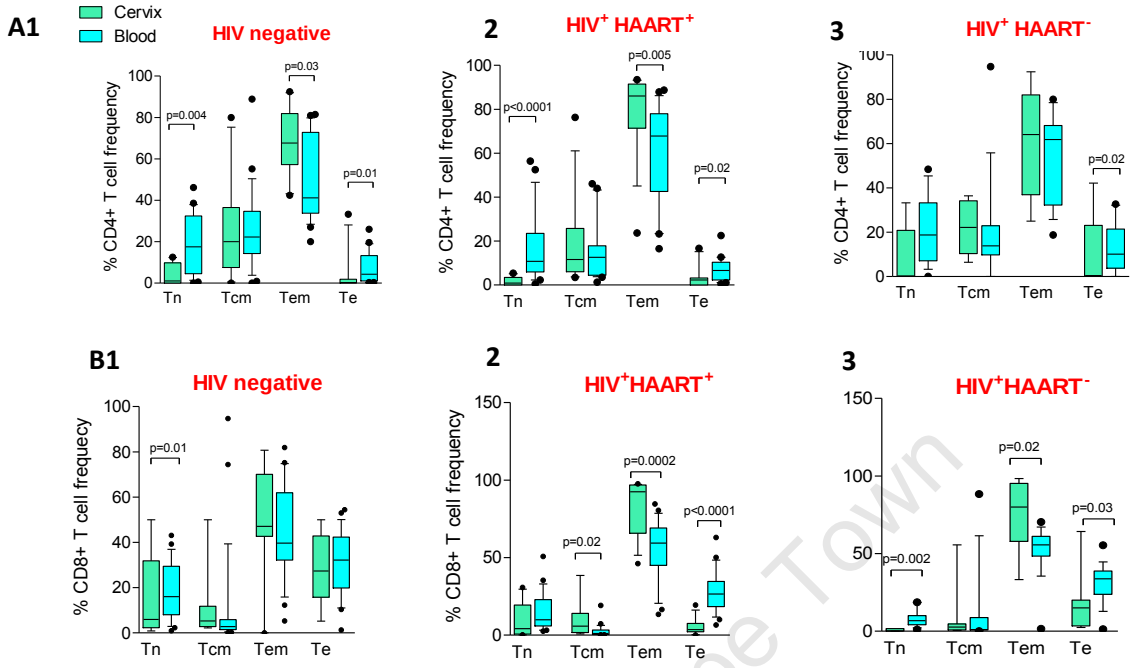


Figure 3.4: Memory differentiation status of CD4 (A) and CD8 (B) T cells in the cervix and blood of uninfected, HIV^+HAART^+ and HIV^+HAART^- women. Green bars represent the cervix memory phenotypes while blue bars represent blood memory phenotypes. Tn: naive ($CD45RA^+CCR7^+$), Tcm: central memory ($CD45RA^-CCR7^+$), Tem: effector memory ($CD45RA^-CCR7^-$), Te: effector ($CD45RA^-CCR7^-$). Horizontal lines in box and whisker plots indicate median values. $P \leq 0.05$ were considered significant.

3.6 Memory status of Th17 cells at the cervix and in blood

As with total CD4+ T cells, Th17 $CD4^+$ T cells were predominantly effector memory in phenotype ($CD45RA^-CCR7^-$; Figure 3.5).

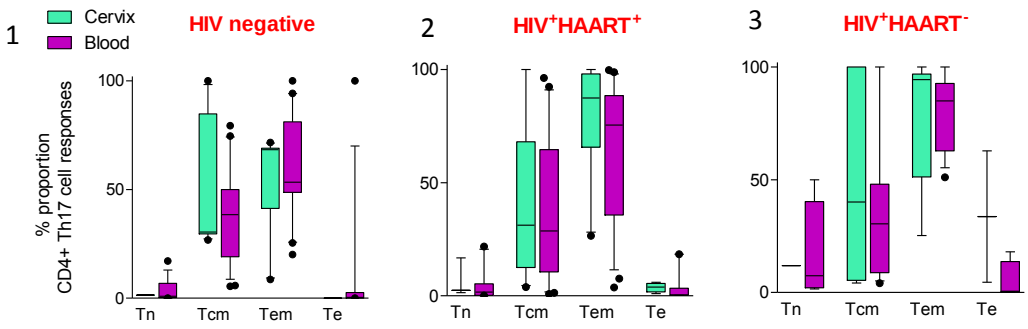


Figure 3.5: Maturational status of CD4$^+$ Th17 cells from the cervix and blood of uninfected, HIV$^+$HAART$^+$ and HIV$^+$HAART$^-$ women. Frequency of memory Th17 cell expression by CD4$^+$ memory T cell subsets in the cervix and blood of HIV uninfected (1), HIV$^+$HAART$^+$ (2) and HIV$^+$HAART$^-$ (3) women was evaluated. Green bars represent the cervix and purple bars represent blood. Tn: naive (CD45RA$^+$CCR7$^+$), Tcm: central memory (CD45RA$^-$CCR7$^+$), Tem: effector memory (CD45RA$^-$CCR7$^-$), Te: effector (CD45RA$^-$CCR7$^-$). Horizontal lines indicate median values. Statistically significant differences between mean values were considered if the P-value was ≤ 0.05.

3.7 Evaluation of HIV Gag-specific T cell responses at the cervix and in blood of HIV-infected women

A subset of the HIV-infected women in this study were evaluated for cervical and blood Th17 (intracellular production of IL-17) or Th1 (intracellular production of IFN-γ) responses to HIV Gag-specific peptides (Table 3.2). Generally, Th17 responsive T cells were detected at very low frequencies in the CD4 compartment and were absent from the CD8 compartment in both blood and at the cervix. CD4$^+$ Th1 responses to Gag were detected at higher frequencies than CD4 Th17 responses confirming that Th17 cells generally do not recognize viral antigens.

Despite being detected at low frequencies, cervical Th17 responses by CD4$^+$ T cells were significantly higher in magnitude than responses detected in matching blood samples. Because this part of the analysis was performed on only a small number of women and because CD4 T cells are depleted in the cervix during HIV infection and therefore represent only a minor population in cervical cytobrush samples, detailed conclusions from this data would not be reliable.

TABLE 3.2: Net[a] HIV Gag-specific Th17 and Th1 responses at the cervix and in blood of HIV-infected women

T cells	Th Subset	HAART$^-$ (n=6) Median % (IQR)[b]		HAART$^+$ (n=16) Median % (IQR)[b]	
		Cervix	Blood	Cervix	Blood
CD4$^+$	Th17	1.1 (0.1-9.2)*	0.0 (0.0-0.04)	1.1 (0.2-2.3)**	0.0 (0.0-0.0)
	Th1	4.9 (3.0-6.7)[c]	0.06 (0.02-1.9)	1.7 (0.5-13.0)	0.09 (0.0-4.1)
CD8$^+$	Th17	0.02 (0.0-0.1)	1.3 (0.1-2.3)	0.0 (0.0-0.1)	0.3 (0.1-0.8)
	Th1	0.9 (0.4-1.3)	0.6 (0.1-2.0)	0.7 (0.4-1.1)	0.1 (0.03-0.2)

[a]Net percentages of CD4 or CD8 T cells producing IL-17 (Th17) or IFN- (Th1) in response to stimulation with HIV Gag peptide pool. Net = Gag-specific cytokine responses minus background cytokine responses (unstimulated control cells). [b]Median (interquartile range). [c]Only 2 women were included in measurement of Th1 CD4+ T cell responses at cervix in HAART- women. * and ** denote that cervical Th17 responses were significantly higher in frequency than Th17 responses in blood (p=0.02* and p<0.0001**).

3.8 Evaluation of CEF-specific T cell responses at the cervix and in blood of HIV-infected and uninfected women

HIV uninfected and infected women were evaluated for CEF-specific T cell responses at the cervix and the blood (Table 3.3). Although Th17 responses were generally absent from both uninfected and HIV-infected women in the cervix and in blood, cervical CD4$^+$ Th17 cell response frequencies to CEF peptides were significantly higher than those detected in blood. CD4$^+$ Th17 cell responses were not measurable in the blood of either uninfected or HIV-infected women. As observed with the Gag-specific responses, blood Th1 responses to CEF were detected less frequently than cervical Th1 responses.

TABLE 3.3. Net[a] CEF-specific Th17 and Th1 responses at the cervix and in blood of uninfected and HIV-infected women

T cells	Th Subset	Uninfected (n=6) Net median % (IQR)[b]		HIV-infected HAART- (n=8) Net median % (IQR)[b]	
		Cervix	Blood	Cervix	Blood
$CD4^+$	Th17	1.5 (0.1-6.1)*	0.01 (0.0-0.03)	0.01 (0.0-0.03)	0.0 (0.0-0.01)
	Th1	0.9 (0.6-3.5)	0.3 (0.2-1.3)	0.3 (0.03-1.6)	0.0 (0.0-0.2)
$CD8^+$	Th17	0.04 (0.02-0.1)	0.02 (0.0-0.05)	0.0 (0.0-0.02)	0.0 (0.0-0.0)
	Th1	1.3 (0.3-8.2)	0.5 (0.1-1.2)	0.3 (0.07-1.1)	0.1 (0.04-0.6)

[a]Net percentages of CD4 or CD8 T cells producing IL-17 (Th17) or IFN-γ (Th1) in response to stimulation with CEF peptide pool. Net = CEF-specific cytokine responses minus background cytokine responses (unstimulated control cells). [b]Median (interquartile range). * denote that cervical Th17 responses were significantly higher in frequency than Th17 responses in blood (p=0.0006).

Together, these results indicate that only low frequencies of $CD4^+$ Th17 cells respond to Gag or CEF peptides. Viral antigen responses in both compartments were dominated by Th1-secreting IFN-γ $CD4^+$ and $CD8^+$ T cells in the cervix and the blood.

3.9 Impact of HIV infection on genital tract inflammatory and regulatory cytokines in genital secretions and plasma

To evaluate whether genital tract inflammation or the innate genital cytokine milieu was associated with recruitment of Th17 cells to the cervix, the concentrations of inflammatory (IL-6, IL-8, IL-1β and IL-12p40) and regulatory (TGF-β, IL-23 and IL-10) cytokines were measured in cervical secretions (Figure 3.6A) and plasma (Figure 3.6B) from HIV-infected and uninfected women. In the genital tract, concentrations of IL-12p40, IL-1β, IL-6, and IL-10 were significantly higher in HIV^+HAART^- women compared to uninfected women (Figure 3.6A). $HAART^+$ women generally had similarly elevated inflammatory cytokine concentrations in genital secretions as $HAART^-$ HIV-infected women. Irrespective of HIV status, there was a significantly positive correlation between concentrations of IL-1β and IL-6 (Rho=0.6; p=0.0001 including all women) indicating that production of these inflammatory markers may be co-regulated.

Previous studies have shown that IL-23 stabilizes the final stage of Th17 cell differentiation (Peng *et al.,* 2009). In contrast to the inflammatory cytokines measured, IL-23 concentrations were significantly lower in genital secretions from HIV^+HAART^+ women compared to uninfected women (p=0.03). IL-23 is not an inflammatory but rather a regulatory cytokine (Chen *et al.,* 2006) and may be important in that it is down-regulated in HIV^+ women. These results suggest that the cervical micro-environment of HIV-infected women was enriched with inflammatory cytokines compared to uninfected women. In plasma, IL-10 was significantly higher in HIV^+HAART^- compared to HIV^+HAART^+ women (p=0.02) (Figure 3.6B). IL-6 was significantly higher in HIV^+HAART^- compared to uninfected women (p=0.04).

When concentrations of cytokines were compared in genital secretions and in plasma, significantly higher concentrations of the inflammatory cytokines were detected in genital secretions than in plasma [IL-12p40 (HIV-p=0.02; HIV^+ $HAART^-$ p=0.0008), IL-6 (HIV^+HAART^- p=0.0008; HIV^+HAART^+ p=0.0002), IL-1β (uninfected p=0.003; HIV^+HAART^- p<0.001; HIV^+HAART^+ p<0.001) and IL-8 (HIV^- p=0.001, HIV^+HAART^- p<0.0001; HIV^+HAART^+ p<0.0001)]. In contrast, regulatory cytokines were generally detected at lower concentrations in genital secretions than in plasma [IL-10, HIV^- p=0.006; IL-17 (HIV^- p=0.003, HIV^+HAART^- p<0.0001; HIV^+HAART^+ p<0.0001), and TGF-β HIV^- p<0.0001, HIV^+HAART^- p=0.0007, HIV^+HAART^+ p=0.0005)].

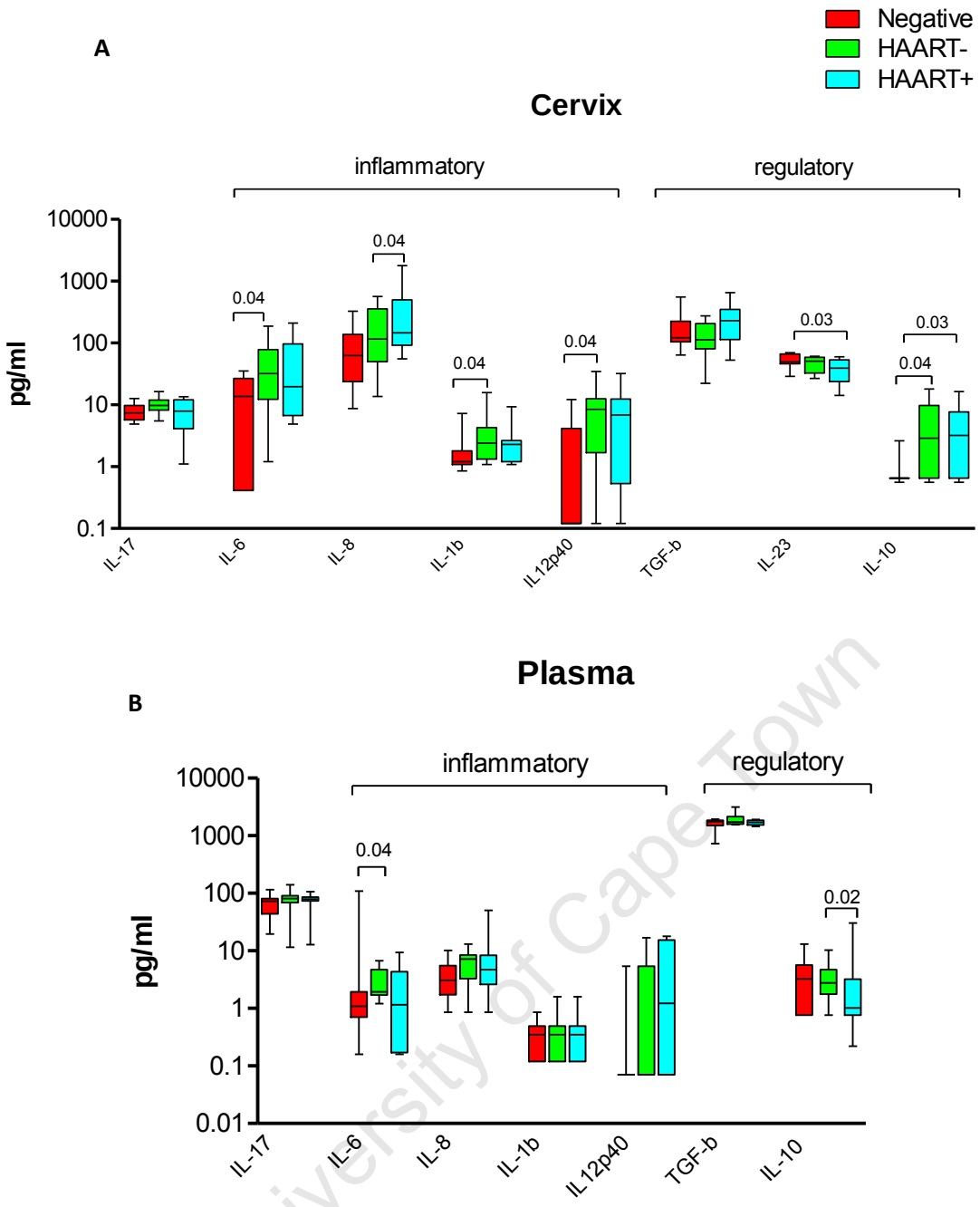


Figure 3.6: Cytokine concentrations in genital secretions (A) and plasma (B) of HIV$^-$, HIV$^+$HAART$^-$ and HIV$^+$HAART$^+$ women. (A) Genital cytokine concentrations (n=7, 14 and 13 for all HIV negatives, HIV$^+$HAART$^-$ and HIV$^+$HAART$^+$ respectively). (B) Plasma cytokine concentrations (n=7, 15 and 12 for all HIV negatives, HIV$^+$HAART$^-$ and HIV$^+$HAART$^+$ respectively). IL-23 was not measured in the plasma. Floating bars show range (min to max) of detectable cytokine concentrations in cervical supernatants from HIV negative (red bars), HIV$^+$HAART$^-$ (green bars) and HIV$^+$HAART$^+$ (blue bars) women. Line within the bars indicates median cytokine concentrations. Significant differences of $p<0.05$ were calculated using an unpaired student t test.

3.10 Relationship between inflammatory and regulatory cytokines in genital secretions and Th17 cell frequencies at the cervix

TGF-β is an important cytokine involved the differentiation of naive T cells to Th17 cells. This process is possible only if TGF-β is combined with IL-1β or IL-6 (Yang *et al.,* 2008). IL-23 aids in further stabilizing the final part of the differentiation process of naive T cells to Th17 cells (Di Cesare *et al.,* 2010) and has also been shown to be important in regulating inflammation (Oppmann *et al.,* 2001). It is believed that IL-10 dampens Th17 cell responses (Bettelli *et al.,* 2006; O' Connor *et al.,* 2010).

At the cervix, a significant positive relationship was observed between inflammatory cytokines and IL-17 concentrations [Figure 3.7; IL-1β versus IL-17 (r=0.5; p=0.004) and IL-12p40 versus IL-17 (r=0.4, p=0.03)]. In contrast, a negative relationship was found between concentrations of the regulatory cytokine IL-10 and IL-17 (Figure 3.7C; r=0.4, p=0.02). These results indicate IL-17 could be driving the increase in key cytokines involved in Th17 cell differentiation (IL-12p40 and IL-1β).

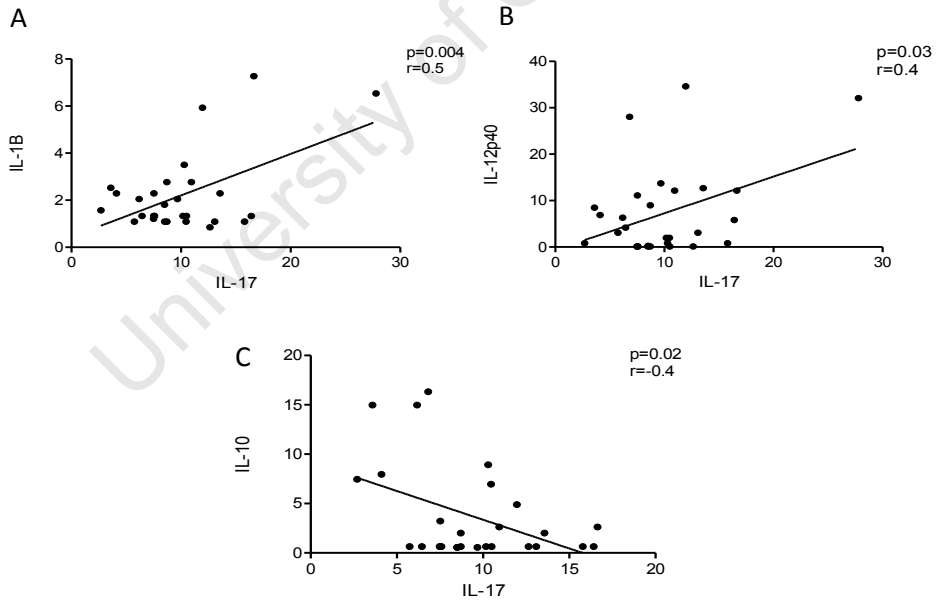


Figure 3.7: Association of inflammatory cytokines with IL-17 at the cervix of HIV uninfected and HIV positive women. Scatter plots show relationship between concentrations of IL-17 and IL-1β (A), IL-12p40 (B) and IL-10 (C). Pearson R values indicate the strength of the relationship and P values were considered statistically significant if p≤ 0.05.

In contrast, a significant negative correlation was found between the concentrations of genital inflammatory cytokines and IL-23 [Figure 3.8 IL-1β Rho=0.4, p=0.02; IL-6 Rho=0.6, p=0.006; IL-12p40 Rho=0.5, p=0.006)]. These results indicate that the concentrations of IL-23 and genital tract inflammation may be counter-regulated.

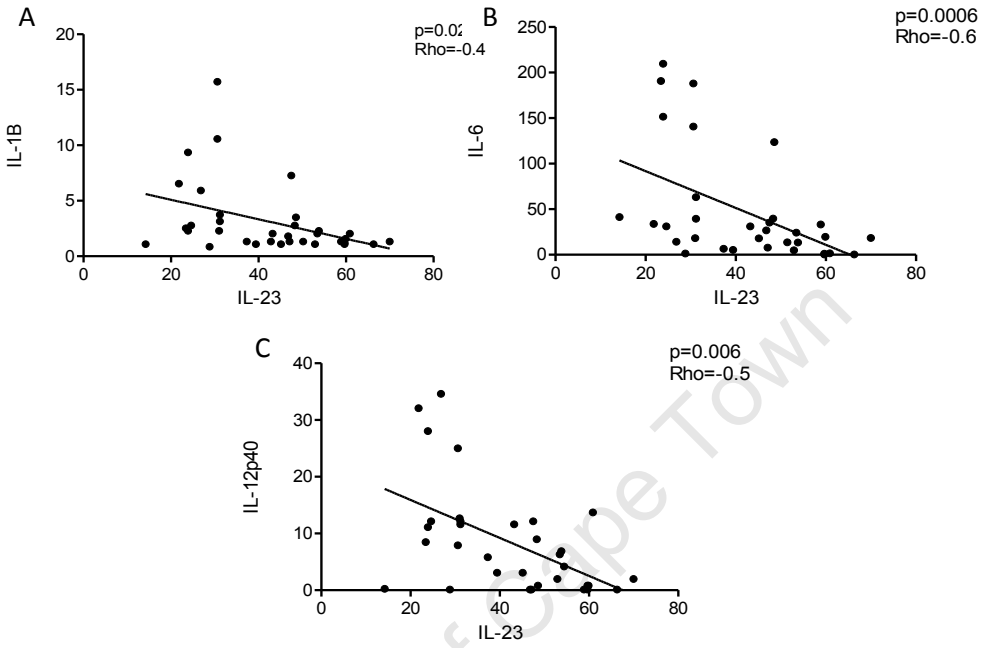


Figure 3.8: Association of inflammatory cytokines with IL-23 at the cervix of HIV uninfected and HIV positive women. Scatter plots show relationship between concentrations of IL-23 and IL-1β (A), IL-6 (B) and IL-12p40 (C). Spearman Rho values indicate the strength of the relationship and P values were considered statistically significant if $p \leq 0.05$.

Interestingly, Th17 cell frequencies in the genital tract or blood did not correlate with the amount of IL-17 measured in cervical supernatant or plasma, respectively (data not shown). In addition, the frequencies of Th17 cells detected at the cervix or in blood were not associated with the concentration of inflammatory cytokines detected in genital secretions or plasma (data not shown). In contrast, the frequency of Th17 cells in blood from $HIV^{+}HAART^{+}$ and $HIV^{+}HAART^{-}$ women was significantly positively associated with the concentration of TGF-β in plasma (Figure 3.9; r=0.6, p=0.04). This result could indicate that TGF-β is possibly driving Th17 cell differentiation in the blood. This relationship between genital tract TGF-β concentrations and Th17 cell frequencies was not observed at the cervix, however.

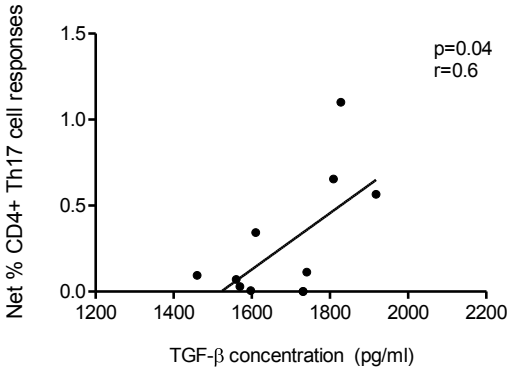


Figure 3.9: Relationship between Th17 cell responses and TGF-β concentrations in the blood of HIV positive women. Scatter plot shows relationship between concentrations of TGF-β and Th17 cell responses in the blood. Pearson R values indicate the strength of the relationship and P values were considered statistically significant if $p \leq 0.05$.

3.11 Relationship between Th17 cell frequencies at the cervix and Candida albicans infection

The concentration of (1-3)-β-δ-glucan from *Candida albicans* was measured in genital supernatants to determine the prevalence of yeast infections in the women included in this study. The concentration of (1-3)-β-δ-glucan was significantly higher in HIV^+HAART^+ women compared to uninfected women (p=0.04) (Figure 3.10A) and HIV-infected women on and off HAART had similar concentrations of (1-3)-β-δ-glucan. Furthermore, there was a significant negative correlation between the frequency of Th17 cells measured at the cervix and the concentration of (1-3)-β-δ-glucan in the genital tract (Rho=-0.34, p=0.04) (Figure 3.10B) in HIV^+HAART^+ and HIV^+HAART^- women, although this relationship is very weak. These results indicate that an increasing concentration of (1-3)-β-δ-glucan at the cervix results in a decrease in the number of Th17 cells present in this compartment.

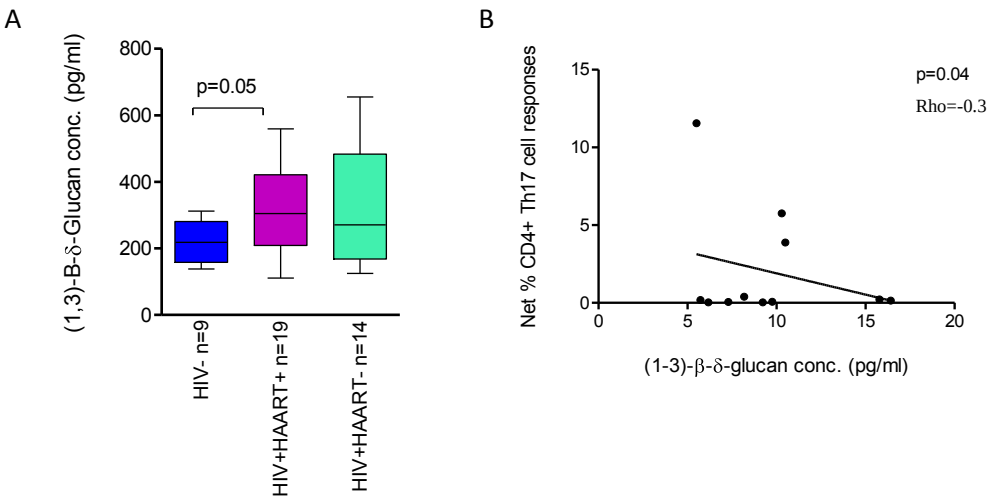


Figure 3.10: Effect of HIV infection on (1-3)-β-δ-glucan concentrations at the cervix and association with cervical Th17 frequencies. (A) (1-3)-β-δ-glucan concentration in cervical supernatants from uninfected, HIV-infected HAART$^-$ and HAART$^+$ women; (B) Correlation between CD4$^+$ Th17 frequencies and (1-3)-β-δ-glucan concentrations in cervical supernatants. Box and whisker plots show median and interquartile range of glucan concentrations in the cervical supernatant of HIV negative (blue bars), HIV$^+$HAART$^+$ (purple bars) and HIV$^+$HAART$^-$ women (green bars). P≤0.05 were considered significant. Spearman Rho values indicate the strength of the relationship.

3.12 Relationship between Th17 cell frequencies at the cervix and concentrations of LPS or soluble CD14

LPS binds to membrane bound or soluble CD14 (sCD14) (Scherberich *et al.*, 2000). Binding of LPS to sCD14 is important for initiating an inflammatory response and promoting the secretion of pro-inflammatory cytokines (Hershberger *et al.*, 1968). The concentration of LPS and sCD14 were measured as surrogate markers for genital tract bacterial burden and immune activation. No differences were found in either the concentrations of LPS and sCD14 for uninfected and HIV-infected women (Figure 3.11A and B, respectively). The frequency of Th17 cells at the cervix was not associated with the concentration of sCD14 or LPS measured in genital secretions (Figure 3.11C).

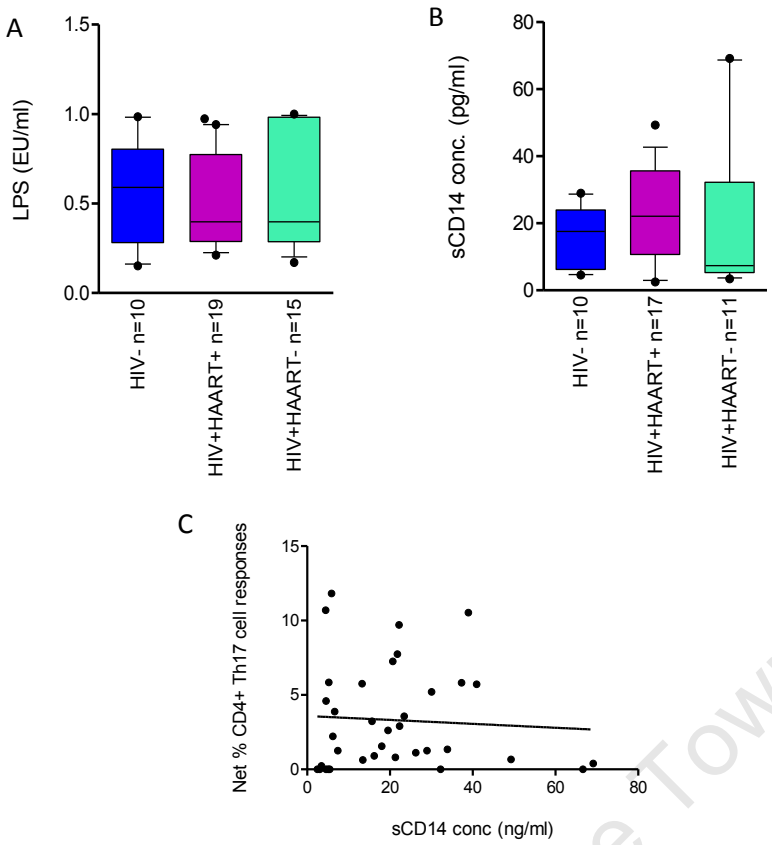


Figure 3.11: Effect of HIV infection on LPS and sCD14 concentrations at the cervix and their correlations with Th17 frequencies. (A) Concentration of LPS and (B) sCD14 in genital secretions. Box and whisker plots showing median and interquartile ranges of LPS and sCD14 concentrations in the cervix of HIV^- (blue bars), HIV^+HAART+ (purple bars) and HIV^+HAART^- women (green bars). (C) Relationship between frequencies of $CD4^+$ Th17 cells at the cervix and sCD14 concentrations. Pearson R value indicates the strength of the relationship and P values ≤ 0.05 were considered significant.

3.13 HIV shedding in the genital tract of HIV-infected women

A third of therapy naïve HIV-infected women in this study were shedding HIV in their genital secretions (Table 3.1). Previous studies from our group have shown that genital tract inflammation is associated with HIV shedding (Gumbi et al., 2008; Anderson *et al.,* 2008). The role of genital tract inflammation, bacterial burden or Th17 cell frequencies in shedding of HIV in this cohort was investigated (Figure 3.12). Concentrations of IL-6, IL-1β and IL-8 were elevated in genital secretions from women shedding HIV compared to those that were not, although not significantly (Figure 3.12A).

Although not significant, HIV-infected women shedding HIV in genital secretions had elevated frequencies of Th17 cells but similar IL-17 concentrations at the cervix than women not shedding HIV (Figure 3.12B and C). The results suggest that there may be some relationship between inflammation, Th17 cells recruitment and HIV shedding in genital secretions from HIV-infected women naïve to therapy.

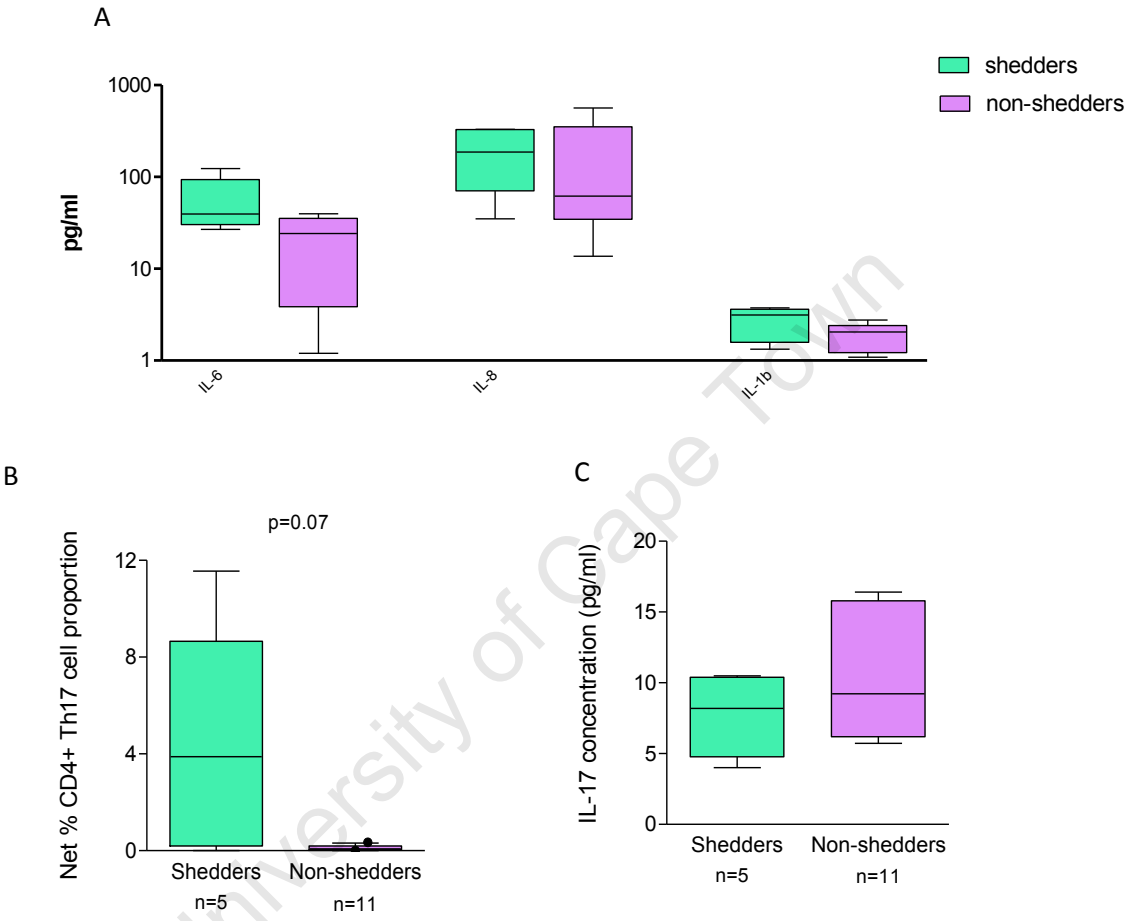


Figure 3.12: Relationship between HIV shedding status in the female genital tract and (A) inflammatory cytokines, (B) CD4$^+$ Th17 cell proportions, and (C) IL-17 concentrations. Floating bars show range (min to max) in HIV$^+$HAART$^+$ and HIV$^+$HAART$^-$ women. n = sample size. Green bars = shedders and purple bars = non-shedders. (A): n = 5. The line within the bars indicates the median concentrations of inflammatory cytokines or Th17 frequencies. P<0.05 were considered significant.

Chapter 4

Discussion

$CD4^+$ T cell depletion is central to HIV pathogenesis and has been shown in both blood and the GALT of HIV-infected individuals who are not on HAART during all stages of disease (Prendergast *et al.,* 2010; Schacker *et al.,* 2008). The extent of CD4 depletion in tissue associated with the FGT is not known. Recent studies have shown that the newly defined Th17 cell subset also becomes depleted during HIV infection in the gut and that this mirrors total CD4 depletion in this compartment (El Hed *et al.,* 2010). In the gut, depletion of Th17 cells has been shown to result in deregulation of epithelial cell tight junction formation which compromises gut mucosal barrier integrity (Dandekar *et al.,* 2010). This has been suggested to contribute to the "leaky gut" phenomenon, which is the major driver of microbial translocation and may lead to high levels of systemic immune activation during HIV infection (Brenchley *et al.,* 2008).

In the genital tract of women, HIV is thought to be preferentially transmitted across the mucosal barrier of the cervix. Studying the relationship between Th17 cells, genital inflammation and other local innate factors may give insight into the role of this novel Th subset in maintaining genital tract mucosal immunity during HIV infection. The impact of HIV on $CD4^+$ Th17 cell numbers in the FGT remains unknown. Because Th17 cells have been shown to recognize fungal and bacterial antigens, this subset may be important in the FGT where fungal and bacterial infections are relatively common. Mucosal immunity in the genital tract may be compromised due to depletion of $CD4^+$ Th17 cells in this compartment during HIV infection.

The hypothesis of this study was that Th17 cells in the FGT, which may be essential for anti-fungal and anti-bacterial responses in this compartment, are recruited to the genital mucosa under an inflammatory gradient but are depleted during chronic HIV infection. There have been no published studies showing that Th17 cells traffic out of blood to tissue sites along an inflammatory gradient. Based on this hypothesis, the impact of inflammatory and regulatory cytokines as well as markers of bacterial and fungal burden (LPS, sCD14 and (1-3)-β-δ-glucan respectively) on $CD4^+$ Th17 cells at the cervix and blood was investigated. The phenotype and function of Th17 cells in the blood and cervix were characterized in uninfected, HIV^+HAART^+ and HIV^+HAART^- women.

This study showed that $CD4^+$ T cells are significantly depleted in both the cervix and the blood of HIV^+HAART^- women compared to uninfected women. Similar to another study from our group (Mkhize *et al.,* 2010), these $CD4^+$ T cells were partially reconstituted at the cervix of HIV-infected women on HAART. Because of this significant depletion of total $CD4^+$ T cells in both compartments, the extent of $CD4^+$ Th17 cell depletion was investigated in this study. While blood $CD4^+$ Th17 cells were significantly lower in HIV-infected women compared to uninfected women, Th17 cell frequencies were significantly higher at the cervix in HIV-infected compared to uninfected women. Elevated Th17 cell frequencies at the cervix during HIV infection may reflect that Th17 cells are either selectively not depleted (possibly because they are not directly infected with HIV) or that they are selectively recruited to the genital mucosa because of increased fungal burden or incidence of bacterial vaginosis and sexually transmitted infections (Yudin *et al.,* 2003; Ernst *et al.,* 1999).

It is important in future studies to investigate the possible mechanisms behind Th17-cell preservation at the cervix during chronic HIV infection. Paiardini *et al.,* (2010) has described the activation state and expression of HIV entry receptors by $CD4^+$ Th17 cells and showed that $CD4^+$ Th17 cells do not express CCR5 in the blood (Sato *et al.,* 2007) but a significant number of $CD4^+$ Th17 cells in the human GIT express CCR5 (Brenchley *et al.,* 2008). $CD4^+$ Th17 cells are highly activated in the gut of HIV-infected individuals and express lower levels of CCR5 in this compartment (Paiardini *et al.,* 2010).

This study showed that $CD4^+$ Th17 cells in the cervix and the blood were predominantly effector memory. It would be interesting to similarly measure the activation phenotype of Th17 cells in the cervix, whether they are actively or latently infected with HIV and whether they express markers of apoptosis.

El Hed *et al.* (2010) showed that Th17 cells were reduced in the blood of HIV^+HAART^+ but not in HIV^+HAART^- individuals. HIV^+HAART^- participants may have preserved Th17 cell numbers to compensate for immune activation associated with HIV disease, keeping in mind that Th17 cells respond to bacterial and fungal antigens. El Hed *et al*. (2010) suggested that HAART may fail to restore Th17 numbers to those present before HIV infection, perhaps because of a compromise in Th17 cell differentiation or survival during HIV infection.

In non-pathogenic primate models of lentiviral infection, despite intense viral replication, there was no depletion of Th17 $CD4^+$ T cells at mucosal sites (Favre *et al.,* 2009 & Campillo-Gimenez *et al.,* 2010). It can thus be hypothesised that indirect mechanisms may be involved in Th17 cell depletion (Campillo-Gimenez *et al.,* 2010). In HIV-infected individuals, Th17 cells are depleted in the GALT, due to ongoing local inflammation, even after initiation of HAART (Brenchley *et al.,* 2008; Guadalupe *et al.,* 2006).

This study showed that concentrations of IL-1β, IL-6, IL-8, IL-10 and IL-12p40 were significantly higher at the cervix of HIV^+HAART^- women than uninfected women, indicating that inflammatory cytokines are elevated in the cervix of HIV-infected women. Nkwanyana *et al.* (2009) also showed that inflammatory cytokines are increased in the cervix of HIV-infected women compared to uninfected women and that HAART did not reduce genital tract inflammation. In this study, the concentrations of IL-1β, IL-6 and IL-12p40 at the cervix were negatively associated with the concentration of IL-23. While IL-23 has been shown to be involved in the final stages of Th17 cell differentiation, IL-1β, IL-6 and IL-12p40 are involved in earlier phases of differentiation (Jäger *et al.,* 2010). The negative association between IL-23 and inflammatory cytokines possibly reflects that Th17 cell differentiation has been completed in blood, before migration to tissue sites under an inflammatory gradient.

Previous studies have shown that TGF-β is associated with Th17 cell differentiation (Zhou *et al.,* 2008). In this study, the concentrations of TGF-β and IL-17 were significantly higher in the plasma of both uninfected and HIV-infected women, compared to the cervix. The frequencies of Th17 cells in blood in HIV^+HAART^- and HIV^+HAART^+ women were also positively correlated with TGF-β concentrations in plasma. These results may further suggest that Th17 cell differentiation takes place in the blood rather than at tissue sites such as the genital mucosa.

The concentration of IL-17 at the cervix was positively associated with IL-1β and IL-12p40, but negatively associated with IL-10. Since IL-17 is recognized as a pro-inflammatory cytokine (Wong *et al.,* 2000), these findings may suggest that IL-17 released at the cervix induces an inflammatory cascade. The negative relationship between IL-10 and IL-17 might suggest that Tregs, which secrete IL-10 (Prendergast *et al.,* 2010), are recruited to the genital tract to suppress inflammation and Th17 cell activity at the cervix. This could be a mechanism to prevent tissue damage as a result of inflammation.

The concentrations of LPS and sCD14 were measured in genital secretions of HIV-infected and uninfected women as a surrogate measurement for bacterial antigenic load at the cervix. While several inflammatory cytokines were shown to be elevated in HIV-infected compared to uninfected women, concentrations of LPS and sCD14 were found to be similar in both groups of women. *Candida albicans* is a common fungal opportunistic infection which is more prevalent in HIV-infected than uninfected women (Wheeler *et al*., 2008) and (1-3)-β-δ-glucan is part of the cell wall of this common fungal pathogen.

The concentration of (1-3)-β-δ-glucan was significantly higher in cervical secretions from $HIV^{+}HAART^{+}$ than uninfected women. Elevated (1-3)-β-δ-glucan concentrations at the cervix during HIV infection may result in recruitment Th17 cells to the cervix in direct response to prevalent fungal infections.

A limitation of this study was that syndromic management was used for the management of STIs and women with active STIs were excluded from the study. Asymptomatic STIs were not taken into account. Some STIs are asymptomatic which could affect LPS and (1-3)-β-δ-glucan concentrations. Furthermore, "good" and "bad" bacteria were not distinguished in this study; and LPS or sCD14 measurement would not be able to distinguish these. Many STIs would also contribute to LPS and/or glucan concentrations in the genital tract (Keane *et al.*, 2006).

Previous work from this laboratory found a relationship between HIV shedding in the genital tract and genital inflammation (Gumbi *et al.*, 2008). In this study, no differences in Th17 cell frequencies were observed between HIV shedders and non-shedders at the cervix, indicating that Th17 cells are not affected by or involved in local HIV replication or trafficking of virus from blood. Surprisingly, no significant differences were found in inflammatory cytokine concentrations or concentrations of bacterial and fungal markers between HIV shedders and non-shedders, suggesting that direct replication of HIV were not associated with Th17 cell depletion in the genital tract. As only a few women were studied, future studies should aim for larger numbers of women to fully evaluate the relationships between shedding and Th17.

Future studies and recommendations

The selective resistance of Th17 cells in the genital tract to depletion during HIV infection was one of the more interesting findings from this study. Unravelling the mechanism behind this resistance should be a priority of future studies in this field. Including markers of proliferation (such as Ki67) and activation (such as HLA-DR) in future *in vitro* studies would allow determination of the activation state of Th17 cells and address whether reduced activation plays a role in resistance to depletion. To further evaluate if HIV directly infects Th17 cells, CCR5-expression levels would need to be analysed, as previous studies have shown a direct correlation between CCR5 expression and HIV infection (Ostrowski *et al.*, 2009).

Th17 cells could also be directly purified from cervical cytobrush samples by FACS sorting and directly investigated for HIV infection by PCR. Investigating the relationship between Th17 cells and Treg cells at the cervix may give insight into Th17 cell preservation in this compartment. Defining Th17 cells by detection of IL-17 secretion might also underestimate the presence of lineage committed Th17 cells (El Hed *et al.*, 2010); and it may be better in future studies to focus on CCR6 and CCR4 as Th17 cell markers.

To accurately assess the concentration of IL-17 in genital secretions, it would be vital in future studies to assess a broader range of cells that secrete IL-17. An increased concentration of TGF-β in an inflammatory environment supports the possible induction of IL-17-secreting populations during HIV infection (Campillo-Gimenez *et al.*, 2010). It has been shown that CD8 T cells (He *et al.*, 2006), γδ T cells (O' Mara *et al.*, 2005), CD4-CD8-TCR^{+} T cells (Shibata *et al.*, 2007) and NK T cells (Michel *et al.*, 2007) are all able to produce IL-17. Furthermore, assessing Th17 cell expression of exhaustion markers, such as PD-1 and Tim-3, in future studies may give insight into the mechanism of Th17 cell persistence in the genital tracts of HIV-infected women. It would also be important to include bacterial and fungal antigens to characterize Th17 cell responses at the cervix. Doing a longitudinal instead of a cross sectional study during earlier phases of HIV infection would also be interesting as this would show which phases of HIV infection Th17 cells in blood become depleted and when Th17 cell enrichment at the cervix occurs.

Ivanov *et al.,* (2007) postulated that Th17 cells play a role in mucous production by mobilizing mucins and inducing neutrophil recruitment. Th17 cells may play a similar role in response to genital infections in the FGT. Other possible functions of Th17 cells in the genital tract could be maintenance of epithelial cell tight junctions, contributing towards mucosal barrier integrity and anti-microbial protein production as has been described in the gut (Artis *et al.,* 2008). It is possible that epithelial tight junctions are compromised during HIV infection due to the depletion of Th17 cells, resulting in subsequent dysregulation of mucous production.

Conclusion

This study provides evidence for Th17 cell preservation or reconstitution at the cervix of HIV-infected women compared to uninfected women. Investigating Th17 cells in the FGT is crucial because Th17 cells have an anti-fungal and bacterial function. Their potential role in combating STIs (perhaps through maintenance of epithelial tight junctions and mucous production) in the genital tract during HIV infection may give important insight into contributing factors to HIV disease progression. While the gut has highly organized lymphoid structures, mucosal tissue associated with the cervix and vagina are tertiary effector sites and lack similarly organized lymphoid aggregates (Brandtzaeg *et al.,* 1997). The role of Th17 cells in the FGT may therefore be very different from their role in the gut-associated lymphoid tissue. The lack of association between inflammation and Th17 frequencies argues against selective recruitment of Th17 cells along a pro-inflammatory gradient, but may suggest selective resistance to cell death of Th17 in this compartment.

References

Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* **8:**942-9.

Adhikary, D., U. Behrends, A. Moosmann, K. Witter, G. W. Bornkamm, and J. Mautner. 2006. Control of Epstein-Barr virus infection in vitro by T helper cells specific for virion glycoproteins. *J Exp Med* **203:**995-1006.

Adler, M., S. Schulz and M. Spengler. 2009. Cytokine Quantification in Drug Development: A comparison of sensitive immunoassay platforms. *Chimera Biotech. (Report).*

Afzali, B., G. Lombardi, R. I. Lechler, and G. M. Lord. 2007. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol* **148:**32-46.

Anderson, B. L., and S. Cu-Uvin. 2008. Determinants of HIV shedding in the lower genital tract of women. Curr Infect Dis Rep **10:**505-11.

Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* **278:**1910-4.

Artis, D., and R. K. Grencis. 2008. The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol* **1:**252-64.

Aujla, S. J., Y. R. Chan, M. Zheng, M. Fei, D. J. Askew, D. A. Pociask, T. A. Reinhart, F. McAllister, J. Edeal, K. Gaus, S. Husain, J. L. Kreindler, P. J. Dubin, J. M. Pilewski, M. M. Myerburg, C. A. Mason, Y. Iwakura, and J. K. Kolls. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med **14:**275-81.

Baroncelli, S., C. M. Galluzzo, M. F. Pirillo, M. G. Mancini, L. E. Weimer, M. Andreotti, R. Amici, S. Vella, M. Giuliano, and L. Palmisano. 2009. Microbial translocation is associated with residual viral replication in HAART-treated HIV+ subjects with <50copies/ml HIV-1 RNA. *J Clin Virol* **46:**367-70.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-71.

Batten, M., J. Li, S. Yi, N. M. Kljavin, D. M. Danilenko, S. Lucas, J. Lee, F. J. de Sauvage, and N. Ghilardi. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nat Immunol* **7:**929-36.

Bearman, G. M., and R. P. Wenzel. 2005. Bacteremias: a leading cause of death. *Arch Med Res* **36:**646-59.

Belyakov, I. M., and J. A. Berzofsky. 2004. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. *Immunity* **20:**247-53.

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. *Nature* **441:**235-8.

Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* **8:**345-50.

Betts, M. R., C. M. Gray, J. H. Cox, and G. Ferrari. 2006. Antigen-specific T-cell-mediated immunity after HIV-1 infection: implications for vaccine control of HIV development. *Expert Rev Vaccines* **5:**505-16.

Blaschitz, C., and M. Raffatellu. 2010. Th17 cytokines and the gut mucosal barrier. *J Clin Immunol* **30:**196-203.

Boasso, A., G. M. Shearer, and C. Chougnet. 2009. Immune dysregulation in human immunodeficiency virus infection: know it, fix it, prevent it? *J Intern Med* **265:**78-96.

Brandtzaeg, P. 1997. Mucosal immunity in the female genital tract. *J Reprod Immunol* **36:**23-50.

Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200:**749-59.

Brenchley, J. M., D. A. Price, T. W. Schacker, T. E. Asher, G. Silvestri, S. Rao, Z. Kazzaz, E. Bornstein, O. Lambotte, D. Altmann, B. R. Blazar, B. Rodriguez, L. Teixeira-Johnson, A. Landay, J. N. Martin, F. M. Hecht, L. J. Picker, M. M. Lederman, S. G. Deeks, and D. C. Douek. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* **12:**1365-71.

Brenchley, J. M., M. Paiardini, K. S. Knox, A. I. Asher, B. Cervasi, T. E. Asher, P. Scheinberg, D. A. Price, C. A. Hage, L. M. Kholi, A. Khoruts, I. Frank, J. Else, T. Schacker, G. Silvestri, and D. C. Douek. 2008. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. *Blood* **112:**2826-35.

Bull, M., D. Lee, J. Stucky, Y. L. Chiu, A. Rubin, H. Horton, and M. J. McElrath. 2007. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *J Immunol Methods* **322:**57-69.

Campillo-Gimenez, L., M. C. Cumont, M. Fay, H. Kared, V. Monceaux, O. Diop, M. Muller-Trutwin, B. Hurtrel, Y. Levy, J. Zaunders, M. Dy, M. C. Leite-de-Moraes, C. Elbim, and J. Estaquier. 2010. AIDS progression is associated with the emergence of IL-17-producing cells early after simian immunodeficiency virus infection. *J Immunol* **184:**984-92.

Campillo-Gimenez, L., C. Elbim, M. Laforge, M. C. Leite-de-Moraes, J. Zaunders, and J. Estaquier. 2010. IL-17 and HIV pathogenesis. *Eur Cytokine Netw* **21:**222-5.

Cecchinato, V., C. J. Trindade, A. Laurence, J. M. Heraud, J. M. Brenchley, M. G. Ferrari, L. Zaffiri, E. Tryniszewska, W. P. Tsai, M. Vaccari, R. W. Parks, D. Venzon, D. C. Douek, J. J. O'Shea, and G. Franchini. 2008. Altered balance between Th17 and Th1 cells at mucosal sites predicts AIDS progression in simian immunodeficiency virus-infected macaques. *Mucosal Immunol* **1:**279-88.

Cecchinato, V., and G. Franchini. 2010. Th17 cells in pathogenic simian immunodeficiency virus infection of macaques. *Curr Opin HIV AIDS* **5:**141-5.

Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim. 1997. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89:**263-73.

Chen, Y., C. L. Langrish, B. McKenzie, B. Joyce-Shaikh, J. S. Stumhofer, T. McClanahan, W. Blumenschein, T. Churakovsa, J. Low, L. Presta, C. A. Hunter, R. A. Kastelein, and D. J. Cua. 2006. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J Clin Invest* **116:**1317-26.

Chen, Z., A. Laurence, and J. J. O'Shea. 2007. Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. *Semin Immunol* **19:**400-8.

Chen, L., L. Lei, X. Chang, Z. Li, C. Lu, X. Zhang, Y. Wu, I. T. Yeh, and G. Zhong. 2010. Mice deficient in MyD88 Develop a Th2-dominant response and severe pathology in the upper genital tract following Chlamydia muridarum infection. *J Immunol* **184:**2602-10.

Coakley, E., C. J. Petropoulos, and J. M. Whitcomb. 2005. Assessing chemokine co-receptor usage in HIV. *Curr Opin Infect Dis* **18:**9-15.

Croitoru, K., J. Bienenstock, and P. B. Ernst. 1994. Phenotypic and functional assessment of intraepithelial lymphocytes bearing a 'forbidden' alpha beta TCR. *Int Immunol* **6:**1467-73.

Crome, S. Q., B. Clive, A. Y. Wang, C. Y. Kang, V. Chow, J. Yu, A. Lai, A. Ghahary, R. Broady, and M. K. Levings. 2010. Inflammatory effects of ex vivo human Th17 cells are suppressed by regulatory T cells. *J Immunol* **185:**3199-208.

Cua, D. J., and C. M. Tato. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* **10:**479-89.

Dandekar, S., M. D. George, and A. J. Baumler. 2010. Th17 cells, HIV and the gut mucosal barrier. *Curr Opin HIV AIDS* **5:**173-8.

Deknuydt, F., E. Scotet, and M. Bonneville. 2009. Modulation of inflammation through IL-17 production by gammadelta T cells: mandatory in the mouse, dispensable in humans? *Immunol Lett* **127:**8-12.

Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381:**661-6.

Di Cesare, A., P. Di Meglio, and F. O. Nestle. 2010. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J Invest Dermatol* **129:**1339-50.

.

DiPaolo, R. J., C. Brinster, T. S. Davidson, J. Andersson, D. Glass, and E. M. Shevach. 2007. Autoantigen-specific TGFbeta-induced Foxp3+ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells. *J Immunol* **179:**4685-93.

Doisne, J. M., A. Urrutia, C. Lacabaratz-Porret, C. Goujard, L. Meyer, M. L. Chaix, M. Sinet, and A. Venet. 2004. CD8+ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. *J Immunol* **173:**2410-8.

Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup. 2002. HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417:**95-8.

Duvall, M. G., A. Jaye, T. Dong, J. M. Brenchley, A. S. Alabi, D. J. Jeffries, M. van der Sande, T. O. Togun, S. J. McConkey, D. C. Douek, A. J. McMichael, H. C. Whittle, R. A. Koup, and S. L. Rowland-Jones. 2006. Maintenance of HIV-specific CD4+ T cell help distinguishes HIV-2 from HIV-1 infection. *J Immunol* **176:**6973-81.

El Hed, A., A. Khaitan, L. Kozhaya, N. Manel, D. Daskalakis, W. Borkowsky, F. Valentine, D. R. Littman, and D. Unutmaz. 2010. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *J Infect Dis* **201:**843-54.

Epple, H. J., T. Schneider, H. Troeger, D. Kunkel, K. Allers, V. Moos, M. Amasheh, C. Loddenkemper, M. Fromm, M. Zeitz, and J. D. Schulzke. 2009. Impairment of the intestinal barrier is evident in untreated but absent in suppressively treated HIV-infected patients. *Gut* **58:**220-7.

Ernst, P. B., F. Song, G. R. Klimpel, H. Haeberle, K. B. Bamford, S. E. Crowe, G. Ye, and V. E. Reyes. 1999. Regulation of the mucosal immune response. Am J Trop Med Hyg **60:**2-9.

Evans, D. T., L. A. Knapp, P. Jing, J. L. Mitchen, M. Dykhuizen, D. C. Montefiori, C. D. Pauza, and D. I. Watkins. 1999. Rapid and slow progressors differ by a single MHC class I haplotype in a family of MHC-defined rhesus macaques infected with SIV. *Immunol Lett* **66:**53-9.

Fahey, J. V., T. M. Schaefer, J. Y. Channon, and C. R. Wira. 2005. Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. *Hum Reprod* **20:**1439-46.

Favre, D., S. Lederer, B. Kanwar, Z. M. Ma, S. Proll, Z. Kasakow, J. Mold, L. Swainson, J. D. Barbour, C. R. Baskin, R. Palermo, I. Pandrea, C. J. Miller, M. G. Katze, and J. M. McCune. 2009. Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS Pathog* **5:**e1000295.

Feinen, B., A. E. Jerse, S. L. Gaffen, and M. W. Russell. 2010. Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection. *Mucosal Immunol* **3:**312-21.

Fenoglio, D., A. Poggi, S. Catellani, F. Battaglia, A. Ferrera, M. Setti, G. Murdaca, and M. R. Zocchi. 2009. Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. *Blood* **113:**6611-8.

Gaffen, S. L. 2009. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* **9:**556-67.

Gentry, L. E., M. N. Lioubin, A. F. Purchio, and H. Marquardt. 1988. Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol Cell Biol* **8:**4162-8.

Ghilardi, N., and W. Ouyang. 2007. Targeting the development and effector functions of TH17 cells. *Semin Immunol* **19:**383-93.

Givan, A. L. 2001. Principles of flow cytometry: an overview. *Methods Cell Biol* **63:**19-50.

Gordon, S. N., N. R. Klatt, S. E. Bosinger, J. M. Brenchley, J. M. Milush, J. C. Engram, R. M. Dunham, M. Paiardini, S. Klucking, A. Danesh, E. A. Strobert, C. Apetrei, I. V. Pandrea, D. Kelvin, D. C. Douek, S. I. Staprans, D. L. Sodora, and G. Silvestri. 2007. Severe depletion of mucosal CD4+ T cells in AIDS-free simian immunodeficiency virus-infected sooty mangabeys. *J Immunol* **179:**3026-34.

Grakoui, A., N. H. Shoukry, D. J. Woollard, J. H. Han, H. L. Hanson, J. Ghrayeb, K. K. Murthy, C. M. Rice, and C. M. Walker. 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science* **302:**659-62.

Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar. 2003. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77:**11708-17.

Guadalupe, M., S. Sankaran, M. D. George, E. Reay, D. Verhoeven, B. L. Shacklett, J. Flamm, J. Wegelin, T. Prindiville, and S. Dandekar. 2006. Viral suppression and immune restoration in the gastrointestinal mucosa of human immunodeficiency virus type 1-infected patients initiating therapy during primary or chronic infection. *J Virol* **80:**8236-47.

Gumbi, P. P., N. N. Nkwanyana, A. Bere, W. A. Burgers, C. M. Gray, A. L. Williamson, M. Hoffman, D. Coetzee, L. Denny, and J. A. Passmore. 2008. Impact of mucosal inflammation on cervical human immunodeficiency virus (HIV-1)-specific CD8 T-cell responses in the female genital tract during chronic HIV infection. *J Virol* **82:**8529-36.

Haak, S., A. L. Croxford, K. Kreymborg, F. L. Heppner, S. Pouly, B. Becher, and A. Waisman. 2009. IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *J Clin Invest* **119:**61-9.

Haas, A., M. Rehr, F. Graw, P. Rusert, W. Bossart, H. Kuster, A. Trkola, H. F. Gunthard, and A. Oxenius. 2010. HIV-1 replication activates CD4+ T cells with specificities for persistent herpes viruses. *EMBO Mol Med* **2:**231-44.

Harari, A., G. P. Rizzardi, K. Ellefsen, D. Ciuffreda, P. Champagne, P. A. Bart, D. Kaufmann, A. Telenti, R. Sahli, G. Tambussi, L. Kaiser, A. Lazzarin, L. Perrin, and G. Pantaleo. 2002. Analysis of HIV-1- and CMV-specific memory CD4 T-cell responses during primary and chronic infection. *Blood* **100:**1381-7.

Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* **6:**1123-32.

Hashimoto, T., K. Akiyama, N. Kobayashi, and A. Mori. 2005. Comparison of IL-17 production by helper T cells among atopic and nonatopic asthmatics and control subjects. *Int Arch Allergy Immunol* **137 Suppl 1:**51-4.

Hazenberg, M. D., J. W. Stuart, S. A. Otto, J. C. Borleffs, C. A. Boucher, R. J. de Boer, F. Miedema, and D. Hamann. 2000. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to

immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* **95:**249-55.

He, D., L. Wu., H.K. Kim., H. Li., C.A. Elmets and H. Xu. 2006. $CD8^+$ IL-17-Producing T Cells Are Important in Effector Functions for the Elicitation of Contact Hypersensitivity Responses. *J Immunol* **177:** 6852-6858.

Herr, W., J. Schneider, A. W. Lohse, K. H. Meyer zum Buschenfelde, and T. Wolfel. 1996. Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor alpha in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J Immunol Methods* **191:**131-42.

Hershberger, C., and S. B. Binkley. 1968. Chemistry and metabolism of 3-deoxy-D-mannooctulosonic acid. I. Stereochemical determination. *J Biol Chem* **243:**1578-84.

Hladik, F., and M. J. McElrath. 2008. Setting the stage: host invasion by HIV. Nat Rev Immunol **8:**447-57.

Hladik, F. and Hope, T.J. 2009. HIV infection of the genital mucosa in women. *Current HIV/AIDS reports* **6:** 20-28

Hossain, Z., and T. Hirata. 2008. Molecular mechanism of intestinal permeability: interaction at tight junctions. *Mol Biosyst* **4:**1181-5.

Ivanov, II, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* **126:**1121-33.

Ivanov, S., and A. Linden. 2007. Th-17 cells in the lungs? *Expert Rev Respir Med* **1:**279-93.

Iwakura, Y., and H. Ishigame. 2006. The IL-23/IL-17 axis in inflammation. *J Clin Invest* **116:**1218-22.

Jager, A., and V. K. Kuchroo. 2010. Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation. *Scand J Immunol* **72:**173-84.

Jiang, W., M. M. Lederman, P. Hunt, S. F. Sieg, K. Haley, B. Rodriguez, A. Landay, J. Martin, E. Sinclair, A. I. Asher, S. G. Deeks, D. C. Douek, and J. M. Brenchley. 2009. Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. *J Infect Dis* **199:**1177-85.

Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **189:**991-8.

Johansson, E.L., Rask, C., Fredriksson, M., Eriksson, K., Czerkinsky, C. & Holmgren, J. 1998. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates. *Infection and immunity* **66:**514-520

Johansson, M., and N. Y. Lycke. 2003. Immunology of the human genital tract. *Curr Opin Infect Dis* **16:**43-9.

Kader, M., X. Wang, M. Piatak, J. Lifson, M. Roederer, R. Veazey, and J. J. Mattapallil. 2009. Alpha4(+)beta7(hi)CD4(+) memory T cells harbor most Th-17 cells and are preferentially infected during acute SIV infection. *Mucosal Immunol* **2:**439-49.

Kahn, J. O. 1998. Post-exposure prevention of HIV-1 infection. *Antivir Ther* **3 Suppl 4:**45-7.

Kammerer, R., A. Iten, P. C. Frei, and P. Burgisser. 1996. Expansion of T cells negative for CD28 expression in HIV infection. Relation to activation markers and cell adhesion molecules, and correlation with prognostic markers. Med Microbiol Immunol **185:**19-25.

Kaul, R., F. A. Plummer, J. Kimani, T. Dong, P. Kiama, T. Rostron, E. Njagi, K. S. MacDonald, J. J. Bwayo, A. J. McMichael, and S. L. Rowland-Jones. 2000. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J Immunol* **164:**1602-11.

Kaushic, C., V. H. Ferreira, J. K. Kafka, and A. Nazli. 2010. HIV infection in the female genital tract: discrete influence of the local mucosal microenvironment. *Am J Reprod Immunol* **63:**566-75.

Kawanishi, H., and K. Joseph. 1992. Effects of phorbol myristate and ionomycin on in vitro growth of aged Peyer's patch T and B cells. *Mech Ageing Dev* **65:**289-300.

Keane, F., C. A. Ison, H. Noble, and C. Estcourt. 2006. Bacterial vaginosis. *Sex Transm Infect* **82 Suppl 4:**iv16-8.

Khader, S. A., S. L. Gaffen, and J. K. Kolls. 2008. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol* **2:**403-11.

Kimbrell, M. R., H. Warshakoon, J. R. Cromer, S. Malladi, J. D. Hood, R. Balakrishna, T. A. Scholdberg, and S. A. David. 2008. Comparison of the immunostimulatory and proinflammatory activities of candidate Gram-positive endotoxins, lipoteichoic acid, peptidoglycan, and lipopeptides, in murine and human cells. *Immunol Lett* **118:**132-41.

Kitchens, R. L. 2000. Role of CD14 in cellular recognition of bacterial lipopolysaccharides. *Chem Immunol* **74:**61-82.

Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* **21:**467-76.

Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2007. IL-17 and Th17 Cells. *Annu Rev Immunol* **27:**485-517.

Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68:**4650-5.

Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* **201:**233-40.

Laurence, A., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E. M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* **26:**371-81.

Lawrence, D. A., R. Pircher, C. Kryceve-Martinerie, and P. Jullien. 1984. Normal embryo fibroblasts release transforming growth factors in a latent form. *J Cell Physiol* **121:**184-8.

Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* **203:**2271-9.

Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C. J. Miller, and A. T. Haase. 2005. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* **434:**1148-52.

Liu, J. Z., M. Pezeshki, and M. Raffatellu. 2009. Th17 cytokines and host-pathogen interactions at the mucosa: dichotomies of help and harm. *Cytokine* **48:**156-60.

Liu, Y., B. Yang, M. Zhou, L. Li, H. Zhou, J. Zhang, H. Chen, and C. Wu. 2009. Memory IL-22-producing CD4+ T cells specific for Candida albicans are present in humans. *Eur J Immunol* **39:**1472-9.

Loesch, K., L. Deng, X. Wang, K. He, J. Jiang, and S. J. Frank. 2007. Endoplasmic reticulum-associated degradation of growth hormone receptor in Janus kinase 2-deficient cells. *Endocrinology* **148:**5955-65.

Lombardi, V., L. Van Overtvelt, S. Horiot, and P. Moingeon. 2009. Human dendritic cells stimulated via TLR7 and/or TLR8 induce the sequential production of Il-10, IFN-gamma, and IL-17A by naive CD4+ T cells. *J Immunol* **182:**3372-9.

Lukacs, N. W., J. J. Smit, S. Mukherjee, S. B. Morris, G. Nunez, and D. M. Lindell. 2010. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. *J Immunol* **185:**2231-9.

Lunemann, J. D., M. Tintore, B. Messmer, T. Strowig, A. Rovira, H. Perkal, E. Caballero, C. Munz, X. Montalban, and M. Comabella. 2010. Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Ann Neurol* **67:**159-69.

Macal, M., S. Sankaran, T. W. Chun, E. Reay, J. Flamm, T. J. Prindiville, and S. Dandekar. 2008. Effective CD4+ T-cell restoration in gut-associated lymphoid tissue of HIV-infected patients is associated with enhanced Th17 cells and polyfunctional HIV-specific T-cell responses. *Mucosal Immunol* **1:**475-88.

MacFie, J. 2004. Current status of bacterial translocation as a cause of surgical sepsis. Br Med Bull **71:**1-11.

Macpherson, A. J., M. B. Geuking, and K. D. McCoy. 2005. Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. *Immunology* **115:**153-62.

Maecker, H. T. 2004. Cytokine flow cytometry. *Methods Mol Biol* **263:**95-108.

Majewski, M., T. O. Bose, F. C. Sille, A. M. Pollington, E. Fiebiger, and M. Boes. 2007. Protein kinase C delta stimulates antigen presentation by Class II MHC in murine dendritic cells. *Int Immunol* **19:**719-32.

Mastro, T.D. & de Vincenzi, I. 1996. Probabilities of sexual HIV-1 transmission. *AIDS* (London, England) **10:**75-82.

Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434:**1093-7.

Mattapallil, J. J., and M. Roederer. 2006. Acute HIV infection: it takes more than guts. *Curr Opin HIV AIDS* **1:**10-5.

McAllister, F., A. Henry, J. L. Kreindler, P. J. Dubin, L. Ulrich, C. Steele, J. D. Finder, J. M. Pilewski, B. M. Carreno, S. J. Goldman, J. Pirhonen, and J. K. Kolls. 2005. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J Immunol* **175:**404-12.

McAuley, J. L., S. K. Linden, C. W. Png, R. M. King, H. L. Pennington, S. J. Gendler, T. H. Florin, G. R. Hill, V. Korolik, and M. A. McGuckin. 2007. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *J Clin Invest* **117:**2313-24.

McKenzie, B. S., R. A. Kastelein, and D. J. Cua. 2006. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol* **27:**17-23.

Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz. 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* **200:**761-70.

Mehandru, S., K. Tenner-Racz, P. Racz, and M. Markowitz. 2005. The gastrointestinal tract is critical to the pathogenesis of acute HIV-1 infection. *J Allergy Clin Immunol* **116:**419-22.

Mesquita, P. M., N. Cheshenko, S. S. Wilson, M. Mhatre, E. Guzman, E. Fakioglu, M. J. Keller, and B. C. Herold. 2009. Disruption of tight junctions by cellulose sulfate facilitates HIV infection: model of microbicide safety. *J Infect Dis* **200:**599-608.

Michel, M.L., A.C. Keller, C. Paget., M. Fujio., F. Trottein., P.B. Savage., C. Wong., E. Schneider., M. Dy and M.C. Leite-de-Moraes. 2007. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* **204**:995–1001.

Miyazono, K., U. Hellman, C. Wernstedt, and C. H. Heldin. 1988. Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J Biol Chem* **263:**6407-15.

Mkhize, N. N., P. P. Gumbi, L. J. Liebenberg, Y. Ren, P. Smith, L. Denny, and J. A. Passmore. 2010. Persistence of genital tract T cell responses in HIV-infected women on highly active antiretroviral therapy. *J Virol* **84:**10765-72.

Moore, J.P. & Shattock, R.J. 2003. Preventing HIV-1 sexual transmission--not sexy enough science, or no benefit to the bottom line? *J Antimicro Chemo* **52:**90-892.

Mowat, A. M., and J. L. Viney. 1997. The anatomical basis of intestinal immunity. *Immunol Rev* **156:**145-66.

Mrabet-Dahbi, S., M. Metz, A. Dudeck, T. Zuberbier, and M. Maurer. 2009. Murine mast cells secrete a unique profile of cytokines and prostaglandins in response to distinct TLR2 ligands. *Exp Dermatol* **18:**437-44.

Nandi, D., and J. P. Allison. 1993. Characterization of neutrophils and T lymphocytes associated with the murine vaginal epithelium. *Reg Immunol* **5:**332-8.

Nazli, A., X. D. Yao, M. Smieja, K. L. Rosenthal, A. A. Ashkar, and C. Kaushic. 2009. Differential induction of innate anti-viral responses by TLR ligands against Herpes simplex virus, type 2, infection in primary genital epithelium of women. *Antiviral Res* **81:**103-12.

Nishimura, Y., T. Igarashi, O. K. Donau, A. Buckler-White, C. Buckler, B. A. Lafont, R. M. Goeken, S. Goldstein, V. M. Hirsch, and M. A. Martin. 2004. Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proc Natl Acad Sci USA* **101:**12324-9.

Nkwanyana, N. N., P. P. Gumbi, L. Roberts, L. Denny, W. Hanekom, A. Soares, B. Allan, A. L. Williamson, D. Coetzee, A. J. Olivier, W. A. Burgers, and J. A. Passmore. 2009. Impact of human immunodeficiency virus 1 infection and inflammation on the composition and yield of cervical mononuclear cells in the female genital tract. *Immunology* **128:**e746-57.

Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* **448:**480-3.

Obayashi, T., M. Yoshida, T. Mori, H. Goto, A. Yasuoka, H. Iwasaki, H. Teshima, S. Kohno, A. Horiuchi, A. Ito, and et al. 1995. Plasma (1-->3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet* **345:**17-20.

O'Connor, W., Jr., L. A. Zenewicz, and R. A. Flavell. 2010. The dual nature of T(H)17 cells: shifting the focus to function. *Nat Immunol* **11:**471-6.

O'Mara, L. A., L. A. Norian., D. Kreamalmeyer., J.M. White, and P.M. Allen. 2005. T cell-mediated delay of spontaneous mammary tumor onset: increased efficacy with in vivo versus in vitro activation. *J Immunol* **174**: 4662-4669.

Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J. S. Abrams, K. W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J. F. Bazan, and R. A. Kastelein. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13:**715-25.

Ostrowski, S. R., T. L. Katzenstein, B. K. Pedersen, J. Gerstoft, and H. Ullum. 2008. Residual viraemia in HIV-1-infected patients with plasma viral load <or=20 copies/ml is associated with increased blood levels of soluble immune activation markers. *Scand J Immunol* **68:**652-60.

Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* **28:**454-67.

Pabst, R., and H. J. Rothkotter. 2006. Structure and function of the gut mucosal immune system. *Adv Exp Med Biol* **579:**1-14.

Paiardini, M. 2010. Th17 cells in natural SIV hosts. *Curr Opin HIV AIDS* **5:**166-72.

Pantaleo, G., and A. Harari. 2006. Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat Rev Immunol* **6:**417-23.

Passmore, J. A., V. C. Burch, E. G. Shephard, D. J. Marais, B. Allan, P. Kay, R. C. Rose, and A. L. Williamson. 2002. Single-cell cytokine analysis allows detection of cervical T-cell responses against human papillomavirus type 16 L1 in women infected with genital HPV. *J Med Virol* **67:**234-40.

Pedron, T., and P. Sansonetti. 2008. Commensals, bacterial pathogens and intestinal inflammation: an intriguing menage a trois. *Cell Host Microbe* **3:**344-7.

Peng, J., X. O. Yang, S. H. Chang, J. Yang, and C. Dong. 2009. IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation. *Cell Res* **20:**62-71.

Petrovas, C., J. P. Casazza, J. M. Brenchley, D. A. Price, E. Gostick, W. C. Adams, M. L. Precopio, T. Schacker, M. Roederer, D. C. Douek, and R. A. Koup. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* **203:**2281-92.

Pircher, R., P. Jullien, and D. A. Lawrence. 1986. Beta-transforming growth factor is stored in human blood platelets as a latent high molecular weight complex. *Biochem Biophys Res Commun* **136:**30-7.

Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker. 1999. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med* **5:**518-25.

Plourd, D. M. 1997. Practical Guide to Diagnosing and Treating Vaginitis. *Medscape Womens Health* **2:**2.

Prendergast, A., J. G. Prado, Y. H. Kang, F. Chen, L. A. Riddell, G. Luzzi, P. Goulder, and P. Klenerman. 2010. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. *AIDS* **24:**491-502.

Probst, H. C., K. Tschannen, A. Gallimore, M. Martinic, M. Basler, T. Dumrese, E. Jones, and M. F. van den Broek. 2003. Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression. *J Immunol* **171:**5415-22.

Pudney, J., Quayle, A.J. & Anderson, D.J. 2005. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biology of reproduction* **73:**1253-1263.

Renu, S., Young, C. and J. Neu. 2010. Molecular Modulation of Intestinal Epithelial Barrier: Contribution of Microbiota. *J Biomedicie and Biotechnology* **Article ID 305879.**

Roederer, M., Z. Darzynkiewicz, and D. R. Parks. 2004. Guidelines for the presentation of flow cytometric data. *Methods Cell Biol* **75:**241-56.

Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278:**1447-50.

Rouvier, E., M. F. Luciani, M. G. Mattei, F. Denizot, and P. Golstein. 1993. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J Immunol* **150:**5445-56.

Rowland-Jones, S., J. Sutton, K. Ariyoshi, T. Dong, F. Gotch, S. McAdam, D. Whitby, S. Sabally, A. Gallimore, T. Corrah, and et al. 1995. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nat Med* **1:**59-64.

Rowland-Jones, S. L., T. Dong, K. R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, K. S. MacDonald, A. J. McMichael, and F. A. Plummer. 1998. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *J Clin Invest* **102:**1758-65.

Rozhkova, E., M. Yurinskaya, O. Zatsepina, D. Garbuz, V. Karpov, S. Surkov, A. Murashev, V. Ostrov, B. Margulis, M. Evgen'ev, and M. Vinokurov. 2010. Exogenous mammalian extracellular HSP70 reduces endotoxin manifestations at the cellular and organism levels. *Ann N Y Acad Sci* **1197:**94-107.

Rychert, J., S. Saindon, S. Placek, D. Daskalakis, and E. Rosenberg. 2007. Sequence variation occurs in CD4 epitopes during early HIV infection. *J Acquir Immune Defic Syndr* **46:**261-7.

Sato, W., Aranami, T., and T. Yamamura. 2007. Cutting edge: human Th17 cells are identified as bearing $CCR2^{+}CCR5^{-}$phenotype. *J Immunol* **178:**7525-7529.

Schacker, T. 2008. The role of secondary lymphatic tissue in immune deficiency of HIV infection. *AIDS* **22 Suppl 3:**S13-8.

Scheibenbogen, C., K. H. Lee, S. Mayer, S. Stevanovic, U. Moebius, W. Herr, H. G. Rammensee, and U. Keilholz. 1997. A sensitive ELISPOT assay for detection of CD8+ T lymphocytes specific for HLA class I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. *Clin Cancer Res* **3:**221-6.

Scherberich, J. E., and W. A. Nockher. 2000. Blood monocyte phenotypes and soluble endotoxin receptor CD14 in systemic inflammatory diseases and patients with chronic renal failure. *Nephrol Dial Transplant* **15:**574-8.

Scriba, T. J., B. Kalsdorf, D. A. Abrahams, F. Isaacs, J. Hofmeister, G. Black, H. Y. Hassan, R. J. Wilkinson, G. Walzl, S. J. Gelderbloem, H. Mahomed, G. D. Hussey, and W. A. Hanekom. 2008. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* **180:**1962-70.

Seabra, V. F., G. Thomas, and B. L. Jaber. 2009. Soluble CD14 and endotoxin levels in hemodialysis patients: a tale of 2 molecules. *Am J Kidney Dis* **54:**990-2.

Shacklett, B.L. 2008. Mucosal immunity to HIV: a review of recent literature. *Current opinion in HIV and AIDS* **3:**541-547.

Shacklett, B.L., Critchfield, J.W. & Lemongello, D. 2009. Isolating mucosal lymphocytes from biopsy tissue for cellular immunology assays. *Methods in molecular biology (Clifton, N.J.)* **485:**347-356.

Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J Immunol* **178:**4466-72.

Smith, J. A., and R. Daniel. 2006. Following the path of the virus: the exploitation of host DNA repair mechanisms by retroviruses. *ACS Chem Biol* **1:**217-26.

Solum, N. O., C. Rigollot, A. Z. Budzynski, and V. J. Marder. 1973. A quantitative evaluation of the inhibition of platelet aggregation by low molecular weight degradation products of fibrinogen. *Br J Haematol* **24:**419-34.

Song, C., L. Luo, Z. Lei, B. Li, Z. Liang, G. Liu, D. Li, G. Zhang, B. Huang, and Z. H. Feng. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* **181:**6117-24.

Srinivasan, N. 2010. Telling apart friend from foe: Discriminating between commensals and pathogens at mucosal sites. *Innate Immun*. **16:** 391-404.

Steinman, L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* **13:**139-45.

Stewart, I., P. J. Schluter, and G. R. Shaw. 2006. Cyanobacterial lipopolysaccharides and human health - a review. Environ Health **5:**7.

Stockinger, B. 2007. Th17 cells: An orphan with influence. *Immunol Cell Biol* **85:**83-4.

Streeck, H., and D. F. Nixon. 2010. T cell immunity in acute HIV-1 infection. *J Infect Dis* **202 Suppl 2:**S302-8.

Sugimoto, K., A. Ogawa, E. Mizoguchi, Y. Shimomura, A. Andoh, A. K. Bhan, R. S. Blumberg, R. J. Xavier, and A. Mizoguchi. 2008. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* **118:**534-44.

Sun, X., H. Yamada, K. Shibata, H. Muta, K. Tani, E. R. Podack, and Y. Yoshikai. 2010. CD30 ligand/CD30 plays a critical role in Th17 differentiation in mice. *J Immunol* **185:**2222-30.

Suni, M. A., L. J. Picker, and V. C. Maino. 1998. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods* **212:**89-98.

Tapping, R.I, and P.S. Tobias. 2000. Cellular Binding of Soluble CD14 Requires Lipopolysaccharide (LPS) and LPS-binding Protein. *J. Biol. Chem.* **272:** 23157-23164.

Tilton, J. C., M. R. Luskin, A. J. Johnson, M. Manion, C. W. Hallahan, J. A. Metcalf, M. McLaughlin, R. T. Davey, Jr., and M. Connors. 2007. Changes in paracrine interleukin-2 requirement, CCR7 expression, frequency, and cytokine secretion of human immunodeficiency virus-specific CD4+ T cells are a consequence of antigen load. *J Virol* **81:**2713-25.

Touraine, J. L., J. W. Hadden, F. Touraine, E. M. Hadden, R. Estensen, and R. A. Good. 1977. Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. *J Exp Med* **145:**460-5.

UNAIDS. 2008. Sub-Saharan Africa AIDS epidemic update: regional summary, 2007.UNAIDS, Geneva, Switzerland. URL: http://data.unaids.org/pub/Report/2008/jc1526_epibriefs_subsaharanafrica_en.pdf.
Last accessed 30 September, 2010.

Unutmaz, D., V. N. KewalRamani, S. Marmon, and D. R. Littman. 1999. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J Exp Med* **189:**1735-46.

Vajdy, M., R. Veazey, I. Tham, C. deBakker, S. Westmoreland, M. Neutra, and A. Lackner. 2001. Early immunologic events in mucosal and systemic lymphoid tissues after intrarectal inoculation with simian immunodeficiency virus. *J Infect Dis* **184:**1007-14.

van Beelen, A. J., Z. Zelinkova, E. W. Taanman-Kueter, F. J. Muller, D. W. Hommes, S. A. Zaat, M. L. Kapsenberg, and E. C. de Jong. 2007. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* **27:**660-9.

van Leeuwen, H. C., J. M. Liefhebber, and W. J. Spaan. 2006. Repair and polyadenylation of a naturally occurring hepatitis C virus 3' nontranslated region-shorter variant in selectable replicon cell lines. *J Virol* **80:**4336-43.

Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner. 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280:**427-31.

Veazey, R. S., P. A. Marx, and A. A. Lackner. 2003. Vaginal CD4+ T cells express high levels of CCR5 and are rapidly depleted in simian immunodeficiency virus infection. *J Infect Dis* **187:**769-76.

Veldhoen, M., K. Hirota, A. M. Westendorf, J. Buer, L. Dumoutier, J. C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* **453:**106-9.

Wakefield, L. M. 1987. An assay for type-beta transforming growth factor receptor. *Methods Enzymol* **146:**167-73.

Wang, P. L., and K. Ohura. 2002. Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Crit Rev Oral Biol Med* **13:**132-42.

Wang, C., S. G. Kang, J. Lee, Z. Sun, and C. H. Kim. 2009. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunol* **2:**173-83.

Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* **25:**821-52.

Weinberg, A., L. Zhang, D. Brown, A. Erice, B. Polsky, M. S. Hirsch, S. Owens, and K. Lamb. 2000. Viability and functional activity of cryopreserved mononuclear cells. *Clin Diagn Lab Immunol* **7:**714-6.

Wheeler, R. T., D. Kombe, S. D. Agarwala, and G. R. Fink. 2008. Dynamic, morphotype-specific Candida albicans beta-glucan exposure during infection and drug treatment. *PLoS Pathog* **4:**e1000227.

Wiekowski, M. T., M. W. Leach, E. W. Evans, L. Sullivan, S. C. Chen, G. Vassileva, J. F. Bazan, D. M. Gorman, R. A. Kastelein, S. Narula, and S. A. Lira. 2001. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol* **166:**7563-70.

Wilson, J. 2004. Managing recurrent bacterial vaginosis. *Sex Transm Infect* **80:**8-11.

Wolk, K., E. Witte, U. Hoffmann, W. D. Doecke, S. Endesfelder, K. Asadullah, W. Sterry, H. D. Volk, B. M. Wittig, and R. Sabat. 2007. IL-22 induces lipopolysaccharide-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease. *J Immunol* **178:**5973-81.

Wong, C. K., C. Y. Ho, E. K. Li, and C. W. Lam. 2000. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. *Lupus* **9:**589-93.

Xie, M. H., S. Aggarwal, W. H. Ho, J. Foster, Z. Zhang, J. Stinson, W. I. Wood, A. D. Goddard, and A. L. Gurney. 2000. Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. *J Biol Chem* **275:**31335-9.

Yang, L., D. E. Anderson, C. Baecher-Allan, W. D. Hastings, E. Bettelli, M. Oukka, V. K. Kuchroo, and D. A. Hafler. 2008. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* **454:**350-2.

Yang, X. O., B. P. Pappu, R. Nurieva, A. Akimzhanov, H. S. Kang, Y. Chung, L. Ma, B. Shah, A. D. Panopoulos, K. S. Schluns, S. S. Watowich, Q. Tian, A. M. Jetten, and C. Dong. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* **28:**29-39.

Ye, P., F. H. Rodriguez, S. Kanaly, K. L. Stocking, J. Schurr, P. Schwarzenberger, P. Oliver, W. Huang, P. Zhang, J. Zhang, J. E. Shellito, G. J. Bagby, S. Nelson, K. Charrier, J. J. Peschon, and J. K. Kolls. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* **194:**519-27.

Yoshiga, Y., D. Goto, S. Segawa, Y. Ohnishi, I. Matsumoto, S. Ito, A. Tsutsumi, M. Taniguchi, and T. Sumida. 2008. Invariant NKT cells produce IL-17 through IL-23-dependent and -independent pathways with potential modulation of Th17 response in collagen-induced arthritis. *Int J Mol Med* **22:**369-74.

Young, N. S., J. Levin, and R. A. Prendergast. 1972. An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. *J Clin Invest* **51:**1790-7.

Yudin, M. H., S. L. Hillier, H. C. Wiesenfeld, M. A. Krohn, A. A. Amortegui, and R. L. Sweet. 2003. Vaginal polymorphonuclear leukocytes and bacterial vaginosis as markers for histologic endometritis among women without symptoms of pelvic inflammatory disease. Am J Obstet Gynecol **188:**318-23.

Yue, F. Y., A. Merchant, C. M. Kovacs, M. Loutfy, D. Persad, and M. A. Ostrowski. 2008. Virus-specific interleukin-17-producing CD4+ T cells are detectable in early human immunodeficiency virus type 1 infection. *J Virol* **82:**6767-71.

Zeldin, D. C., P. Eggleston, M. Chapman, G. Piedimonte, H. Renz, and D. Peden. 2006. How exposures to biologics influence the induction and incidence of asthma. *Environ Health Perspect* **114:**620-6.

Zhang, Z. Q., S. W. Wietgrefe, Q. Li, M. D. Shore, L. Duan, C. Reilly, J. D. Lifson, and A. T. Haase. 2004. Roles of substrate availability and infection of resting and activated CD4+ T cells in transmission and acute simian immunodeficiency virus infection. *Proc Natl Acad Sci USA* **101:**5640-5.

Zheng, Y., D. M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, and W. Ouyang. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* **445:**648-51.

Zhou, L., M. M. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* **30:**646-55.

Zhou, L., J. E. Lopes, M. M. Chong, Ivanov, II, R. Min, G. D. Victora, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, S. F. Ziegler, and D. R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* **453:**236-40.

Zhu, J., L. Guo, C. J. Watson, J. Hu-Li, and W. E. Paul. 2001. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* **166:**7276-81.