EVALUATING THE INTERACTION OF HIV AND THE IMMUNE SYSTEM IN MUCOSAL TISSUES

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in The Graduate Department of Medicine in the Institute of Medical Sciences at The University of Toronto

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ABSTRACT

90% of Human Immunodeficiency Virus (HIV) infections are acquired across the genital or gastrointestinal mucosa, and infection leads to profound depletion of CD4+ lymphocytes. Antiretroviral therapy can restore blood CD4+ T cells. However, immune dysfunction and defects in mucosal antimicrobial defence persist. Some CD4+ T-subsets, particularly antimicrobial Th17 cells, show enhanced susceptibility to HIV infection and are also preferentially depleted in the course of HIV infection; the latter may allow microbial translocation into the bloodstream. Genital infections have been shown to have direct mucosal immune effects and to increase susceptibility to HIV; however, the effect of systemic infections, such as Malaria (which is holo-endemic in some HIV prevalent regions) is unknown. Understanding the relationship between HIV, highly susceptible immune cells, immune activation and malaria infection on mucosal tissues has been the main focus of my thesis.

In HIV-infected individuals, I explored whether HIV antiretroviral therapy restores gut Th17 populations and improves gut antimicrobial defences. Therapy restored gut Th17 populations in some, but not all individuals, but antimicrobial defence remained impaired. I then piloted a novel mucosal-optimized PCR assay to measure cervical immune gene responses, as standard mucosal assays are inadequate. I succeeded in measuring mitogen-induced, but not HIV-specific, cervical immune responses in HIV-infected individuals. Next, using this PCR platform I examined mitogen-induced cervical immune responses in individuals
demonstrating reduced susceptibility to HIV, and found that they had reduced
production of both Th17-associated and pro-inflammatory cytokines from cervical
cells. Finally, in a murine model I found that malaria caused genital and
gastrointestinal mucosal immune activation, and increased both the expression of
mucosal HIV susceptibility immune markers, and mucosal T cell immune activation.

In summary, insufficient gastrointestinal Th17 cells restoration does not
underlie persistent mucosal immune activation and microbial translocation in HIV-
infected people on therapy. A reduced frequency of highly susceptible Th17 cells in
the cervix of HIV-exposed but uninfected individuals was identified as a correlate of
reduced HIV susceptibility. Malaria, a common systemic infection in HIV-endemic
countries, may enhance susceptibility to HIV through increasing putative immune
markers of HIV susceptibility and immune activation in potential mucosal sites of
HIV exposure.
Thesis overview

Chapter-1: This section gives a general overview of HIV, ranging from HIV epidemiology, virology, antiretroviral treatment and recent HIV vaccine trials. Here core emphasis is given to topics I investigated during my thesis including mucosal transmission of HIV, biological factors affecting the susceptibility and transmission of HIV within mucosal tissues including the role of Th17 cells, early pathogenic effects of HIV infection in the gut, and the associated disease pathogenesis that ensues from early gut Th17 immune barrier depletion.

Chapter-2: During HIV infection, loss of highly susceptible gut mucosal Th17 CD4+ cells is associated with increased microbial translocation and increased systemic immune activation during HIV pathogenesis. Antiretroviral therapy can reverse the loss of CD4+ T cells, however immune activation and microbial translocation are never fully resolved and could be due to a Th17 imbalance. I show data here confirming that gut Th17 cells are lost early in HIV infection, and that antibacterial immune defects persist both before and after antiretroviral therapy. However, I also show new data demonstrating that long-term antiretroviral therapy has a heterogeneous effect on gut Th17 restoration; where successful gut Th17 restoration is associated with reduced microbial translocation, and having a reduced HIV proviral reservoir in gut tissues.

Chapter 3: In this chapter I continue to explore how HIV interacts with the immune system at the mucosa through the optimization of a real-time quantitative PCR (qPCR) assay to measure cervical immune responses in HIV-infected individuals. Assay development was necessitated by the absence of standard assays to measure
multiparameter immune responses in the genital mucosa due to recovery of low cell numbers. Here I test and report the most stable reference (housekeeping) gene to use in normalizing immune gene induction levels from cervical tissues. I then show data on the optimization of the qPCR assay in blood cells using cervical-appropriate reduced cell numbers and go on to show that despite thorough optimization, my qPCR assay could only detect mitogen and not low-level HIV-specific immune responses in cervical tissues.

**Chapter 4:** The presence of a critical mass of susceptible cells, particularly CD4+ T cells producing IL17 (Th17) cells, and immune activation at the genital mucosa may be an important determinant of whether HIV will be acquired. Using my optimized qPCR assay and a multiplex ELISA platform, I measure ex-vivo and mitogen-stimulated gene and protein induction in the cervical mucosa of HIV-exposed, seronegative (HESN) individuals to identify possible correlates of immune protection in the blood and genital mucosa. I report that HIV exposure without infection is associated with blunted IL17 and reduced pro-inflammatory immune responses in the cervix further expounding the known correlates of immune protection from HIV.

**Chapter 5:** Malaria and HIV show a high degree of overlap in Kenya, where the bulk of my studies are concentrated, and each of these two infections may enhance transmission of the other. Using a mouse model, I explore the effect of murine malaria infection on common HIV mucosal transmission sites including the gut and genital mucosa. In this chapter I show that systemic malaria infection increases T cell immune activation and expression of putative HIV susceptibility markers on
lymphocytes in both the gut and genital mucosa, shedding some light on how malaria may be increasing susceptibility to HIV in holo-endemic regions.

**Chapter 6:** In this section I summarize and briefly discuss my research findings with useful insight on possible future directions of my work.
ACKNOWLEDGEMENTS

I would like to start by thanking and dedicating this work to my parents, James and Leah Chege who made my studies possible. My academic journey perhaps started at 10 years old with a sneeze that led my parents to my hiding spot under the dinning table where I watched my favourite TV shows in hiding, instead of studying. My parents then embarked on a vigorous academic programme and ensured that I attended any school I wanted in the world at a great financial sacrifice to them, for which I am deeply grateful. I would also like to thank my dad for always reminding me when I faltered in my academics that he would readily 'buy me some cows to raise' if I felt that academics was not for me...Needless to say, I stuck to my academics. I would also like to thank my significant other Linda Samba, who reminded me in the later years of my academic training that I could not quit now as I had ‘eaten the whole elephant, and that I could surely finish the tail too’. To them, I am eternally thankful.

I would also like to thank my Supervisor Dr. Rupert Kaul whose strong mentorship, training and virtuosity made this body of work possible. I would particularly like to thank him for taking me in to his lab as an international student, despite the fact that my student status made me ineligible for external stipend scholarships, which he gladly covered. I will forever be grateful for his benevolence. I would also like to thank members of my program advisory committee, Dr. David Kelvin, Dr. Kelly McDonald and Dr. Gray-Owen who gave me very insightful critique of my work, and also generously extended to me various expertise within their labs to make my work possible.
I am also especially indebted to Prameet Sheth, Sanja Huibner and Joshua Kimani who throughout the years of my studies were always ready to assist me in my research work, offer helpful advice and encourage me along the way when things did not go as planned in Canada or Kenya. Other members in my lab and in other collaborative groups have also been instrumental to my work and for which I am very thankful. They included Lucy Shin, Connie Kim, Kamnoosh Shahabi, Shehzad Iqbal, Lyle McKinnon, Blake Ball, Yijie Chai, Taylor Kain, Sarah Higgins, Chloe McDonald, Jemima Nyakio, and Ann Maingi.

I realize now that it took a ‘village’ for me to complete my studies. There are many other unmentioned ‘giants’ (colleagues, friends and family) on whose shoulders I have stood on to get here, and I will always be eternally thankful to them.
LIST OF ABBREVIATIONS

ACD – Acid Citrate Dextran
AIDS – Acquired Immunodeficiency Syndrome
ANOVA – Analysis of variance
AP – Alkaline Phosphatase
ART- Antiretroviral Therapy
bDNA – Branched Deoxyribose Nucleic Acid Assay
CBA – Cytometric Bead Array
CCR5 – CC Chemokine Receptor 5
CD – Cluster of Differentiation
C.trachomatis – Chlamydia trachomatis
CTL – Cytotoxic T cell
CXCR4 – CXC Chemokine Receptor 4
DC – Dendritic cell
DC-SIGN – Dendritic cell Specific Intracellular Adhesion Molecule-3 grabbing Non-integrin
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribose Nucleic acid
ELISA – Enzyme Linked Immunosorbent Assay
ELISPOT – Enzyme Linked Immunospot Assay
ENV – HIV envelope glycoprotein
ER – Endoplasmic Reticulum
FACS – Fluorescence Activated Cell Sorting /Flow Cytometry
FBS – Fetal Bovine Serum

FCS – Fetal Calf Serum

GAG – Group Specific Antigen

Gut – Gastrointestine

GP160 – Envelope Glycoprotein 160

GP120 – Envelope Glycoprotein 120

GP41 – Envelope Glycoprotein 41

Group M – Major HIV strain

Group O – Outlier HIV strain

GUD – Genital Ulcer Disease

HAART – Highly Active Antiretroviral Therapy

HESN – HIV Exposed Seronegative

HIV – Human Immunodeficiency Virus-1

HIV-2 – Human Immunodeficiency Virus -2

HLA – Human Leukocyte Antigen

HSV-2 – Herpes Simplex Virus type 2

IEL – Intestinal Epithelial Lymphocyte

LPL – Lamina Propria Lymphocyte

IgG – Immunoglobulin G

IL – Interleukin

IE-1 – Immediate Early protein-1

IFN-g - Interferon-gamma

KIR – Killer Immunoglobulin Receptors
LTR- Long Terminal Repeat
MALT – Mucosa Associated Lymphoid Tissue
MBL – Mannose Binding lectin
MCP – Macrophage Chemotactic Protein
MHC – Major Histocompatibility Complex
MIP-1b – Macrophage Inflammatory Protein 1-beta
MSM – Men who have Sex with Men
mRNA – Messenger Ribonucleic Acid
*N. gonorrhea* – Neisseria gonorrhea
NK cell – Natural Killer cells
NRTI – Nucleoside Reverse Transcriptase Inhibitor
NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitor
NEF – HIV-1 nef gene
p24 – Gag Capsid protein
PAMP – Pathogen Associated Molecular Pattern
PBMC – Peripheral Blood Mononuclear Cells
PCR – Polymerase Chain Reaction
PI – Protease Inhibitor
POL – HIV Polymerase gene
PRR – Pattern Recognition Receptors
RANTES – Regulated Upon Expression Normally T cell Expressed and Secreted
REV – Regulator of Expression of Viral proteins
RPMI 1640 – Roswell Park Memorial Institute media
RRE – Rev Response Element
RNA – Ribonucleic Acid
RTC – Reverse Transcription Complex
SEB – Staphylococcus Enterotoxin - B
SIV – Simian Immunodeficiency Virus
SLPI – Secretory Leukocyte Protease Inhibitor
SM – Sooty Mangabey
STI – Sexually Transmitted Infection
Th – T helper cell
TAT – Transactivator of Transcription
TBP – TAT Binding Protein
TCR – T-cell Receptor
TLR – Toll-like receptor
TNF-a – Tumor necrosis factor – alpha
*T. pallidum* – Treponema pallidum
T Reg – T Regulatory Cells
UNAIDS – Joint United Nations Program on HIV/AIDS
VIF – Viral Infectivity Factor
VPR – Viral Protein R
VPU – Viral Protein U
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Chapter 1: INTRODUCTION TO HIV
1.1 The Global HIV Pandemic

Human immunodeficiency virus (HIV), which leads to the acquired immunodeficiency syndrome (AIDS), is a serious viral infection that has significant impact on public health globally. The World Health Organization and The United Nations Program on HIV/AIDS (UNAIDS) estimates that since the start of the epidemic, over 46 million people have died following infection by HIV[1]. By some estimates, a new HIV infection occurs every 7 seconds while death from this infection occurs every 10 seconds [2]. In 1996, mathematical modelling suggested that by the prevalent trends of HIV infections at the time that, in the year 2000 over 100 million people would be infected with HIV [3]. However, following the introduction of highly effective antiretroviral therapy and widespread prevention interventions, only about 33.3 million people are currently infected with HIV [1]. Nevertheless, despite the introduction of these measures approximately 1.8 million people died from HIV/AIDS related complications in 2009 alone [1].

An overwhelming majority of new HIV infections (95%) occur in low and middle-income countries [1]. Sub-Saharan Africa (SSA) bears the brunt of the HIV epidemic where 22.5 million people (68%) are currently living with HIV/AIDS, and 1.8 million of them acquired the infection in 2009 alone [1]. Currently, South Africa has the highest number of HIV infected individuals in the world with over 5.6 million infected [1]. HIV infection rates, though fewer in total number, remain extremely high in other parts of SSA. For instance, 1 in 4 (25.9%) of people in Swaziland are currently infected [1]. Nonetheless, there are
also some disparities in prevalence of HIV/AIDS within SSA. In Kenya, a country in East Africa where part of my research studies were conducted, there are approximately 1.5 million people living with HIV/AIDS [1, 4] within a population of roughly 40 million people. In North America, where another subset of my research was also conducted, a similar number (approximately 1.5 million) of adults are currently living with HIV/AIDS but in a population of roughly 500 million [1].

Reasons underlying the higher HIV infection rates in SSA are multifactorial and complex to unravel. However, it is clear that two-thirds of HIV infected individuals reside in Africa and are of black ethnicity [1]. Epidemiologic studies conducted in the United States find that HIV/AIDS rates are 10 times higher in African-Americans than in white Americans [5], while in Ontario, Canada HIV-infected individuals of African ethnicity account for 22.5% of provincial HIV infection rates despite making up only 3.9% of the overall population [6]. In SSA, the per-contact-risk of HIV-transmission is 3 times greater than that in developed countries [7]. The reasons for higher HIV infection rates in SSA are unknown. However various social-economic and cultural factors in SSA such as higher per-capita rates of commercial sex work, increased history of sexual partners/concurrent relationships, and traditions such as wife inheritance may contribute to the high HIV incidence in individuals of African ethnicity [6]. Focus on only these socio-demographic factors may lead to stigmatization of HIV infection, and may not helpfully address biological issues that might be responsible for higher HIV infection rates here such as bacterial vaginosis (BV)
or douching [9] which are both common in Africa. In addition, systemic immune activation rates, host genetics and viral factors may also contribute to altered susceptibility to HIV infection in SSA in individuals of African ethnicity [6]. These biological factors are addressed in more detail in section 1.5 of this thesis.

Women are also disproportionately afflicted by the HIV/AIDS epidemic. Greater than 50% of individuals living with HIV/AIDS globally are women, and this statistic is worse in SSA where 76% of all HIV-positive women live [1]. Estimates suggest that for every 13 women infected with HIV in SSA, only 10 men will be infected [1]. Given such grim statistics, it is clear that the HIV epidemic not only impacts women more. Therefore, characterizing the events occurring in the female genital mucosa may help us better understand this disparity, and will contribute to the development of strategies to stem the tide of global infections. A significant proportion of my research work discussed here will focus on understanding mucosal immune events that underpin HIV susceptibility/acquisition in the female genital tract.

1.2 Discovery and Origin of HIV

1.2.1 Discovery and Identification of HIV and its Subtypes

The first cases of AIDS were reported in 1981 in the United States, after opportunistic infections in healthy young homosexual men with general immune deficiency were frequently observed. In 1983, seminal studies by Drs. Barré-
Sinoussi and Montagnier recovered a virus capable of reverse transcription activity from a patient with persistent generalized lymphadenopathy (PGL) [10]. They initially named it the lymphadenopathy-associated virus (LAV). For their discovery Drs. Barré-Sinoussi and Montagnier were awarded the 2008 Nobel Prize in physiology and medicine [11]. About a year later, Dr. Gallo and colleagues were working on an isolated virus with properties similar to LAV, and named it human T-cell leukemia virus (HTLV-III), and showed that this retrovirus was capable of infecting and killing CD4+ T cells, and thus linked the virus to CD4+ T cell immunodeficiency [12, 13]. Due to the similarities between the identified retroviruses from these two research groups, in 1986 the International Committee on Taxonomy of Viruses recommended naming the AIDS causing virus HIV.

The viral strain of HIV discovered by Barré-Sinoussi et al was later renamed HIV-1, after the discovery of another subtype of HIV named HIV-2 shortly thereafter. HIV-2 was identified in Portugal after its discovery in a patient of Senegalese and Cape Verde origin [14]. Subsequently, HIV-2 isolates were recovered from other regions of West Africa where this subtype predominates. HIV-1 and HIV-2 make up the two main subtypes of HIV discovered.

Further diversity is observed within both HIV subtypes. HIV-1 is phylogenetically divided into three groups named M (main), Outlier (O) and N (non-m, non-O) [2]. The M group, as the name implies, is the predominant HIV-1 subtype globally, and can be further divided into 9 distinct clades with designations ranging from A to D, F to H, J and K. These clades often show geographical spatial distribution with clade C being the most prevalent globally. Clade C is mostly found
in Southern Africa and India, clade B predominates in North America, Europe and Australia, while clade A predominates in East Africa, including Kenya. Similarly, HIV-2 also has several (eight) subtypes with designations ranging from A to H. However, these HIV strains are mostly relegated to distinct regions globally including West Africa and India [2].

1.2.2 Origin of HIV

Phylogenetic analysis of viral sequences suggests that HIV in humans is caused by two lentiviruses of zoonotic origin. HIV-1 is most likely derived from SIVcpz from the Pan Troglodytes chimpanzee while HIV-2 is thought to originate from SIVsm from the sooty mangabey. HIV is thought to have crossed over into humans multiple independent times, which may account for the various subtypes (M, O and N etc).

From geographical mapping of SIVcpz in non-human primates, HIV-1 is believed to originate from the Central Africa region lying between Cameroon, Gabon, Congo, Central Africa Republic and Equatorial Guinea. How zoonotic predecessors of HIV may have infected humans is unknown. However, some have suggested that transmission of HIV to humans may have occurred in part following exposure to blood and meat from infected animals sourced from bushmeat [15]. Alternatively, conspiracy theorists have suggested that HIV may have originated from a massive oral polio vaccine campaign carried out in the Congo in 1959. As SIVcpz was endemic in the region at the time, and monkey kidney cells were used to grow the polio vaccine used in the Congo then, this gave some partial credence to this theory.
However, sequence analysis on historical blood samples showed that the circulating virus in this region was phylogenetically distinct from all previous HIV-1 strains disproving this theory [16].

Estimation of the exact time of HIV-1 acquisition in humans is controversial, and at best inconclusive [17]. Most of the techniques utilized used assume that a constant rate of evolutionary trend exists between different viral strains where then the genetic distance from the root of the phylogenetic tree (timeline) can be estimated and extrapolated. Analysis of genetic changes from current viral strains up to those obtained in the Democratic Republic of Congo as far back as 1959 suggest that HIV-1 group M subtype may have been the first to infect humans as far back as in the 1930s [18]. Alternative molecular clock data interpretation suggest that HIV-1 M infections may have been present as far back as the end of the 17th century [19].

1.3 The HIV Virion

1.3.1 Structure of HIV

HIV comes from the family of Retroviridae known as lentiviruses. Retroviruses are so named as their RNA genome is transcribed into DNA within the cell, while in Latin the word lentus, means ‘slow’, which is derived from HIVs gradual induction of disease. The HIV virion comprises of an outer spiked envelope membrane with external glycoprotein trimmers made up of gp120 and gp41, as well as an inner nucleocapsid membrane (Figure 1.1). The nucleocapsid membrane houses two
copies of an RNA genome attached to reverse transcriptase enzymes, as well as integrase and several other viral proteins that mediate viral replication [2, 20].

The HIV genome consists of nine genes flanked by long terminal repeat sequences known as (LTR) [2, 20].

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>gag</td>
<td>Group-specific antigen</td>
</tr>
<tr>
<td>pol</td>
<td>Core proteins and matrix proteins</td>
</tr>
<tr>
<td>env</td>
<td>Reverse transcriptase, protease, and integrase enzymes</td>
</tr>
<tr>
<td>tat</td>
<td>Transmembrane glycoproteins. gp120 binds CD4 and CCR5; gp41 is required for virus</td>
</tr>
<tr>
<td>rev</td>
<td>Fusion and internalization</td>
</tr>
<tr>
<td>vif</td>
<td>Positive regulator of transcription</td>
</tr>
<tr>
<td>vpr</td>
<td>Allows export of unspliced and partially spliced transcripts from nucleus</td>
</tr>
<tr>
<td>vpu</td>
<td>Affects particle infectivity</td>
</tr>
<tr>
<td>nef</td>
<td>Transport of DNA to nucleus. Augments virion production. Cell-cycle arrest</td>
</tr>
<tr>
<td></td>
<td>Promotes intracellular degradation of CD4 and enhances release of virus from cell</td>
</tr>
<tr>
<td></td>
<td>membrane</td>
</tr>
<tr>
<td></td>
<td>Augments viral replication <em>in vivo and in vitro</em>. Decreases CD4, MHC class I and II</td>
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<td>expression</td>
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Figure 1.1 The structure of the HIV virion (top), the HIV genome structure (middle), and the function of respective HIV genes (bottom).
Within the genome are three major genes, gag, pol, and env, which are transcribed by all retroviruses including HIV. The gag gene encodes for structural proteins within the viral core, pol encodes for proteins responsible for viral replication and integration into host DNA, while env encodes for the envelope glycoproteins [2, 20]. Figure 1.1 gives a detailed summary of all HIV genes and their respective functions, which are described in more detail below.

1.3.2 HIV Infection and Replication in Activated Target cells

To initiate HIV infection, HIV must first come into close proximity to its target cells. Here, the virus may bind with higher affinity (and preferentially to) CD4+ T lymphocytes directly or may first attach to target CD4 T cells expressing α4β7- a molecule that has a similar binding domain to the CD4+ cell surface molecule that allows the virus to remain in close proximity to the CD4 molecule [2, 20, 21]. Next, HIV binds to one of several other chemokine/HIV co-receptors, primarily CCR5, CXCR4, or less frequently to its other co-receptors (CCR3, CCR2b, CCR1 and CCR4), to initiate viral entry into the target cell.

The co-receptor used by HIV to gain entry in to its target cell, determines HIV’s cellular tropism. One of the two main variants of HIV binds to CCR5 chemokine receptor (R5 strain) and requires only a low level of CD4 surface marker expression to infect, while the other variant binds to CXCR4 chemokine receptor (X4 stain) and requires a high level of CD4 [2]. These HIV variants are distinguishable by the fact that R5 viral strains can only infect macrophages but not T cell in vitro, while X4
strains infect only CD4+ T cells in vivo. Therefore these two HIV viral strains were initially referred to as macrophage-tropic (R5) and lymphocyte-tropic (X4) viruses respectively. Although both viral strains can be detected following primary HIV infection, the R5 HIV strain is transmitted in virtually all sexual transmission events and predominates throughout the clinical course of infection [22], while the X4 strain emerges mainly in the later stages of infection in approximately half of HIV infected individuals. The reasons underlying this viral transmission 'bottleneck' remain unclear [22], particularly because CXCR4 is expressed in relatively high levels on mucosal cells at sites of exposure and is also present in genital secretions of an HIV-infected individual [23, 24].

After binding of viral gp120 to CD4 and its relevant entry co-receptor, the viral gp120 trimer changes conformation exposing the underlying gp41 glycoprotein that subsequently binds to the target cell membrane and initiates viral fusion with the target cell. Once in the cell cytoplasm, viral reverse transcriptase transcribes a complementary viral strand to create viral cDNA. Viral protein R (vpr) then transports viral DNA from the cell membrane to the cell nucleus where the viral integrase enzyme inserts viral cDNA into the host DNA, thus becoming a provirus [2, 20].

Experimental studies in non-human primates have repeatedly shown that HIV can infect both resting and activated CD4+ T cells. However, following exposure, it is necessary for localized viral replication and amplification to take place, and this happens exclusively in activated CD4+ T cells (discussed next in more detail) [2, 20]. Indeed, molecular studies confirm that infectious virus particles from integrated
HIV proviral DNA require CD4+ T cell activation and describe the mechanism by which this happens. Here, CD4+ T cells activation results in induction of the NFκB transcription factor which binds to both the host and proviral DNA transcription promoters, harnessing host RNA polymerase to express both cellular and proviral RNA [2, 20]. Viral transactivator (tat) and regulator of viral expression (rev) are amongst the first proviral expressed genes to overcome cellular gene regulation mechanisms. Firstly, to control host gene expression, host cells insert negative elongation factors to block excessive RNA transcriptional activity. Viral tat protein inhibits this cellular regulation mechanism allowing enhanced viral genome expression in activated CD4+ lymphocytes. Second, cellular gene mechanisms prevent the transport of unspliced or partially spliced products out of the nucleus to ensure that gene transcripts are fully processed before transport into the cytoplasm for translation. This self-regulatory cell mechanism can be detrimental to virus production as gag-pol proteins are produced from unspliced mRNA, while env is produced from singly spliced mRNA. Therefore, viral rev directly binds and activates the host nucleocytoplasmic transport proteins bypassing host regulation mechanisms, and allows transport of viral mRNA out into the cytoplasm [2, 20] (Figure 1.1).

Finally, the late proteins gag, pol and env are then translated in the cytoplasm and assembled into complete viral particles, which can either bud from the cell membrane and be released as infectious cell-free virus or the viral components can be passed on to adjacent CD4+ T cells via an infection synapse [2, 20].
1.3.3 Why Immune Activation Favours HIV Replication

The HIV life cycle is closely related to the extent of immune activation, which in turn drives the subsequent replication of the virus. Cellular immune activation can enhance each of the three key stages of the viral life cycle including viral cellular entry, reverse transcription and proviral transcription that ultimately result in increased viral replication from activated CD4+ T cells [2].

HIV entry typically requires the binding of viral particles to CD4 and one CCR5 or CXCR4 chemokine/HIV co-entry receptor. These chemokine receptors are inducible, and immune activation will lead to their transcriptional upregulation and increased surface expression on HIV target cells [2]. This is another reason why increased immune cell activation such as sexually transmitted infections, may enhance viral entry.

Completion of HIV single strand reverse transcription into cDNA is less likely if the host cell is immunologically quiescent [25]. In quiescent T cells, HIV entry may still occur, however, HIV core proteins and virus nucleic acids can be harboured in the cytoplasm for up to 2 weeks without virus production [25-27] until the T cells are activated by the viral envelope itself or other antigenic co-stimulatory processes [28]. In this quiescent state host cell viral transcription results in the functional blockade of the reverse transcription of viral single RNA strands. Conversely, increased cellular activation enhances viral RNA reverse transcription by increasing cytoplasmic concentrations of transcriptional mediators such as nuclear factor of activated T-cells (NFATc). NFAT belongs to the family of genes related to the NFkB transcription factor and plays an important role in the transcriptional regulation of
inflammatory cytokines. NFAT can then facilitate reverse transcription of the HIV genome through an unknown mechanism [29] and can also bind to NFAT receptor elements upstream of HIV proviral LTR to enhance viral replication [30] making activated cells more likely to support productive viral infection.

Finally, the rate of HIV proviral DNA transcription is highly associated with the activation state of the cell. Viral LTR sequences within the 5’ end of the viral genome regulate proviral transcription and contain receptors for host encoded transcription factors, including NFkB [31]. Activation of T cells by external stimuli may lead to the production of immune cytokines such as TNFa, which in turn signals through a cascade and results in NFkB transcription [31]. NFkB will in turn bind to the host cell DNA as well as proviral DNA regions in the viral LTR and result in both host and proviral viral gene transcription [31]. Essentially, HIV harnesses the host cell transcription machinery of a recently activated cell to promote its own replication.

1.3.4 HIV Evolution and Genetic Diversity

HIV is a fast evolving virus, which poses a significant challenge to either its prevention by vaccines, its control by the immune system or its suppression by antiretroviral therapeutics [17]. The HIV virion can survive for ~2.6 days per generation, and replicates once every 18 hours [32]. As a result, over $10^9$-$10^{12}$ virions are produced each day [20, 32]. However, the rapid evolution of HIV is as a result of a combination of factors. First, viral reverse transcriptase is highly error...
prone and makes about 0.2 errors every 1.04 bases during each replication cycle [33]. During proviral DNA transcription in mRNA, RNA polymerase makes further errors, while supressing host cellular transcriptional and mRNA transcriptional factors that may correct for these errors [17]. These factors drive HIV to rapidly evolve away from host immune responses during the course of infection.

1.4 SEXUAL TRANSMISSION OF HIV

1.4.1 Epidemiology of mucosal HIV infection and transmission

Approximately 85% of global HIV infections have been acquired through sexual transmission [34]. Therefore, on a population level it is important to first understand the potential for HIV epidemic to spread sexually within a defined population. The dynamics of HIV transmission can be described using the basic reproductive rate ($R_0$) equation;

$$R_0 = \beta c D.$$  

Here, the variables describing $R_0$ describe the probability that an individual will infect their sexual partners over the course of a relationship ($\beta$), the average number of sexual partners per unit time ($c$), and the duration of infectiousness ($D$) [35].

An $R_0$ of $>1$ will mean that a person living with HIV will pass on the virus to at least one another individual, leading to virus propagation within the population. Indeed,
on a population level, it has previously been observed that approximately 90% of transmissions originate from just 20% of previously infected individuals [36]. These individuals have been described as ‘core transmitter groups’, and may have increased numbers of sexual partners (c), or increased infectiousness (β), due to an increased level of genital HIV shedding at the time of transmission, and their transmission duration (D) may be altered by other external factors such as having other sexual co-infections (see section 1.5.2) and antiretroviral therapy use [35, 36].

A more ‘real-world’ view of how HIV is sexually transmitted is captured by from the numbers depicting the contribution of each mucosal exposure site to global HIV infection numbers (Table 1.1). The female genital tract accounts for the single highest anatomical HIV-infection site, with 12.6 million cases out of a total of 33 million global HIV infections [1, 34, 37]. The male penis accounts for the second highest sexual HIV transmission site with 10.2 million occurring here from both heterosexual transmission and men who have sex with men (MSM) [1, 34, 37]. Lastly, the intestinal tract (rectum and upper gastrointestinal tract) accounts for the third highest global HIV-infection site with 3.9 million infections that includes anybody having receptive anal or oral sex [1, 34, 37].
Table 1.1 The contribution of HIV invasion sites to global HIV infections.

<table>
<thead>
<tr>
<th>HIV invasion site</th>
<th>Anatomical sub-location</th>
<th>Type of epithelium</th>
<th>Transmission medium</th>
<th>Transmission probability per exposure event</th>
<th>Estimated contribution to HIV cases worldwide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female genital tract</td>
<td>Vagina</td>
<td>Squamous, non-keratinized</td>
<td>Semen</td>
<td>1 in 200 – 1 in 2,000</td>
<td>12.6 million</td>
</tr>
<tr>
<td></td>
<td>Ectocervix</td>
<td>Squamous, non-keratinized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endocervix</td>
<td>Columnar, single layer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Various</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male genital tract</td>
<td>Inner foreskin</td>
<td>Squamous, poorly keratinized</td>
<td>Cervicovaginal and rectal</td>
<td>1 in 700 – 1 in 3,000</td>
<td>10.2 million$^a$</td>
</tr>
<tr>
<td></td>
<td>Penile urethra</td>
<td>Columnar, stratified</td>
<td>secretions and desquamations</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Various</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal tract</td>
<td>Rectum</td>
<td>Columnar, single layer</td>
<td>Semen</td>
<td>1 in 20 – 1 in 300</td>
<td>3.9 million$^t$</td>
</tr>
<tr>
<td></td>
<td>Upper GI tract</td>
<td>Various</td>
<td>Semen</td>
<td>1 in 2,500</td>
<td>1.5 million</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maternal blood, genital secretions(intrapartum)</td>
<td>1 in 5 – 1 in 10</td>
<td>960,000$^l$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast milk</td>
<td>1 in 5 – 1 in 10</td>
<td>960,000$^l$</td>
</tr>
</tbody>
</table>

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Exposure to HIV at the female and male genital tract leads to between 1:200 to 1:2000, and 1:700 to 1:3000 chance of transmitting the virus respectively [34, 38]. Surprisingly, the intestinal tract accounts for the third highest mucosal site of global HIV infections despite its relatively higher transmission probability rate of between 1:20 to 1:300 chance of transmission per exposure. This observation might be accounted for by several factors including the number of individuals globally exposed to HIV rectally, sexual transmitted infections at the site of exposure and other environmental factors (discussed in more detail in the next few sections). It is also prudent to acknowledge that other sites of HIV exposure do exist such as the blood (needle-stick injuries and blood products), and through mother-to-child transmission through the placenta (rarely) or through exposure to a HIV-infected mother’s blood at birth. These routes account for 2.6 million (needle-stick/blood products) and 480 thousand (mother-to-child) HIV transmission cases globally [1]
and are a minority of global HIV infections. Therefore in this thesis I focus more on sexual mucosal transmission of HIV [1, 34, 37].

1.4.2 Sexual Acquisition of HIV

1.4.2.1 HIV entry across the female genital tract

Women now account for nearly 60% of all new HIV infections in Africa [39], and around 30-40% of all global HIV transmissions events occur across the female genital tract (FGT) following heterosexual exposure[34]. Therefore a closer examination of HIV transmission at this particular mucosal site is critical to understanding the spread of this infection.

The FGT is generally divided into two main regions comprising the lower genital tract (vagina, ectocervix and endocervix) and the upper genital tract (endometrium and fallopian tubes)[40]; Figure 1.2. A single columnar epithelial layer covers the upper genital tract while the lower genital tract consists of a squamous epithelial layer [40]. Although it remains to be proven, the cervical transformation zone that lies between the endocervix and ectocervix is thought to be the area most susceptible to HIV infection [34, 41, 42]. Recent macaque SIV challenge models mapping early FGT infection show initial focal SIV infections around the transformation zone and endocervix [43], while in humans observational studies demonstrate increased HIV acquisition in individuals with cervical ectopy – the extension of the columnar cell (single-layer) epithelium to the ectocervix [44, 45]. Furthermore, this region between the ecto- and endo- cervix
area is also relatively enriched for HIV target cells including antigen presenting cells and T cell lymphocytes at both the sub-mucosal and intra-epithelial layers [40].

However, the cervical transformation zone is not the only site of HIV acquisition. HIV animal models show that SIV can be transmitted in hysterectomized animals [46], and HIV infections have also been observed in individuals with a congenital absence of a cervix [47]. While the preferential area of HIV entry remains unknown, the vagina may play as much of a significant role in HIV acquisition. The vaginal tract offers a much larger surface area [40] for HIV to infect and may allow greater access to underlying HIV target cells if the epithelium integrity is compromised by ulcerations following genital co-infections or following mechanical abrasions caused by coitus [48].
Figure 1.2: The anatomical structure of the female genital tract (a) and HIV transmission across this mucosa (b). Figure 1.2a illustrates the anatomical features of the female genital tract which is generally divided into two main regions comprising of the lower genital tract (vagina and ectocervix) and the upper genital tract (endocervix, endometrium and fallopian tubes); A single columnar epithelial layer covers the upper genital tract while the lower genital tract consists of a squamous epithelial layer, and the transformation zone consisting of both cell types is particularly rich in HIV target cells and may be a key site in HIV transmission. Figure 1.2b illustrates the infection of both resting and activated HIV target cells in the lamina propria of the genital tract. Activated cells are thought to be necessary to allow for productive viral replication and onwards broadcast of the infection into the lymph nodes and eventual establishment of infection.
HIV crosses the FGT mucosal epithelium through several pathways to establish infection. HIV may transytose across an intact epithelium to the luminal surface [49, 50] or may penetrate into gaps between the epithelium wall or through abrasions to gain access to the sub-mucosa, where target cells can then be infected. In the subepithelium layer resides non-langerin expressing dendritic cells (DCs). Intact virions can also bind to sub-mucosal DCs expressing DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) and internalized, whereupon the virus is stored within an intact endosome and can be transmitted to CD4+ T cells [51]. HIV may also be taken in from the FGT by Langerhans cells (LCs), which are found within the superficial epithelial layers from where they extend protheses to the lumen to sample invading pathogens [52]. Both LC and DCs express both CCR5 and CXCR4 HIV entry co-receptors in addition to CD4+ [53]. Indeed both R5 and X4 virus can be transmitted through the genital tract whereupon they are mostly passed on to adjacent cells via an infection synapse to infect sub-mucosal CD4+ T cells. Alternatively, these Langerhans cells may migrate to the draining lymph nodes to do the same [54, 55]. It had generally been assumed that Langerin, an adhesion molecule expressed in Langerhans cells plays a similar role as DC-SIGN in facilitating HIV transmission. However, Langerin in LCs has more recently been shown to play a greater role in preventing HIV transmission. Here HIV captured by Langerin is internalized and directed into Birbeck granules and degraded instead of onward viral transmission to CD4+ T cells during priming [56]. However, regardless of how HIV crosses the epithelial barrier, SIV rhesus macaque model studies suggest that
the earliest foci of HIV infection are in CD4+ T cells, and these cells serve as the best substrate that facilitates the establishment of productive infection [57].

Once acquired, HIV in the FGT mucosa propagates itself via both free and cell-associated HIV/SIV virions [58-60] and this has been shown directly *in vivo* in female macaques [61], mice [62] and inferred in humans from genetic sequencing data comparing viral isolates from acutely infected women with cells present in seminal plasma and blood plasma from their infected partners [63]. *Ex-vivo* studies using human cervical explants have also confirmed the transmission of cell-free and cell-associated HIV [64, 65].

**1.4.2.2 HIV entry in the male genital tract**

Approximately 15 million men are currently infected with HIV and over 70% of these infections were acquired following heterosexual vaginal exposure making the penis the second most frequent site for HIV acquisition [34]. Here viral acquisition mainly occurs following penile-vaginal or penile-anal intercourse [34].

The male genital tract (MGT) primarily consists of the urethra and is mostly lined by a thinly stratified columnar epithelial cell wall, with the exception of the fossa navicularis located at the urethral meatus, which is lined by a non-keratinized squamous epithelial lining. The glans penis (penile tip) and the outer foreskin are covered by a keratinized squamous epithelium, while the inner foreskin is covered with a similarly keratinized epithelium [66, 67]. Figure 1.3.
Three large randomized control trials have demonstrated that foreskin removal (circumcision) reduces HIV acquisition by 50-60% [68-70], demonstrating that the foreskin is the initial site of HIV infection [71, 72] and may be highly permissive to HIV infection [73]. Mechanisms of viral entry in the foreskin remain controversial. Some studies suggest that the inner foreskin is most vulnerable to HIV infection as it has a thinner keratin layer (barrier) than the outer foreskin or penis, making it more vulnerable [74, 75]. However, this initial observation was done primarily using hematoxilin and eosin staining (non-specific for keratin) and disproven with more specific histochemical staining of keratin in foreskin tissue by more rigorous recent studies [66, 67], casting doubt on whether keratinization influences susceptibility to HIV. Alternatively, others propose that the enrichment of HIV target cells in the penile foreskin might be a key driver for increased HIV susceptibility in uncircumcised men. The inner foreskin is relatively enriched for...
HIV target cells, including Langerhans cells and CD4+ T cells in the squamous epithelial lining, as well as CD4+ T cells, macrophages and dendritic cells in the submucosa underlying the foreskin [74, 76, 77], which might increase susceptibility of the inner foreskin to HIV. In addition, more recent studies from our lab demonstrate that the foreskin is enriched with effector memory CD4+ T cells, and has cells with a much higher expression of CCR5 HIV entry co-receptor than blood lymphocytes [78].

The penile urethra is another anatomical site that may play a role in HIV acquisition. This anatomical site has a thinly stratified, non-keratinized epithelial layer and its also possesses a relative enrichment of HIV target cells [34, 79]. However, only anecdotal evidence supports this hypothesis where studies show a 100% success rate of infection with SIV in male macaques through the urethra [80].

Limited information exists regarding in vivo HIV transmission across the penis [79]. This is perhaps owing to the difficulty in sampling the male genital tract [34, 81]. However, in situ hybridization and immunohistochemical techniques have determined that SIV-infected T cells and macrophages are present at all levels of the reproductive tract, and most consistently in the urethra[82]. In addition, HIV is also detectable in the ejaculate of vasectomized, HIV-infected men and in pre-ejaculatory fluid, which suggests that HIV infected cells may be present at other less accessible sites behind the urethra [83, 84].
1.4.2.3 HIV entry across the intestinal mucosa

HIV acquisition across the intestinal mucosa occurs following rectal exposure during receptive anal intercourse and may also be acquired through the upper intestinal mucosa either orally at birth or following fellatio [34]. HIV acquisition in the rectal and the lower gastrointestinal tract occurs following receptive anal intercourse and remains the most common means of HIV transmission in North America, Western Europe and Australia [85-87]. The risk of acquiring HIV through receptive anal intercourse is approximately 1.4%-1.8% per contact [7, 88], which is over 4-fold higher than the risk of genital tract acquisition [7]. Given the predominance of recto-gastrointestinal mucosal transmission of HIV in the developed world, a closer look at how transmission occurs across this surface is warranted.

Surprisingly, despite several reports detailing the impact of HIV in the rectal-gastrointestinal mucosa during acute and chronic infection, relatively little is known on how HIV is acquired across this mucosa [89]. The rectal-gastrointestinal mucosa consists of a simple columnar epithelium, and the gastrointestinal lamina propria houses 50-80% of the entire body’s T cells. These factors would therefore also make the rectal mucosa a prime site for HIV acquisition.

It is currently not known which viral transmission medium in semen (cell-associated or cell-free virus) is most responsible for HIV transmission across the rectal mucosa [7, 90-92]. However, infection here could occur following direct interaction of the virus with CD4 and CCR5 cellular receptors, and/or following formation of virus-antibody complexes to FC receptors on mucosal
monocytes/macrophages [93, 94]. Additionally, intestinal M-cells (mucosal lymph-node like cells), that house organized immune environments similar to lymph nodes in the bowel epithelium, may also permit HIV entry [95]. Finally, HIV acquisition at this site may also occur following viral transytosis (crossing) through epithelial cells and infection of target cells in the underlying lamina propria [49, 96].

1.5 Biological Factors Altering Transmission/Susceptibility to HIV

1.5.1 Viral factors

To date, HIV RNA viral load in blood remains the best predictor of HIV transmission. In a large prospective cohort study in Uganda involving over 15,000 individuals, a stepwise increase in the relative risk of transmission of 2.45 was observed for each log increment in blood viral load, while no transmission events were observed amongst discordant couples when the infectious partner had less than 1500 HIV RNA copies/ml [97]. This association between plasma viral load and transmission events may probably be attributed to the fact that viremia levels are about 70% predictive of genital viral loads [98]. However, even independent of blood viral load, cervical-rectal mucosal viral loads also demonstrate a stepwise increase in transmission risk between cervical-vaginal and semen viral loads. Specifically, every log increase in genital viral load is associated with either a 2.2-fold or 1.79-fold increase in HIV transmission risk in cervical-vaginal and semen viral loads respectively [99]. Furthermore, the highest risk for HIV transmission occurs during the acute infection phase (up to 1:10 per contact) when viral load is
highest, in comparison to the chronic untreated asymptomatic phase (up to 1:1000).

Nonetheless, temporary viral blips in mucosal secretions occur despite effective antiretroviral therapy that can maintain undetectable blood viral loads (<50 viral copies/ml) for prolonged periods [100, 101]. Additionally, statistical modelling suggests that transmission rates might actually increase at a population level despite effective antiretroviral therapy if no condoms are used [102]. Therefore, the amount of virus in either the blood and the genital tract or whether one is on antiretroviral therapy will influence the transmission probability of HIV.

HIV subtypes differ in entry co-receptor usage, viral fitness and disease pathogenesis [2]. Similarly, emerging studies suggest that viral subtypes also possess differing rates of infectivity. Studies from Thailand observe that HIV-1 clade E (now SRF01_AE) demonstrates higher rates of heterosexual transmission than subtype B [103, 104]. Elsewhere, a Ugandan study demonstrated that HIV-1 clade A subtype has a higher rate of transmission than subtype D [105]. However, limited information exists on the variability of viral clade transmission rates due to the fact that few populations have different circulating subtypes transmitted using the same route [105].

It is also prudent to again note that there is a virus genetic 'bottleneck' during the sexual transmission of HIV (see section 1.3.2). Although both R5 and X4 viral strains can be transmitted, R5 strains are typically observed during early and chronic immune infection [2, 20]. This suggests that R5 viruses are more easily transmitted than their X4 counterparts. This preferential transmission of R5 virus is perplexing, since in culture X4 viruses replicate faster than R5 viruses, and nearly all
circulating naïve CD4+ T cells are CXCR4 but not CCR5 positive [106, 107]. However, studies in rhesus macaques have shown that an R5-utilising simian/human HIV hybrid (SHIV) induced a greater loss of CD4+ intestinal cells than CD4+ T cells than an X4 SHIV strain [108]. In addition, unlike X4 viral strains, R5 viral strains can more readily infect non-activated CD4+ T cells and can also infect a broader range of target cells (DCs and macrophages), further increasing the chances that productive viral infection will result from R5 viruses [20, 109]. As such, it can be postulated that the predominance of R5 viral strains during transmission/early infection may in part be due to the high substrate availability of R5 susceptible cells in the gut mucosa and is perhaps less likely an issue of enhanced R5 viral fitness/transmission capability [2]. Nevertheless, there is no clear consensus in the field as to why R5 viruses predominate in early infection in vivo [2].

1.5.2 Co-infections

1.5.2.1 Sexually transmitted and other genital infections

Sexually transmitted infection (STIs)- such as genital herpes/Herpes Simplex Virus type 2 (HSV-2), gonorrhoea, chancroid, syphilis, cytomegalovirus, - and other non-sexually acquired genital infections, - such as bacterial vaginosis, are associated with an increased risk of HIV transmission and acquisition. Pooled estimates in a recent meta-analysis of 31 longitudinal studies indicate that having any STI will increase the risk of acquiring HIV by at least 2-3 fold [110]. Furthermore, impact on the risk of acquiring HIV also varies between two broad STI categories. Specifically
those that cause genital ulcers, such as HSV-2, syphilis, and chancroid, pose a higher risk of HIV acquisition (OR 2.8), compared to those that are non-ulcerative (OR 1.7), such as gonorrhoea, candidiasis and trichomonas [110]. However, studies may have a limited ability to delineate which genital infections contribute the most to HIV transmission/susceptibility, due to high rates of co-infection and their ability to remain asymptomatic in most individuals. For instance, cytomegalovirus and HSV-2 are chronic infections with a prevalence in SSA approaching 100% and 60% respectively [98], and both may independently increase shedding of HIV RNA in co-infected people, and by extension both may also increase the risk of HIV acquisition.

Among the STIs causing genital ulceration STIs, HSV-2 merits special mention, due to its ability to establish lifelong infection, and its relatively high prevalence in sexually active adults in both SSA (60%) and in North America (20-30%) [111, 112]. Consequently, HSV-2 and HIV have a dramatic epidemiological overlap [113]. Epidemiological studies indicate that HSV-2 infection is associated with a three-fold increase in HIV acquisition within the general population [114], and a five-fold increase in the same within a high-risk sex worker cohort [115]. In HIVHSV-2 co-infected individuals, HSV-2 is associated with a two to five-fold increased chance of transmitting HIV [42].

Increased susceptibility to HIV acquisition is in part attributed to the disruption of the genital epithelial barrier by HSV-2 induced macro or micro-ulceration, which may enhance HIV crossing into the genital sub-mucosa. Additionally, HSV-2 infection may lead to localized inflammation and the recruitment of activated HIV target cells to the genital mucosa increasing
susceptibility to HIV [42]. Indeed, studies from our lab showed that HSV-2 infection was associated with a three-fold increase in cervical CCR5+CD4+ T cells, as well as a ten-fold increase in Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) expressing dendritic cells [116]. Increased secondary transmission of HIV in co-infected individuals may be driven by the ability of HSV-2 to induce HIV viral replication and shedding in the genital mucosa [117, 118], which in turn may increase HIV transmission events. Placebo controlled studies provide further support of the association between HSV-2 and HIV transmission by showing that suppression of HSV-2 using acyclovir treatment leads to a decrease in the frequency and level of HIV genital shedding, as well as reduced HIV viremia in HSV-2/HIV co-infected individuals [119].

Non-ulcerative STIs and other genital infections such as gonorrhoeae, chlamydia, trichomonas and bacterial vaginosis also pose a risk towards increasing susceptibility to HIV or its transmission [120]. Although not discussed in detail here, it is important to mention that these types of infections are also capable of inducing inflammation and having a similar impact on HIV transmission as HSV-2 [42]. Non-ulcerative genital infections also induce the recruitment of HIV target cells and/or the induction of HIV replication in co-infected individuals, which ultimately increases both the susceptibility of an individual towards acquiring HIV or the transmission of HIV in co-infected individuals [120]. In addition, some infections like trichomonas and bacterial vaginosis can increase the risk of vaginal bleeding by more than 12-fold further increasing the risk that HIV-transmission will occur during sexual activity [121].
These associations have provided a strong rationale for the evaluation of STI control as a HIV prevention strategy. With the exception of one study that managed to show a reduction in HIV transmission rates following STI treatment, results from other studies have so far been disappointing and showed that treatment of STIs does not reduce HIV transmission rates at a population level (reviewed in [122]). These discrepancies in findings might reflect the notion that STIs are a key driver of HIV transmission in the early phases of the epidemic, and might be more prevalent in core high-risk groups, while in the later phase of the HIV epidemic transmission occurs mainly within stable lower-risk groups [123]. Failure in HSV-2 therapeutic trials to show a reduction in HIV transmissions is attributed mainly to the persistence of HIV target cells in genital ulcerations long after healing of HSV-2 induced lesions [124]. Sub-optimal adherence/dosage may also have contributed to the results obtained in these studies [122]. In particular, one long-term HSV-2 suppression study using 400mg twice daily of acyclovir showed a modest (0.25 Log) reduction in HIV viral load in the HIV infected partner, and showed no efficacy in reducing HIV transmission in serodiscordant couples [125]. Other studies show that valacyclovir, a pro-drug of acyclovir, shows 3-5 fold greater bioavailability than acyclovir [126, 127], and that high dose valacyclovir (1500 mg instead of standard 500mg dose twice daily) can reduce HIV viral load by 1.23 log in another study [128]. Therefore, the type of drug used in earlier studies (mostly acyclovir or low dose acyclovir) may have led to conclusions that HSV-2 suppression does not alter HIV-transmission. Further studies are underway to see if high dose valacyclovir can prevent HIV transmission.
1.5.2.2 Other Common Infections in Africa as possible drivers of HIV Transmission

In addition to the relatively high prevalence of STIs in Sub-Saharan Africa, a huge burden of other parasitic and bacterial diseases also overlaps with the HIV epidemic in this region. These infections include malaria, tuberculosis and, helminthic infections: together they afflict almost a third of the world's population, and occur mostly in the developing world [129-131]. Infection with these parasitic and bacterial pathogens causes increased systemic immune activation, in the case of malaria and tuberculosis[132, 133], and the skewing towards a Th2-type (IL4-inducing) immune response in the case of helminthic infections [134]. This has in turn been associated with increases in HIV viremia in HIV co-infected individuals [134]. A recent systematic review evaluating the effect of tuberculosis, malaria and helminthic co-infection treatment on HIV showed decreases of >3log_{10} HIV DNA copies/ml following tuberculosis treatment, ~0.3 log_{10} HIV DNA copies/ml after malaria treatment and ~0.2 log_{10} HIV DNA copies/ml following geohelminth treatment [135]. As yet, no clinical studies have evaluated the effect of treating these co-infections on HIV susceptibility. However, there is some preliminary data on the effect of malaria on HIV transmission. Here, mathematical modelling suggests that changes in HIV plasma load may have a significant effect on the risk of viral transmission. A 1-log drop in viremia halves the risk of transmission, while a similar drop in viremia also delays the time to an AIDS defining event by 2 years [136]. Therefore, these chronic infections may considerably impact on the host immune system, and may increase the transmission of HIV from co-infected individuals.
1.5.2.3.1 Malaria (epidemiology and geographic distribution with HIV in Africa)

Approximately 300-500 million episodes of malaria occur each year [137], and 1.2 million deaths annually can be attributed to this parasitic infection [138]. Roughly half of the global population are at risk of malarial infection and approximately 90% of these infections occur in Africa [139]. Interestingly, the distributions of HIV and malaria overlap in many regions of the world, particularly in sub-Saharan Africa, where approximately 22.5 million are infected with HIV. In particular, the high level of geographical overlap translates into a high incidence of malarial and HIV co-infection, particularly in Zambia, Zimbabwe, Mozambique, Malawi and the Central African Republic where the HIV prevalence is over 10% and 90% of the population is regularly exposed to malaria [140]. Additionally, in countries such as Kenya where both infections are endemic, there is a high degree of geographical overlap [4, 141-143] (Figure 1.4). South Africa however, is an exception to this rule, as it has a very low malaria prevalence despite very high HIV rates. On an individual basis, limited information is available on the level of co-infection between these two diseases.
Figure 1.4: The geographic distribution of HIV and malaria (*plasmodium falciparum; PfPr*) in Kenya. The geographic distribution of HIV is illustrated by the regional means shaded from pale blue to purple, as well as by the size of each beige dot showing the prevalence from each HIV cluster survey. The size of the cluster dot indicates the HIV prevalence within each cluster survey. The geographical distribution of malaria in Kenya is indicated by the each dot from an individual cluster survey. The intensity of each dot (cluster) color indicates the prevalence of malaria (*plasmodium falciparum; PfPR*) from pink (0%) to red (100%).

1.5.2.3.2 Immune responses to Malaria Infection

Human malaria is most often caused by four different types of plasmodium parasites; *falciparum, malariae, ovale* and *vivax* [139]. Each one of these infections can cause severe illness, although, *P. falciparum* causes the most morbidity and mortality, and can also cause asymptomatic chronic infection for several years [139].
Host control of malarial infection is mediated by both the innate and adaptive immune systems [132]. Briefly, in response to parasites, dendritic cells and macrophages recognize malarial antigen through toll like receptors 2 and 9 (TLR 2, TLR9) or CD36 scavenger receptor on their surface, leading to their migration to the spleen, the primary site of host anti-malarial immune responses. Here, these innate cells mature and produce several cytokines including IL12, which in turn activates NK cells to produce IFNg. IFNg production results in the activation and differentiation of naïve CD4+ T cells into Th1 cells, which along with activated CD8+ T cells inhibit malarial parasite development in hepatocytes through IFNg release. Further, IFNg production activates macrophages in hepatocytes to produce more TNFa, more nitric oxide and to increase phagocytic clearance of malarial parasites [132]. Antibodies and compliment-mediated factors are also involved in mediating the clearance of malarial parasites [132]. In effect, malarial is a parasitic infection that induces the production of several pro-inflammatory cytokines, and that results in the increase of immune activation of blood lymphocytes.

1.5.2.3.3 Interactions Between Malaria and HIV

HIV and malaria interact in several different ways on both epidemiologic and molecular levels. HIV infected individuals, particularly during advanced HIV stages (AIDS) show a reduced ability to mount adequate anti-malarial immune responses. As a result of this negative interaction in areas where both infections are endemic, HIV infection has been estimated to increase cases of clinical and severe malaria by
1.3% (3 million cases), and to increase mortality following malarial co-infection by 4.9% (65,000 individuals) in Sub-Saharan Africa [144]. Furthermore, mathematical models based on data from co-infected individuals suggest that people living in high malaria-HIV holo-endemic regions are twice as likely to be HIV infected [145]. Another study estimated that in excess of 8500 new HIV infections occurred in Kisumu city, a western Kenyan community of only 200,000 individuals due to this interaction over a period of a decade [141].

Conversely, malaria may also impact on HIV infection, and this is mainly through induction of HIV replication. In a large prospective cohort of HIV-infected individuals in Malawi, malarial infection resulted in an almost 1 log increase in HIV viremia within individuals who had clinical symptoms of infection and high parasitemia [146]. However, this elevated HIV blood viral loads were normalized in participants 8-9 weeks after anti-malarial treatment was completed, suggesting that malarial infection was the driver of increased HIV viremia [146]. Similarly, several in vitro studies have shown that malaria increases HIV replication [147, 148].

The biological interaction link between HIV and malaria may lie in the activation of the immune system. Immune responses mounted to clear malarial infections results in activation of several components of the innate and adaptive immune system [132]. TNFa is one of the key cytokines produced following infection by malaria; and during HIV infection, the same cytokine has been implicated in inducing viral replication by activating viral LTR sequences [132, 149]. Indeed, in vitro monoclonal antibody blocking of malarial antigen-induced TNFa cytokine can block malarial-parasite induced HIV viral replication [147].
The effect of malaria on HIV susceptibility in adults is unknown. However, malaria is thought to increase the likelihood of HIV acquisition in HIV-uninfected infants through elevation of systemic immune activation. Here, in vitro studies have demonstrated that cord blood cells obtained from in utero malaria-exposed babies expressed higher levels of the immune activation marker HLA-DR, and were preferentially infected by HIV in culture [150]. Similarly, another study demonstrated a 3-fold increase in mRNA expression of the HIV entry co-receptor CCR5 in the placenta of malaria-infected women compared to uninfected participants [151]. In accordance with these findings in pregnant women, neonates were shown in another study to be approximately three times more likely to acquire HIV (relative risk 2.9) if their mothers had a placental malarial infection [152]. Nonetheless, it remains unknown whether malaria might influence heterosexual acquisition of HIV, which is the predominant means of HIV acquisition in high HIV-malarial regions.

1.5.3 HIV Immune Susceptibility Markers

The likelihood of productive HIV infection may not just be a stochastic event after one is exposed. Several lines of evidence suggest that several immune parameters may play a role in the preferential infection of particular target cells by HIV; these include the expression of particular immune markers on the surface of cells, the differentiation subset of target cells, and the activation state of particular cell types [153]. In this section we discuss known surface markers and
differentiation phenotypes that may increase susceptibility to HIV or propagate productive HIV infection and disease progression.

1.5.3.1 CCR5

As discussed in previous sections, expression of CCR5 might predispose HIV target cells to infection. The best evidence implicating CCR5 as a susceptibility marker is obtained from individuals who do not express the CCR5 receptor on the cell surface following a base-pair deletion at allele marker 32 (CCR5-Δ32) [154]. Individuals with a homozygous CCR5-Δ32 mutation demonstrate near-absolute protection from HIV R5 strain infection [154], and to date the only demonstrated functional ‘cure’ for HIV has been observed in ‘The Berlin patient’ who had a stem cell transplantation from a donor with the homozygous CCR5-Δ32 mutation [155]. Conversely, the presence of sexually transmitted genital infections induces T cell activation and increased CCR5 expression on immune cells and results in increased susceptibility to HIV [156-158]. Therefore, increased CCR5 expression on HIV target cells may significantly increase ones susceptibility to HIV infection.

1.5.3.2 α4β7

Alpha-4, beta-7 (α4β7) integrin is expressed on lymphocytes and facilitates their migration from gut-inductive sites, where initial immune responses are first induced, to the gut lamina propria. More recently, α4β7 positive cells have been observed at the genital mucosa [24] suggesting that α4β7 might allow for broader
targeting of immune cells to other mucosal sites. $\alpha 4 \beta 7$ migration to mucosal tissues is mediated by its natural ligands MADCAM1, VCAM and fibronectin [159].

Arthos et al demonstrated that gp120 viral envelope could still bind to CD4+ T cells even in the presence of monoclonal antibody bound to CD4 surface molecule [21]. This attachment of viral gp120 could be abrogated by MADCAM1 and VCAM immunoglobulin protein implying that the virus envelope protein was binding to $\alpha 4 \beta 7$ molecules on CD4+ T cells. Binding of viral envelope to $\alpha 4 \beta 7$ surface molecules is possible due to the resemblance of the gp120 V2 loop to the natural $\alpha 4 \beta 7$ ligands binding conformation [21]. Therefore, the net effect of $\alpha 4 \beta 7$ expression on T cells would be to bring the virus and target cell into constant close proximity, making it more likely that infection would occur. Indeed, non-human primate studies show that $\alpha 4 \beta 7^+$ CD4+ T cells are predominantly infected during acute infection and contain approximately 5-times more SIV-gag DNA than $\alpha 4 \beta 7^-$ CD4+ T cells [160]. Human studies also corroborate this finding and show that $\alpha 4 \beta 7^{\text{high}}$ CD4+ T cells have more HIV infection compared to $\alpha 4 \beta 7^{\text{low/negative}}$ CD4+ T cells [161]. Furthermore, $\alpha 4 \beta 7$ cells express higher levels of CCR5 both in vitro in peripheral blood mononuclear cells [161], and in vivo in cervical mucosal T cells [24], than $\alpha 4 \beta 7^-$ CD4+ T cells, which may further enhance the susceptibility of these cells to HIV infection at mucosal sites of exposure [153].

Finally, $\alpha 4 \beta 7$ integrin is part of a multi-step adhesion cascade that also upregulates lymphocyte function-associated antigen 1 (LFA-1) on CD4+ T cells. This cascade allows for the extravasation of leukocytes into mucosal tissue [162]. LFA-1 is also critical in inducing the formation of immunological synapses [163].
Engagement of $\alpha 4\beta 7$ results in the activation of LFA-1 on CD4+ T cells and has been shown to enhance the efficiency of viral infection by promoting synapse formation [21]. Therefore, not only does $\alpha 4\beta 7$ predispose CD4+ T cells to initial HIV infection, but also its induction of synapse inducing LFA-1 may enhance viral propagation via cell-to-cell synapses [21].

1.5.3.3 Th17 and Th22 CD4+ T cell Subsets

CD4+ T cells differentiate into several different T cell subtypes including Th1, Th2, Th17 and Th22. Susceptibility to HIV infection is heterogeneous amongst CD4+ T cell subsets. Studies from non-human primates that develop pathogenic disease (AIDS) show that gut Th17 CD4+ T cell subsets are preferentially depleted in SIV infection while gut Th1 are not significantly depleted during acute infection. This contrasts with non-pathogenic SIV infection of primates such as sooty mangabeys and African green monkeys who are naturally infected by SIV in the wild (natural hosts). SIV infection in natural hosts is absent of preferential depletion of Th17 CD4+ subsets, and SIV infection is not pathogenic or lethal [164].

Th17 cells can be characterized by their surface expression of CCR6+CCR4+ markers and defined by the production of the cytokine IL17, while Th1 cells are mostly CXCR3+ and produce IFNg [165]. In vitro experiments suggest that HIV preferentially infects CCR6+ Th17 cells demonstrating that these Th17 might be more susceptible to HIV [166, 167]. More evidence on the same is provided by in vivo primate experiments that show that the blockade of the CCR6 ligand (CCL20)
reduces recruitment of target cells to the genital tract, and can protect macaques from an otherwise lethal SIV challenge [43].

Th17 (IL17+) CD4+ T cells are also capable of producing the cytokine IL22, as are Th22 cells (IL22+IL17-IFNg-) cells. Th22 cells are a fairly new defined CD4+ T cell subset and are responsible for maintaining gut wall integrity by promoting epithelial cell homeostasis and regeneration [168]. Following infection, emerging studies from our lab show that both Th17 and Th22 CD4+ subsets are preferentially depleted in the genital and gut mucosa, while normal Th1 cell frequencies are maintained [24, 169]. Therefore, it can be concluded that the presence of Th17 cells at the mucosal site of exposure may increase susceptibility to HIV. Further discussion on the role of Th17 and their preferential depletion during HIV infection is provided in section 1.6.3.2.

1.5.3.4 Surface Immune Activation Markers (CD38, HLA-DR)

Immune activated T cells can easily be identified by their expression of various surface immune phenotypic markers such as CD38, HLA-DR and CD69 [20]. During HIV infection, a clear link has been established between immune activation (CD38 and HLA-DR lymphocyte expression) and faster disease progression [170, 171], as well as immune activation and increased HIV replication (section 1.3.2.1). Evidence also points to an association of immune activation and increased HIV susceptibility from studies examining the expression of these immune activation markers in HIV exposed-uninfected individuals. Here, cross-sectional studies show that these individuals have a relatively reduced expression of HLA-DR, CD38 and CD69 surface expression in comparison to HIV-unexposed cohorts [172, 173].
Further, a retrospective analysis of samples from a high-risk cohort of men who have sex with men in Amsterdam demonstrated that individuals who went on to acquire HIV expressed higher levels of HLA-DR and CD38 immune activation markers on both CD4+ and CD8+ T lymphocytes than those who remained uninfected [174]. (HIV exposed-uninfected individuals are separately discussed in more detail in the next section; 1.5.5). These results imply that lower T cell immune activation results in reduced susceptibility to HIV infection.

1.5.4 Host Genetics

Susceptibility to HIV infection has also been associated with a number of genetic influenced factors [175]. The most striking is the CCR5-Δ32 mutation. A 32-base pair deletion in the promoter and coding the CCR5 gene results in a frame-shift mutation and eventual truncation of gene transcription by a premature stop codon. The net effect is the production of a truncated non-functional CCR5 protein and the absence of CCR5 expression on the cell surface [154]. The significance of the absence of CCR5 expression is discussed in section 1.5.3.1. About 1% of individuals of European descent are homozygous, and 10% of the same individuals are heterozygous for the CCR5-Δ32 mutation. However, this mutation is less common in non-Europeans [176].

Several other polymorphisms have been linked to decreased susceptibility in several cross-sectional and prospective cohorts including MCP-1-2578G, CCL3, HLA A2/6802, HLA A0205/6802, IRF-1-619A/179 genes [177]. However, unlike the
CCR5-Δ32 mutation, they have failed to demonstrate an ability to entirely protect against HIV acquisition.

1.5.5 HIV Exposed but Seronegative (HESN) Individuals

1.5.5.1 Historical perspectives

Varying susceptibility to infectious disease has been noted repeatedly throughout history. A classical example is that of Edward Jenner’s milkmaids, who remained uninfected by smallpox having previously been occupationally exposed to cowpox. Similarly, there is evidence that some individuals have reduced susceptibility to HIV, since they remain uninfected despite repeated exposure to the virus. These individuals are collectively referred to as HIV exposed, but seronegative (HESN). This phenomenon has been observed in several different settings including: homosexual men exposed to HIV-1 infected partners, uninfected children born to HIV-1 infected mothers, occupationally exposed health care workers and uninfected heterosexual partners of HIV infected individuals [178].

Obviously, the definitive experiment proving that HIV exposure can occur without seroconversion cannot be done in humans. However, non-human primate studies suggest that HIV exposure without seroconversion is possible, and that protection may be immune mediated [179]. As part of a virus titration experiment, Clerici et al inoculated macaques with low-dose intravenous SIV (0.1 or 0.001 animal infectious doses [AID]) [179]. With the exception of 2 animals, all animals in this experiment were successfully infected as confirmed via PCR and viral isolation,
and succumbed to infection during follow up. The two animals that did not get infected with SIV were later ‘recycled’ and received a higher dose (100 or 10 AIDS) of SIV via intra-rectal challenge in another set of experiments 16 months later, along with another set of primates. Within 4 weeks of infection, previously non-exposed macaques had confirmed SIV infection via PCR and developed AIDS within 80 weeks. Interestingly however, ‘recycled’ macaques only had transient detectable viral blips both via PCR and viral isolation, which later became undetectable, and the animals did not develop AIDS. Although this study was not specifically designed to explore the phenomenon of exposure without infection, the authors concluded from this pilot data that exposure to SIV without productive infection is possible [179].

There is also epidemiological evidence that HIV exposure without seroconversion can occur [180]. In a high-risk commercial sex worker cohort (CSW) in the Majengo-Pumwani area of Nairobi Kenya where a portion of my thesis research was conducted, it was estimated that some female HESN CSWs had repeated unprotected exposures to HIV in a year and several hundred exposures over a decade of prospective follow up [180]. In this cohort, most female CSWs were already HIV-infected at study enrolment, but approximately 80-85% of the remaining women acquired HIV within 3 years of cohort enrolment. A small number (approximately 25% of those HIV negative women) remained uninfected, and for each year that a woman remained uninfected despite engaging in CSW, there was a stepwise decrease in their chances of seroconversion of 1.2 fold/year [180]. It was from these findings that the operational definition of HIV ‘resistance’ was coined. Here, resistant individuals; (i) must be followed up for a period of greater than three
years in the Pumwani cohort and be actively engaged in active sex work, (ii) must be HIV-1 negative by HIV-1 serology and PCR, and (iii) must remain in general good health and have normal CD4 counts. This study is now almost two decades old, and the terminology of ‘HIV-resistant’ may not be entirely true as later studies have shown that some of these women do eventually go on to acquire infection, suggesting that not all women who meet this definition are ‘absolutely’ resistant to HIV infection [181]. In addition, a majority of these women may already be engaged in sex work for several years (and are perhaps already ‘relative-resistant’ to HIV) by the time they are enrolled into the large prospective cohort. Therefore, it is arguably more accurate to describe these women as simply high-risk HESN individuals rather than by their duration of follow up in the cohort.

1.5.5.2 Correlates of Immune Protection

The identification of HESN individuals within CSW populations has generated intense interest in elucidating potential correlates of immune protection, particularly because knowledge gained here would be critical towards informing HIV vaccine or protective microbicide design. In the Majengo CSW cohort, initial studies asked whether it was by chance alone that these women remained uninfected, or whether these women engaged in lower risk behaviors that put them at a reduced chance of acquiring HIV. Mathematical modeling showed that if all women were at equal risk of acquiring HIV, then if followed long enough, they should all get infected [180]. However, even after 16 years of follow up some
women still had not acquired HIV [180, 181]. From standardized questionnaires, HESN CSWs were also noted to engage in as much high-risk behavioral activities as those who went on to acquire HIV [180]. Studies in our lab also showed that there was a high prevalence of sexually transmitted infections, such as genital herpes, in these female CSWs; providing clinical proof of their high-risk taking behavior [182]. Therefore, reduced risk-taking behavior is not responsible for the HESN phenotype, and it is plausible that biological mechanisms may mediate protection from HIV acquisition in HESN individuals.

1.5.5.2.1 Innate Correlates of Protection

Several reports have suggested a role of innate immunity in mediating the HESN phenotype. A proteomics approach that screened vaginal mucosal samples from HESN CSWs identified several cationic proteins including the anti-leukoprotease secretory leukocyte protease inhibitor (SLPI), trappin-2, lactoferrin and several other proteins not previously described, which were collectively able to inhibit viral infection in vitro [183, 184]. How these proteins work is not entirely clear, but they are proposed to inhibit the early steps of HIV infection and/or integration in the host cell genome [185]. The innate antiviral protein, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex (APOBEC), has the ability to prevent HIV proviral integration and reduce viral fitness by introducing hypermutations within the proviral genome [186]. Recent reports indicate that elevated levels of APOBEC3G are present in monocyte/macrophages
from HESN individuals suggesting that this endogenous antiviral factor could play a role in protecting HESN individuals from HIV acquisition [187]. Finally, increased expression of the activating killer inhibitory receptor 3DS1 (KIR3DS1), and a corresponding increase in natural killer (NK) cell activity has also been observed in other HESN cohorts suggesting that these cells also play a role in mediating protection against HIV [188, 189].

1.5.5.2.2 CD8 Correlates of Protection

HIV specific CD8+ T cell responses are perhaps the single best characterized immune response in HESN individuals besides CCR5-Δ32 [190]. HIV-specific immune responses have been described in the blood compartment of Pumwani female CSWs, injecting drug users, and in discordant heterosexual couples [191-196]. In these studies, HIV-specific IFNg, TNFa and IL2 cytokines production as well as proliferative immune responses were detected in HESN individuals, and their CTLs produce elevated levels of perforin and granzyme (CTL lysis effector molecules) compared to HIV infected individuals [197] [196, 198]. In addition, HESN CTLs appear to recognize distinct epitopes from those recognized by individuals already infected with HIV [197] [198] suggesting that protective immune responses may be distinct from those required to control viral production once infection is established.

HIV-specific CD8+ T cell responses are also present in the cervix of HESN women, and they recognize similar epitopes as those in blood indicating that
immune responses observed in blood are reflective of those at the mucosa [191, 199]. Furthermore, within HESN CSWs, genital mucosal immune responses are present at a higher frequency than in blood, implying that stronger CD8 immune protection at the site of exposure may be required to maintain protection from HIV at the genital mucosa following heterosexual exposure [191]. Nonetheless, cervical HIV-specific immune responses in HESN women are still ‘weaker’ than those in HIV-infected individuals and are found at approximately 10 fold lower frequencies than those in HIV-specific individuals [192].

1.5.5.2.3 CD4+ T cell Correlates of Protection

Generally, CD4+ T cells are capable of producing antiviral cytokines such as IFNg, and are vital for the maturation and functional activity of CD8 T cells. However, the role of HIV specific CD4 responses in controlling or preventing HIV infection remains unclear given that activated CD4 cells are the primary targets for viral infection. Nonetheless, increased frequencies of CD4+ T cells have been reported in the genital mucosa of HESN CSWs compared to unexposed controls [200]. Additionally, HESN CD4+ T cells have been demonstrated to produce IL-2 and proliferate after stimulation with HIV peptides [201, 202]. Further, HIV-specific CD4+ T cells have been shown to produce high levels of β-chemokines, in particular RANTES and MIP-1b, and are capable of suppressing R5 HIV-strains in vitro [203, 204]. As such, HIV-specific CD4+ T cells in HESN individuals may mediate protection from HIV infection by supporting HIV-specific CTLs maturation and function, and
may themselves block viral entry through the production of β-chemokines, which bind to and block access to the CCR5 HIV entry co-receptor [177, 203].

1.5.5.2.4 Humoral Correlates of Protection

Two types of humoral immune responses have been reported in HESN individuals so far: antibodies to cellular and viral proteins involved in the HIV infection/entry process, and HIV-specific and/or non-HIV-specific neutralizing IgA mucosal antibody immune responses [177]. In order to confer sterile immunity similar to that observed in HESNs, it is likely that that the latter type of humoral immune response are required, whereby antibodies protecting against sexual HIV acquisition are located at the genital mucosa. Indeed, IgA neutralizing antibodies, which are exclusive to mucosal tissues, have been identified in the genital mucosa of HESN individuals [205-207]. Neutralizing antibodies are thought to confer protection by binding and neutralizing cell free virus on the mucosal surface thus preventing the virus from crossing the genital tract epithelial barrier where it can infect target cells [208].

Cellular-mediated immune correlates of protection in HESN, fundamentally suggest that HIV has already managed to infect the host, and cellular immune mechanisms contained it. Induction of humoral immunity on the other hand would be more preferable as this may confer sterile protection, whereby the infection is prevented before it happens, and the virus has the least chance of immune escape.
1.5.5.3 Role of Continuous Exposure and Immune Correlates of Protection

The likelihood of detecting HIV-specific cell-mediated immune responses increases with the duration of continuous HIV exposure and decreases in the absence of it. For instance prospective cohort studies indicate that there is a stepwise incremental chance of detecting HIV-specific CTLs in HESN CSWs with increasing years of continued sex work (and presumably continued HIV exposure) [192]. Conversely, in the absence of HIV exposure during CSW breaks, decreases in CTL responses have also been demonstrated, which in turn are associated with HIV acquisition [181, 192]. Similarly, HIV-specific immune responses in CD4+ T cells are more likely in recently exposed but uninfected individuals [195], and these responses also tend to wane after cessation of exposure [209, 210]. In the same context, HIV-specific IgA titers were significantly diminished in HESN women who underwent counseling and adopted safe-sex procedures [211]. These findings suggest that the maintenance of immune memory is perhaps required in order to keep the HESN phenotype.

1.5.5.4 Role of Immune Quiescence in HIV Protection

Several HIV-specific immune and genetic mechanisms have been put forth to explain why some individuals in the general population may remain uninfected despite repeated exposure, and they primarily revolve around the need to have HIV-specific immune responses to ward infection off. Immune quiescence is an
alternative hypothesis on how HIV exposed individuals may remain uninfected despite repeated exposure. This hypothesis proposes that the immune system in HESN individuals generally remains in a ‘resting’ or quiescent state upon exposure, which may be the key to remaining uninfected [173, 212].

Several groups have reported an association between decreased T cell activation and reduced HIV susceptibility in the Pumwani CSW cohort [173, 213], CSWs from Co’té d’Ivoire [214], men who have sex with men in Amsterdam [174], and discordant couples in the Central African Republic [172]. Indeed, unstimulated blood from HESN individuals with lower immune activation has been demonstrated to correspond with reduced in vitro susceptibility to HIV in one study[172]. However, this relatively reduced susceptibility to HIV infection was lost after mitogenic activation of the same samples in culture [172]. Additionally, a retrospective study in high-risk HESNs further demonstrated that individuals who went on to get infected had higher immune activation in their blood compared to those that remained uninfected implying that the state of reduced immune activation is associated with remaining uninfected [174]. Surprisingly however, none of these studies have established whether immune quiescence extends to the genital mucosa, which would probably be the first site for host-virus interaction following heterosexual exposure.

Immune quiescence is proposed to maintain the HESN phenotype by reducing cellular activation and gene transcription required for HIV replication, and therefore resulting in a reduced pool of susceptible CD4+ T cells [173, 212]. However, in the unlikely event of infection, the reduced substrate of target cells for HIV will facilitate
very limited localized infections, which may allow enough time for HIV-specific immune responses (previously reported in HESNs) to quickly contain and clear the infection [173, 212] Figure 1.5.

* © Duncan Chege. Figure adapted from Cohen et al., Science 2009. 326: 1476-7

**Figure 1.5: The immune quiescence hypothesis of reduced susceptibility to HIV.** Activated HIV target cells may offer more target for HIV upon exposure and increase the chances of productive infection (top figure). Cellular immune quiescence in the genital mucosa may result in a reduced pool of susceptible CD4+ T cells after initial exposure, reducing HIV acquisition risk (lower figure).

1.5.5.5 Issues and Confounders in HESN Studies

Elucidating mucosal immune correlates of protection is an important focus of HIV vaccine and microbicide research. Studying individuals at a high risk of HIV infection, particularly commercial sex workers, confers the best opportunity to identify these correlates. Identifying the right controls for such studies is
problematic due to the fact that usually low-risk individuals (non-CSWs) do not permit control for the effects of sex work [98, 178]. Sex work may repeatedly induce micro abrasions, localized inflammation, not to mention the effects of seminal plasma itself, which has known immuno-modulatory activity. To properly control for the effects of sex work one would need to enrol a control group of CSWs who are not HESNs. Identifying such a group of individuals is extremely difficult to achieve as HIV has a poor transmission rate (<1%), which means by chance alone, most people are somewhat resistant; this doesn’t mean however that they are ‘true HESNs’.

Distinguishing between a ‘true’ HESN CSW vs. a ‘susceptible’ CSW would require one to predict those that will acquire infection (susceptible) vs. those that will remain uninfected. Thus, it can be argued that there are no ideal/feasible control subjects in such studies [98, 178].

HESN individuals are typically defined by repeated unprotected HIV sexual exposure without infection. This naturally corresponds with a higher prevalence and incidence of other STIs [182]. Therefore, STIs are a potential confounder in HESN CSW studies, as they are known modulators of the genital immune milieu (discussed in section 1.5.2.1), and may confound ‘true’ mucosal protective immune parameters in such individuals.

Finally, identifying ‘truly’ resistant individuals and their associated correlates of protection may be confounded in cross-sectional studies. Again, much less than 1% of genital exposures result in productive infection, as previously discussed. Therefore, chance alone may explain low-level HIV exposure without infection.

Indeed it has been illustrated that some individuals previously described as having
the HESN protective phenotype do go on to acquire HIV infection despite having pre-existing immune responses [181]. This however does not disprove the HESN phenomenon altogether. Perhaps the best way to control for this confounder in order to identify the ‘true’ correlates of protection would be to conduct prospective studies, where the end point is HIV acquisition [98].

### 1.6 STAGES OF HIV INFECTION

#### 1.6.1 Acute HIV Infection

Acute HIV infection is characterized by an exponential rise in viremia and is typically defined as the first 21 days after infection [215]. Within the first few hours after exposure, cell free or cell-associated virus cross the mucosal barrier into the lamina propria and establishes infection in a small population of target mucosal cells (discussed in section 1.4.2) [39]. Macaque animal model studies of HIV suggest that ‘resting’ CD4+ T cells are predominantly infected following initial vaginal exposure to SIV [57]. This finding is somewhat surprising given that in culture, resting cells cannot be productively infected [26, 216]. However, this statistic may be more of a consequence of the fact that there are seven times as many ‘resting’ cells as activated cells and they similarly outnumber dendritic cells and macrophages [57, 217]. Furthermore, although conventional flow cytometry markers suggest that most of these infected cells are at ‘rest’, they actively allow viral replication and have much higher concentrations of CCR5 mRNA than
uninfected naïve T cells, hence some argue that these mucosal infected cells may not truly be at rest [218].

To fully establish infection, antigen presenting cells carrying virus migrate to proximal lymph nodes within 2 days, where they disseminate virus to surrounding CD4 T cells across the follicular dendritic cell network [219]. Within 5-7 days, the abundant availability of target cells allows the virus to expand exponentially, peaking at >10^7 viral RNA copies/ml by 21 days post infection [2, 220].

![Graph showing the phases of HIV/AIDS and immune responses](image)

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**Figure 1.6:** The phases of HIV/AIDS and the magnitude of immune responses and viral levels throughout infection.
The rise in viremia coincides with several immune changes in the host. During the second week of acute infection, CD8+ T cell populations begin to increase and their peak corresponds to a decline in overall CD4+ T cell frequencies in blood [2, 20]. By the end of the second week of acute infection, seroconversion occurs and allows the early detection of anti-HIV antibodies. These antibodies however, appear to have little added effect in lowering viremia levels [2, 20]; Figure 1.6.

1.6.2 Chronic HIV infection and AIDS

The chronic phase of HIV infection is typically defined as the period between 3 months - 10 years following infection and at this stage HIV is largely asymptomatic. During this period blood CD4+ T cell levels initially return to near-normal levels after acute infection, and then begin to decrease steadily at the rate of about 25-60 cells/ul per year throughout chronic progressive infection [221]. In addition, following acute infection, resulting antiviral immune responses (discussed separately in section 1.7) mediate the reduction of peak viremia levels to a lower viral set-point level, where viral replication is largely maintained.

Within 2-10 years of infection, individuals may begin to acquire opportunistic infections after their CD4+ T cell levels decrease significantly. This symptomatic phase or drop in CD4+ T cells below 200 counts/ul is referred to as AIDS [2]; Figure 1.6. During this stage, severe immune dysfunction emerges following destruction of the lymph node follicular network that results in the poor priming of immune responses [2]. A switch in antiviral CD4+ Th1-type to ineffective Th2-type immune responses may occur, and the loss of killing activity in HIV
antiviral CD8+ T cells further exacerbate this dysfunction. Concomitant with this loss in antiviral immune function during AIDS is the unchecked rise in HIV viremia, and a switch in the predominant viral strain from X5 to a more virulent X4 strain in some individuals [2]. If left untreated, the resulting severe immunodeficiency will eventually result in death, generally from overwhelming opportunistic infections [2].

1.6.3 Chronic HIV infection and the Gastrointestinal Mucosa

1.6.3.1 Gut CD4 Depletion During Acute Infection

The gastrointestinal mucosa (gut) is a unique structural and immunological organ that serves as a barrier against foreign pathological microorganisms, while allowing for continuous absorption of nutrients across its surface. The gut, houses 50-80% of the body’s lymphocytes and the early events during HIV infection here set the stage for progressive immune dysfunction [222]. During acute infection, almost 90% of gut cells are depleted within two weeks of infection [223-226]. Interestingly, this profound depletion of gut CD4+ T cells occurs irrespective of the route of HIV infection [224, 227], and continues gradually thereafter throughout the chronic phase of infection.

The cause of preferential gut CD4+ T cell depletion during acute infection is unknown. However, several mechanisms have been put forth to explain the profound depletion of CD4+ T cells in the gut mucosa. First, the high microbial load in the gut leads to the maturation and enrichment for activated memory immune
cells at this site [224]. As HIV preferentially replicates in activated CD4+ T cells, the higher expression of the HIV co-receptor and activation marker, CCR5, and other activation markers (CD69, CD38) in the gut, relative to blood, may drive the increased viral entry, replication and depletion at the gut mucosa. Increased virus replication in turn is associated with induction of CD4+ T cell apoptosis through multiple direct mechanisms including: increased induction of Fas-Fas ligand death receptors, HIV-induced cell membrane degeneration and subsequent cell apoptosis, as well as direct immune lysis of infected cells. Indirect mechanisms such as activation induced cell death (AICD) of naïve CD4+ T cells, which are adjacent to infected cells, may also contribute significantly to CD4 cell depletion [224, 228].

1.6.3.2 Preferential Depletion of Th17 & Th22 CD4+ Subsets in the gut

Maintenance and restoration of the gut structural integrity is dependent on the local immune system. Lymphocyte cells in the gut expressing the retinoic acid orphan receptor (ROR)γt are important both for maintaining the structural barrier in the gut and directing the defense against microbial pathogens [229]. RORγt+ cells produce IL17 and IL22 immune proteins that recruit neutrophils to areas of bacterial infection where they kill invading pathogens. In addition, these cytokines induce the proliferation, homeostatic maintenance and repair of enterocytes, as well as stimulate the production of antimicrobial defensins from epithelial cells [230-232]. Although several different types of RORγt+ immune cells are capable of producing IL17 and IL22, CD4+ Th17 cells are the major producers of these immune proteins. However, plasticity in cytokine production exists, and some Th17 cells may
simultaneously secrete IL22, and IFNg as well as a host of other cytokines in addition to IL17 [230].

HIV infection has heterogeneous effects on different CD4+ T cell subsets. In both rhesus and humans high SIV/HIV viremia during chronic infection is associated with significant decreases in gut Th17 (IL17+CD4+) but not Th1 (IFNg+CD4+) CD4+ T cell subsets [164, 233]. Similarly, studies from our group indicate that there is a preferential depletion of IL22 cells in the genital mucosa during HIV infection [24, 169]. This suggests that Th17/Th22 cells are preferential targets for depletion in the gut mucosa in vivo. In keeping with this hypothesis, Th17 cells, identified by expression of CCR6 chemokine receptor, have been shown to harbour more integrated HIV proviral DNA than CCR6-negative cells [166].

The mechanism underlying the heterogeneous infection of depletion of CD4 T cell subsets is unknown. However, Th17 cells in the blood, cervical and gut mucosa express higher levels of putative HIV susceptibility markers such as α4β7 and CCR5 [24, 160], which might make them more permissive to infection. Nonetheless, not all studies find that Th17 cells are preferentially infected by HIV [164], and this debate remains unresolved. Nevertheless, loss of Th17/Th22 but not Th1 T cells in the gut and genital mucosa during chronic is a consistent finding [24, 164, 233].
1.6.3.4 HIV Disease Progression: Linking The Gut, Microbial Translocation and Immune activation

HIV itself is an obvious culprit for the induction of immune activation following infection. Indeed, either the virus itself or viral proteins cause activation of innate antigen presenting cells through Toll-Like receptors and result in proinflammatory cytokine and chemokine secretion [234]. Additionally, it is well described in the literature that HIV induces HIV-specific adaptive immune responses resulting in activation of B-cells and T lymphocytes. However, individuals who can suppress virus to levels below the limit of detection of conventional assays (elite controllers) still demonstrate elevated immune activation compared to HIV-uninfected controls, suggesting that more than just the virus induces immune activation [235]. Furthermore, individuals who receive completely suppressive prolonged HIV antiretroviral therapy still show increased immune activation compared to uninfected individuals [236]. Therefore, additional drivers of immune activation exist.

Inflammatory bowel disease (IBD) offers some useful insight on other potential drivers of immune activation. IBD is an autoimmune disease of the gut that results in severe damage of the mucosal wall, leading to the development of a 'leaky' gut, permitting microbes to translocate across the gut wall and into systemic circulation; this is known as microbial translocation [237]. Microbial translocation directly results in activation of the immune system, which attempts to control the influx of foreign microbes into systemic circulation.
Similarly, during HIV infection, microbial translocation may contribute to increased immune activation. Following HIV infection, a profound depletion of the gut CD4 lymphocyte immune protective barrier occurs. Additionally, gene expression profiles from gut tissue indicate that genes associated with cell cycle regulation, lipid metabolism, epithelial cell barrier and digestive functions are downregulated in chronic HIV infection [238]. Ultimately, this leads to increased intestinal permeability and subsequent microbial translocation. In turn, microbial translocation directly results in increased activation of the immune system [239].

Additional, animal models of HIV infection demonstrate that antibiotic treatment reduces elevated microbial levels in rhesus macaques following SIV infection, while antiretroviral mediated viral suppression decreases levels of microbial markers in plasma [239]. Taken collectively, the above findings suggest a link between HIV infection, microbial translocation and systemic immune activation. However, to date, there is a lack of empirical evidence showing the directional relationship between these associations; is the virus driving immune activation, or is immune activation driving virus replication or both? [240].

1.6.3.5 Consequences of Immune Activation during HIV Infection

Studies in non-human primates perhaps give us the best opportunity to understand the consequence of immune activation on HIV pathogenesis. SIVsm infection can occur in both its natural hosts such as Sooty Mangabeys, or in non-natural hosts such as rhesus macaque monkeys. Acute SIV infection leads to a early, severe and irreversible depletion of gut mucosal CD4+ T cells and the induction of
SIV-specific immunity in both monkey species. However, a key distinguishing aspect between infection of these two types of SIV hosts is the fact that chronic SIV infection in sooty mangabeys is non-pathogenic, and does not lead to AIDS. In these natural hosts of SIV near normal systemic CD4+ T cell counts are present throughout infection and low levels of immune activation are maintained [241]. Interestingly however, throughout SIV infection Sooty Mangabeys maintain high viremia, suggesting that viral infection in itself is not sufficient to induce pathogenic infection. In contrast, chronic SIV infection in rhesus macaques is characterized by a reduction in peak viremia after acute SIV infection. However, the resulting chronic phase of infection is pathogenic, and is coupled with chronic immune activation that eventually leads to AIDS in rhesus macaques. Taken collectively, the above findings in non-human primates suggest that although depletion of gut CD4+ T cells resulting viral infection occurs following infection, SIV/HIV pathogenesis may largely be driven by chronic immune activation [241].

In humans, it can be argued that immune activation temporarily has some beneficial consequences such as inducing T cell proliferation and partial restoration for memory CD4+ T cells. However, several studies have linked immune activation with detrimental outcomes during HIV infection. In one prospective cohort study of individuals with less than 50 CD4+ T cells counts/ul, expression of CD38 - a late stage lymphocyte activation marker, on both CD4+ and CD8+ T cells - was associated with shorter subsequent survival [170]. In fact, in several studies immune activation has been identified as a better prognostic marker for disease progression/outcome than either systemic CD4+ T cell counts or viremia [170, 242, 243].
The contribution of microbial translocation towards immune activation is profound. Microbial translocation into systemic circulation (blood) can be measured by several methods including the use of lipopolysaccharide (LPS – component of gram-negative bacterial cell walls) or soluble CD14 (sCD14, an adhesion molecule released by macrophages to bind LPS and help activate TLR immune signalling) [229, 240]. Both LPS and sCD14 have been shown to correlate positively with immune activation in blood during HIV infection [239]. In addition, use of antibiotics to treat microbial translocation in an SIV macaque animal model has been shown to reverse increased systemic immune activation levels during the course of chronic SIV infection [239]. Therefore in addition to HIV itself, microbial translocation also leads to increased immune activation [229, 240].

Primarily, immune activation leads to the proliferation and generation of activated CD4+ T cell targets that further increase the pool of infected cells and viral replication. In addition, immune activation leads to a high turnover of T lymphocytes posing a severe strain on T cell homeostatic mechanisms leading to clonal exhaustion, and an overall decrease in T cell half-life [228, 240].

Production of inflammatory cytokines by activated immune and epithelial cells may also further exacerbate gut ‘leakiness’ [244]. Proinflammatory cytokines, such as TNF, in the gut activate the myosin light chain kinase (MLCK) leading to the cytoskeletal rearrangement of tight junction proteins such as zonula occludens 1 (ZO-1) and occludin, which decreases the gut wall integrity [245, 246]. Furthermore, TNF increases apoptosis of epithelial cells, which can lead to increased porousness of the gut mucosa [247]. Alternatively, production of anti-inflammatory TGF-b
mediates fibrosis of lymph nodes, which is in turn associated with architectural
damage to the lymph nodes and the abnormal retention of effector T cells [226, 248,
249]. This damage eventually leads to decreased T cell regeneration in the lymph
nodes and a diminished capacity to resupply CD4+ T cells to mucosal surfaces. CD4+
T cells that do migrate to mucosal sites or proliferate therein become targets for
direct HIV infection and depletion thereby driving disease progression [240].
Therefore immune activation during the course of HIV infection fuels a vicious
negative cycle of viral replication, T cell infection and depletion and immune
dysfunction which eventually drives disease progression.

1.6.3.6 The latent Gut HIV Proviral DNA Reservoir

Circulating HIV virions have a half-life of only a few hours, thus levels of virus
are reflective of the pool of virus-producing cells [250]. Following acute infection,
host immune responses are responsible for reducing viremia to a stable set point
until advanced disease or initiation of antiretroviral therapy. Antiretroviral therapy
is capable of reducing viremia to levels below the limit of detection of most
conventional assays (~50 HIV copies/ul). However, highly sensitive PCR viral
detection assays indicate that low level viremia (<50 copies/ul) still persists despite
prolonged treatment [251]. Estimates suggest that approximately $10^7$ CD4+
memory T cells have integrated viral DNA (proviral DNA), and they persist even in
individuals with <50 HIV copies/ul [252]. These latently infected CD4+ lymphocytes
have a half-life of up to 44 months [253], and by some estimates will require over 5
decades to eradicate using antiretroviral therapy. Thus the CD4+ HIV reservoir is a major hindrance towards eradicating HIV [253].

The gut is host to the single largest collection of lymphoid cells in the body and collectively represents the single largest source of SIV during chronic untreated infection in rhesus macaques [254]. Similarly, a cross-sectional study in humans indicates that the gut plays host to a relatively higher frequency of proviral DNA in comparison to both resting and activated CD4+ T cells in blood, despite 10 years of successful antiretroviral therapy [255]. This implies that low-level viral replication persists in the gut, and that this may allow for continued localized infection and viral persistence at this site [256]. This fact is further supported by findings in aviremic individuals receiving antiretroviral therapy where most [257, 258], but not all studies [259], show incomplete recovery of gut CD4+ T cells even after restoration of systemic CD4+ populations to normal or near normal levels. In summation, the gut mucosa plays host to a significant number of long-lived memory CD4+ lymphocytes containing HIV provirus that allow HIV infection to persist despite antiretroviral therapy. Therefore, once infection is established, the gut latent reservoir poses a significant barrier to HIV eradication [256].

1.7 IMMUNE RESPONSES TO HIV

1.7.1 Innate immune responses

The innate immune system represents the first line of defence against infection. The existence of innate immunity in primordial organisms suggests that that it
evolved before the adaptive immune system. Innate immunity differs in several ways from the adaptive immune system. Innate immunity responds very rapidly to pathogens (minutes to days) whereas the adaptive immune system is delayed (days to weeks). Additionally, the innate immune system detects infections through general cognate patterns of organisms unlike the adaptive immune system, which is major histocompatibility complex (MHC) restricted and responds to a specific antigen. Lastly, the innate immune system generally has no recollection of previous exposure to an organism (immunological memory), and responds in a similar manner each time it encounters the infectious organism. On the other hand, the adaptive immune system retains immunological memory and upon antigen re-exposure it can respond faster and can alter the magnitude of response [20].

The innate immune system comprises of a variety of cellular components including dendritic cells, and natural killer cells, as well as soluble factors including cytokines, chemokines and small molecules, which we discuss in more detail below. These features of innate immunity facilitate the direct engagement of foreign organisms, while also allowing the innate immune arm to recruit and prime the adaptive immune response to help clear the infection.

1.7.1.1 Toll like receptors

The innate immune system recognizes organisms through evolutionarily conserved pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs) on foreign microbes. One such PRR is the Toll-Like receptors (TLRs), of which 10 have been defined in humans. TLRs are located on the
surface (TLR-1, -2, -4, and-6) or within (TLR-3, -7, -8 and -9) innate cells, and are found on a variety of cell types including non-immune cells (epithelial cells, endothelial cells), innate immune cells (macrophages, dendritic cells) as well as adaptive immune cells (T and B cells) [20]. Each TLR can recognize different repeating patterns from microbes. Of particular relevance here is TLR-7, which recognizes organisms containing single-stranded RNA, including HIV [2].

TLR activation leads to a downstream signalling cascade through the adaptor proteins (MyD88 or TRIF) and the eventual activation of NFkB. that then induces the transcription of both chemokine and cytokines. Production of chemokine proteins in turn elicits migration and activation of new immune cells to the vicinity of infection. The role of induced cytokines is two fold. First, produced cytokines mediate the maturation of APCs and the surface expression of T cell co-stimulatory molecules (CD80 and CD86) that are essential for effective induction of adaptive immune responses. Secondly, these cytokines also rev up the innate immune system to increase phagocytosis and killing of the invading microorganism [20].

1.7.1.2 Dendritic Cells

Dendritic cells (DCs) are a major type of APCs that function as patrolling sentinels in tissues where they efficiently capture antigens by phagocytosis, macropinocytosis or endocytosis using their PRR. Capture of microorganisms triggers DC maturation and migration to secondary lymphoid organs. As the DCs migrate, captured pathogens are internalized and processed into antigen to later be
presented on Major Histocompatibility Complexes (MHC) to adaptive immune cells [20].

Immature dendritic cells (iDCs) express both CD4 and the HIV entry co-receptors CCR5 and CXCR4 at their cell surface thus making them directly susceptible to HIV infection. Some iDCs can also capture HIV through the PRR, DC-specific intracellular adhesion molecule-grabbin non-integrin (DC-SIGN), resulting in viral entry into the cell [2]. Once internalized, HIV is stored in a lysosomal compartment where it is degraded into peptides that are then placed on MHC complexes and presented to T cells.

Some DC’s, in particular Langerhan’s cells (LC’s), can also function as ‘Trojan horses’ acting to enhance HIV infection. Here, LC’s capture intact HIV virions either on their surface through DC-SIGN, or as intact virus in endosomes. Viral-laden DCs then migrate to the lymph nodes where this virus can then directly be transferred on to adjacent CD4+ T cells (trans-infection) across cell synapses. In addition, DCs that capture intact virus may have ongoing viral replication (productive infection) that can later be released and proceed to infect nearby target cells. As such, DCs seem to play contrasting roles in HIV infection: they can degrade HIV and activate the adaptive arm of the immune system to help control infection, while on the other hand DCs can serve as targets, ‘couriers’ and enhancers of HIV infection [260]. Molecules involved in deciding influencing whether DCs will transmit HIV or clear the virus, DC-SIGN and Langerin are discussed in section 1.4.2.1.
1.7.1.3 Natural Killer Cells

Natural killer (NK) cells represent another important cellular component of the innate immune defense and account for about 15% of peripheral blood mononuclear cells [2]. These cells recognize and kill virus-infected cells in a non-MHC dependent manner, and instead depend on cell surface triggered inhibitory or activating molecules to select their targets. In this regard, NK cells have killer immunoglobulin inhibitory receptors (KIR) and killer activating receptors (KAR). Upon encountering a microbe infected cell NK cells are able to detect absent or altered surface expression of HLA class I complexes caused by the infection, and in the absence of a KIR will secrete perforin and granzyme to lyse the infected cell [20]. Although the ligands that activate NK cells have yet to be exhaustively defined, NK cells can recognize and lyse cells that have changes in cell surface glycoproteins induced by viral or bacterial infection and also those cells that produce ‘stress/danger’ signals such as MHC class I chain-related (MIC) A or B antigens [260]. Alternatively, upon encountering healthy uninfected cells with adequate expression of surface HLA-I complexes, both KAR and KIR bind to the MHC complex of the target cell; however, this dual engagement of receptors on the NK cell inhibits NK mediated killing [20].

NK cells are also involved in mounting antiviral immune responses against HIV [261]. In order to avoid CD8-mediated killing, HIV downregulates the expression of MHC class I expression on the surface of infected target cells in vitro. Low level of MHC expression then makes these cells classic targets for NK mediated lysis following KAR interaction [261]. However, HIV-infected cells may still avoid
NK-mediated lysis through the selective downregulation of some HLA (HLA-A, HLA-B), but not all HLA molecules (HLA-C, HLA-E) [262]. NK cells also recognize antibodies bound to the viral envelope proteins on infected cell surfaces and induce antibody dependent cell cytotoxicity (ADCC) [261]. Finally, NK cells also secrete β-chemokines, which are ligands for the HIV co-receptor CCR5. NK production of large amounts of MIP1B, MIP1a and RANTES have been shown to suppress HIV replication in vitro [263].

1.7.1.4 Soluble Innate Factors in the Genital Mucosa.

An intact genital epithelium perhaps represents the best innate mechanical defence against HIV infection. However, several soluble innate biological factors in the genital mucosa have also been shown to mediate host defence against HIV. Genital epithelial cells secrete a thick glycoprotein mucus that can efficiently trap HIV, slowing its diffusion across the membrane by 1000-fold more than in water [264]. Genital epithelial cells as well as surrounding leukocytes also secrete proteins with antiviral properties such as secretory leukocyte protease inhibitor (SLPI), lactoferrin, β-defensins and trappin-2/elafin [41, 185]. SLPI and trappin-2/elafin are innate molecules from the family of serine proteases. How these molecules mediate their antiviral properties is unclear. However, epidemiological studies show that the secretion of SLPI and trappin-2 is associated with protection from HIV acquisition [41, 184]. Further, in vitro studies have shown that SLPI binds to HIV target cells and confers a potent inhibitory activity on the HIV prior to the reverse transcription phase of infection [265]. Lactoferrin is an iron-biding glycoprotein found in genital
secretions that has demonstrated in vitro antiviral activity against both DNA and RNA viruses, including HIV. Similarly, how lactoferrin mediates its HIV antiviral properties remains to be fully understood. However, in vitro studies suggest that lactoferrin may control HIV by inhibiting viral entry into target cells by either blocking viral entry receptors on HIV target cells, or by directly binding to gp120 envelope virus and blocking its facilitation of viral infectivity [266]. Lastly, β-defensins are small cationic molecules that have been demonstrated to protect against a wide range of microorganisms including bacteria, fungi and virus including HIV. These molecules antiviral properties come from its ability to bind to the viral envelope and disrupt HIV membrane integrity, which destroys the virus [267]. Nevertheless, despite the demonstrated antiviral activity of both lactoferrin and β-defensins, prospective in vivo studies suggest that the presence of these immune factors correlates with increased HIV levels questioning their HIV antiviral activity in a ‘real world’ setting [268].

1.7.2 Linking Innate and Adaptive Immunity

1.7.2.1 Antigen Presentation and T Cell Activation

Adaptive immune responses are initiated in secondary lymphoid tissues where naïve T cells are continually circulating. Upon activation, innate APCs migrate downstream from tissues where pathogen is present through the proximal draining lymph system into lymphoid tissue to present antigen to naïve T cells. This includes mucosal APCs in the genital mucosa that drain into the proximal inguinal lymph
nodes, gut APCs that drain mostly into Payer’s patches, and those APCs that enter the bloodstream (rare) which are trapped in the spleen [2].

As APCs migrate, internalized pathogen can be processed in one of two ways for presentation to T cells as antigen in the context of the major histocompatibility complexes (MHC). Pathogen peptides from the cytosol (e.g. intracellular viruses) are degrade into smaller fragments by proteasome proteases in the cytoplasm and transported into the lumen of the endoplasmic reticulum (ER) via the transporters associated with the antigen processing (TAP) ‘gateway’ [20]. Within the ER, processed peptides that are approximately 8 amino acids long are then loaded onto the peptide-binding cleft of the MHC class I (MHC-I) molecule. This peptide-MHC complex is then transported to the APC cell surface from the ER via Golgi vesicles for presentation to T cell receptors on CD8+ T cells. A second antigen processing and presentation pathway involves the APC-mediated uptake of extracellular pathogens into vesicles. These vesicles then acidify to activate resident protease enzymes and degrade the pathogen into smaller peptide fragments. Vesicles containing peptides fuse with vesicles containing MHC class II (MHC-II) molecules and enclosed peptides that are up to 15 amino acids long are then mounted to the peptide-binding groove of the MHC-II molecule before the complex is transported to the APC surface to be presented to the cognate CD4+ TCR [20].

1.7.2.2 T Cell Activation

To activate naïve T cells, APCs present a dual signal; first, antigen is presented in the context of the relevant MHC molecule to the cognate TCR on the T
cell. Secondly, the binding of CD80/CD86 co-stimulatory molecules to the CD28 surface molecule on the cognate naïve T cell specific for the antigen confers the second activation signal [20]. T cell antigen specificity is conferred through the T cell receptor (TCR) on its surface. TCRs have a vast potential ability to detect specific antigens, which is brought about by somatic recombination in the thymus of the variable regions (termed V, D and J) from where they undergo positive or negative selection before relocating to secondary lymphoid tissues awaiting APC activation with the specific antigen [269].

1.7.3 Adaptive Immune responses

1.7.3.1 Activated T cell Differentiation Phenotypes

After activation, naïve CD4+ T cells can mature into several subtypes, mainly dependent on the prevalent cytokine immune milieu [270]. Principally, these cells can differentiate either into either T helper 1 (Th1) cells in the presence of IFNg and IL12, Th2 cells in the presence of IL4, Th17 in the presence of IL6 and TGFb or T regulatory cells (Tregs) in the presence of TGFb alone [270, 271]. Other CD4 T cell subsets exist and continue to be discovered and described in the literature mostly according to the cytokine they produce such as Th21 (IL21 producing) and Th22 (IL22 producing) cells [271]. On the other hand, naïve CD8 T cells are predestined to mature only into cytotoxic T cells [20].
1.7.3.2 A General Overview of Armed Effector T Cell Functions

Activation of T lymphocytes leads to their proliferation and differentiation into armed effector T cells. These armed effector cells typically require no additional signal apart from TCR-antigen recognition of target cells or molecules to initiate effector immune responses [20]. Generally, CD8+ T lymphocytes can either secrete effector molecules (perforin and granzyme) or engage the FAS-FAS ligand molecules to induce cell death of virus infected target cells. Th1 cells ‘license’ the activation of macrophages, which in turn revs up the macrophage anti-pathogenic immune responses against engulfed pathogens [20]. The same Th1 cell ‘licensing’ of B-cells also induces the production of opsonizing antibody to help clear invading pathogen [20]. Th2 cells specialize in B cell activation and they actively secrete several B cell activating effector molecules to induce B-cell proliferation and antibody isotype class switching to enable broader antibody recognition of antigens. Th17 cells facilitate the recruitment of neutrophils to areas of mucosal bacterial infection and mediate bacterial and fungal clearance [231, 272]. Lastly, Treg cells act to dampen the magnitude of immune responses in order to limit excessive immune responses that might otherwise be harmful to the host [20, 270, 271].

1.7.3.3 CD8 Immune Responses in HIV

There is strong evidence implicating CD8+ cytotoxic T cells (CTLs) in the control of HIV infection. After blood viremia peaks during acute infection an inverse relationship is then observed between increasing HIV-specific CTL responses and decreasing viral load [273]; Figure 1.6. Macaque animal models provide further
experimental evidence of the importance of CTLs in HIV clearance. In particular the
depletion of CD8+ T cells in vivo using monoclonal antibodies results in uncontrolled
SIV viremia during acute infection [274]. Extended monoclonal antibody-mediated
depletion of CD8+ T cells leads to increased HIV viremia and a plateau in the viral
load set point until the antibody wears off [275]. Furthermore, the persistent
emergence and selection of CTL HIV escape mutants both in vivo and in vitro
suggests that CTLs exert significant immune pressure driving evolution of the virus
[276, 277].

CD8+ T cells control HIV through several different mechanisms (Figure 1.7).
First, virus-specific CTLs secrete granules containing perforin and granzyme
molecules, which make the cell membrane permeable and induce target-cell death
respectively [278]. Alternatively, a minority of CTLs can also induce cell death
through the engagement of CD8 FAS-ligand with the FAS-receptor on the virus-
infected target-cell to trigger apoptosis [279]. CD8+ T cells may also control HIV
infection through non-cytotoxic pathways [280]. Non-cytotoxic CD8-mediated
antiviral activity was first observed in HIV-infected but asymptomatic individuals
who did not shed virus in their cultured blood lymphocytes [281]. Monoclonal
antibody-mediated depletion of CD8+ T cells in these cultures resulted in increased
viral shedding, while their replacement resulted in complete viral suppression
[281]. This immune function is now described as the CD8+ T cell antiviral factor
(CAF) [2]. However, the mechanism by which CD8+ T cells suppress viral replication
remains unknown [282]. Experimental evidence suggests that CAF may function by
blocking HIV LTR-mediated transcription in infected cells, which halts virus
production and encourages viral latency [283]. Finally, CTLs produce cytokines and chemokines that can potentially control HIV. HIV-specific CTLs produce IFNg [284], which inhibits HIV replication and produce β-chemokines including MIP-1a, MIP-1b and RANTES which may suppress HIV replication by competition for and downregulation of the HIV entry co-receptor CCR5 [285, 286].

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**Figure 1.7. The antiviral functions of HIV-specific CD8+ T cells.**

In acute HIV infection, as many as 10% of the body’s CD8+ T cell immune responses are HIV-specific, and their breadth is restricted to a few clones [190]. During chronic HIV infection, the frequency of HIV-specific CD8+ T cells decreases to 1-2% of all circulating CD8+ T cells and these frequencies are maintained even after the onset of AIDS [190]. Despite the relative decrease of HIV-specific CD8+ T cells during chronic infection this cell population still remains important in HIV viral
control, as evidenced by macaque monoclonal CD8 depletion studies during chronic SIV infection where removal of CD8+ T cells results in viral rebound [274].

HIV-specific CTL activity has been demonstrated against cells expressing several different HIV proteins including reverse transcriptase, envelope, gag and the viral accessory proteins (vif, nef and pol). Indeed, broadly reactive HIV-specific immune responses have been identified in individuals who do not progress to AIDS (long-term nonprogressors), suggesting that broad immune responses are important in preventing disease progression [287]. However not all HIV-specific CTL immune responses are ‘equal’. CD8+ T cell responses directed against gag protein are associated with lower viremia in chronic infection, while those against env protein are associated with elevated viremia [287]. In addition, it appears that the breadth but not the magnitude of these gag-specific antiviral immune responses are particularly critical in controlling viremia [288]. The functional mechanism linking enhanced CTL viral control to gag-targeting remains to be elucidated, but it may be due to increased conservation of Gag sequence epitopes in the HIV virion, whereby mutations in the gag gene may come at a significant cost to viral fitness [289].

When HIV infection progresses to AIDS, a specific loss in HIV-specific CD8+ antiviral cytolytic activity [290], but not antiviral CD8+ activity to other viral pathogens such as cytomegalovirus (CMV) or Epstein Barr virus (EBV), is observed [291, 292]. This specific loss in HIV-specific CTL activity in AIDS in progressive HIV infection is attributed in part to their inability to secrete perforin [290], and reduced expression of IFNg [293]. In addition, these poorly functioning HIV-specific CD8+ T
cells are phenotypically identified by their expression of T-cell exhaustion markers PD-1 [294] and Tim-3 [295] in addition to continued expression of T cell central memory (CD27) but not later stage effector T cell markers of maturation [290, 296]. Therefore in summary, CD8 T cells are essential in the control of HIV during early and chronic stages of infection. In advanced HIV disease functional loss in HIV-specific CTL activity occurs and may be due to T cell exhaustion and a consistent failure to mature into terminal end-stage effectors. The failure in CD8+ T cell maturation may be a consequence of CD4+ T helper cell loss during HIV [190].

### 1.7.3.4 CD4 Immune Responses in HIV

HIV-infected CD4+ T cell responses are readily detected in early infection; however these immune responses rapidly disappear during chronic untreated infection [297, 298]. This is to be expected given that the hallmark of HIV infection is the steady depletion of CD4+ T cell populations. Nevertheless, the significance of these immune responses is still unclear given that HIV preferentially depletes HIV-specific CD4+ T cells [299]. In a recent HIV animal model study, CD4+ T cells were depleted in macaques prior to SIV infection, and viral kinetics was characterized [300]. Here, peak viremia was comparable between differently treated macaque groups. However, after attaining peak viremia, viral levels remained elevated in CD4+ depleted animals, but declined in CD4+ competent animals, and all this occurred despite the maintenance of normal SIV-specific CD8+ T cell and B-cell antiviral responses in the prior group [300]. Furthermore, SIV infection progressed more rapidly in CD4+ T cell depleted animals compared to non-depleted macaques.
These findings in animal studies suggest a strong role for CD4+ T cells in controlling HIV infection. Indeed, CD4+ T responses are a prognostic marker of the clinical course of HIV infection [297, 298, 301, 302] and are maintained in non-progressive infection [303].

CD4+ T cells can control HIV infection via one of several mechanisms. HIV-specific CD4+ T cells secrete antiviral cytokines such as IFNγ and β-chemokines (previously discussed) which may inhibit HIV growth and entry respectively [20, 304]. In addition, CD4+ T cells may also directly control viremia through cell-cell dependent lysis of HIV-infected cells, which is mediated by perforin and/or through a Fas-Fas ligand mediated pathways in a similar manner to CTLs [303, 305, 306]. Finally, the role that CD4+ T cells play in facilitating HIV-specific CD8+ antiviral activity is now becoming apparent. Studies in mice show that CD40L surface expression on activated CD4+ T cells is vital in triggering dendritic cells to secrete IL12, which in turn is required to initiate antiviral HIV-specific CD8+ T cell responses [307, 308]. CD4+ T helper cells are also crucial in maintaining CD8+ T cell functions through the secretion of IL21 [309]. Production of IL21 by CD4+ T cells enhances perforin production by HIV-specific CD8+ T cells in vivo, and these HIV-specific CD8+ T cells show an enhanced ability to inhibit viral replication in vitro after IL21 binding [309]. Lastly, CD4+ T cells are also necessary for the maintenance of HIV-specific CD8+ T cell memory during chronic infection [310, 311], and this is mediated in part through CD40L on CD4+ T cells which activates APCs and makes them better able to 'license' CTL activation [312, 313]. Collectively, the above studies show how CD4+ T cells are relevant to the control of HIV infection either
through direct antiviral mechanisms or through maintenance and functional enhancement of HIV-specific CD8+ T cells.

HIV-specific CD4+ T cells can target various HIV proteins expressed during infection [2]; however, until very recently, no study had examined which targeted viral components were most crucial towards viral control. Ranasinghe et al were the first to demonstrate on a population level that the breadth and magnitude of HIV-specific CD4+ T cells targeting gag protein were inversely correlated with viral load [314]. In addition, targeting of HIV-specific CD4+ T cells to conserved gag epitopes was most strongly predictive of immunologic control of viral replication, and this was independent of host HLA types. Conversely, targeting env was associated with high viral load and was most likely to be predominant in HIV progressors [314]. Therefore, taken together, these data imply that HIV-specific CD4+ T cells responses focused on gag are most critical in HIV control during chronic infection.

1.7.3.5 Humoral Immune Responses in HIV

HIV-specific antibodies are detected in infected individuals as early as 1-2 weeks following infection, before the termination of the acute burst in viremia [2, 315]. Initial antibody responses during HIV infection are targeted against the gag protein followed by nef, rev and finally env. Subsequently, there is a stable B-cell repertoire during the first year of infection [316].

Antibodies can effect antiviral properties through several mechanisms. Neutralizing antibodies (NAb) can control infection by binding to and preventing HIV target cell entry. Alternatively, non-NAb may reduce viremia via phagocytosis
of immune complexed virus, lysis of virions by complement and through lysis of infected cells via activation of antibody-dependent cellular cytotoxicity (ADCC) [315, 317].

NAbs humoral immune responses arguably hold the most potential to limit HIV infection [315, 317]. Primate models of HIV infection show that HIV infection via oral, vaginal and intravenous routes can be prevented by passive administration of NAbs [318]. However, NAbs consist typically only a fraction of elicited antibody titres, and when induced during chronic infection these NAbs demonstrate weak neutralizing activity [319, 320]. Furthermore, the presence of NAbs during HIV infection does not correlate with HIV viral load or survival [321-324]. Nonetheless, generation of NAbs may be a key component for a protective HIV vaccine.

Several mechanisms may underpin poor viral neutralization by NAbs during HIV infection. Primarily, NAbs may be ever chasing behind a rapidly evolving virus that has a high mutation rate of 0.2-2 mutations per genome [325, 326], such that contemporaneous long-lasting antibodies have little or no effect on contemporaneous virus [327]. Secondly, the majority of antibody responses are ineffective due to masking of conserved epitopes, and instead most antibodies generated are specific for decoy viral antigen abundantly produced by glycosylation of the viral envelope [317]. Finally, a significant amount of viral transmission events occur across a virological cell-to-cell synapse [163]. Thus antibodies in general have little effect in hindering this form of viral transmission. Nevertheless, broadly neutralizing monoclonal antibodies against HIV have been noted in infected
individuals (such as b12, 17b, 2g12, and 2f5 NAbs), and their presence has been associated with viral control in some [315, 317] but not most studies [321-324].

1.8 HIV TREATMENT

1.8.1 Introduction to Antiretroviral therapies

As of 2009, 5 million individuals were currently receiving antiretroviral (ARV) therapy globally [1]. The widespread introduction ARVs has tremendously reduced the morbidity and mortality associated with HIV infection. In most developed countries this infection has now been transformed from a death sentence to a chronic, manageable infection with a life expectancy that can approach that of uninfected individuals with other chronic ailments such as diabetes [328, 329].

The US Food and Drug Administration approved the first antiretroviral drug in 1987 with the introduction of azidothymidine (AZT), a nucleoside reverse transcriptase inhibitor drug class (NRTI) [330]. Since then, over 20 different drugs from several drug classes have been licensed including non-nucleoside inhibitors (NNRTI), protease inhibitors (PI), entry inhibitors and fusion inhibitors [331]. As suggested by their classification nomenclature, these drugs work by interfering with the various stages of the HIV life cycle to control viral replication. On their own, most approved ARVs can reduce plasma viremia by 0.5-2.0 Log_{10} HIV copies/ml. However, this can quickly result in viral mutation and selection to escape suppression. Therefore, a combination of ARV drug classes is recommended and typically consists of a dual NRTI with an NNRTI or a boosted PI regimen [331, 332].
1.8.1.1 Nucleoside reverse Transcriptase Inhibitors (NRTIs)

NRTIs are perhaps the most commonly prescribed treatment at ARV initiation today, and inhibit viral reverse transcription of the viral complimentary DNA strand. Specifically, NRTIs are initially phosphorylated by cellular kinases before incorporation into the complementary viral DNA molecule during reverse transcription. However, because the incorporated NRTI molecule lacks a 3’ hydroxyl group, the subsequent deoxynucleotide cannot form a phosphodiester bond, and this culminates in premature termination of viral DNA synthesis [333]. In addition to inhibition of viral reverse transcriptase, NRTIs are also specific inhibitors of human mitochondrial DNA polymerase, resulting in reduced mitochondrial DNA replication, cellular dysfunction and eventual cellular death [334]. Examples of current NRTIs include Zidovudine (AZT), Lamivudine (3TC), Abacavir (ABC) and Tenofovir (TDF) [331].

1.8.1.2 Non-Nucleoside reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs act at the same step in the HIV life cycle as NRTIs to bring about viral suppression. However, these two drug classes differ in that NNRTIs specifically target the functional activity of HIV reverse transcriptase, and do not require intracellular phosphorylation to be active. Additionally, NNRTIs also do not inhibit human DNA polymerases and are therefore not associated with the toxic side effects observed in NRTIs.

NRTIs specifically interact with the HIV reverse transcriptase molecule resulting in the physical repositioning of amino acids in the active site, preventing its activity.
The NNRTI-inactivated reverse transcriptase enzyme can therefore not bind and engage in transcription of the complementary viral DNA strand resulting in viral suppression [333, 335]. Specific examples of NNRTI drugs include Efavirence (EFV) and Nevirapin (NVP).

1.8.1.3 Protease Inhibitors (PIs)

The viral protease enzyme is critical for production of smaller functional viral proteins from larger cleaved polyproteins. For instance viral protease is responsible for cleaving the gag/pol polyproteins into viral reverse transcriptase, integrase and P24 peptides; and also for cleaving env gp160 polyprotein into gp120 and gp41 that are expressed on the viral surface [2]. PIs bind to the active site of the viral protease enzyme, inhibiting its ability to cleave viral polyproteins into their functional subset. Due to the lack of viral polyprotein cleavage by protease, non-functional viral proteins are produced and result in the production of immature virions that are unable to productively infect other cells [336]. Examples of approved drugs in the PIs category include lopinavir, ritonavir, tripanavir and darunavir [331].

1.8.1.4 Fusion Inhibitors

This is a new drug class of HIV ARVs, and currently only Enfuvirtide/Fuzion (T20) is approved for use. In order for HIV to infect a CD4+ cell, the envelope protein gp120 must first bind to the CD4 receptor, and a resulting viral envelope conformational change exposes the hidden gp41 viral peptide that is then ‘injected’
into the cell to begin cell-virion fusion [2]. T20 binds to viral gp41 glycoprotein and prevents the shape changes that enable virus-cell fusion. Currently, T20 is degraded by stomach acids if delivered orally, and therefore it is administered via subcutaneous injection [331]. Addition of T20 to standard ARV regimen is associated with faster viral decay in blood. However, this drug is currently not used in treatment-naïve patients, but is approved for use in ‘salvage’ therapy where classical ARV regimens are failing [331].

**1.8.1.5 Integrase Inhibitors**

Viral integrase is also another essential protein for viral replication, and this enzyme is responsible for integrating viral DNA into the host cell’s genome. Viral integration is an ordered process that involves the assembly of integrase enzyme to form a stable complex with viral DNA, the endonucleolytic processing of the viral DNA ends and the subsequent viral DNA strand transfer and joining of the viral and cellular DNAs [337]. Raltegravir is the first and only approved integrase inhibitor ARV drug that functions by binding the integrase enzyme and inhibiting the strand transfer step of integration [337]. Similar to fusion inhibitors, this new ARV drug class is typically only used in salvage therapy, but it can also be used as a supplementary ARV regimen to standard ARV treatments [338, 339].
1.8.2 Kinetics of ARV Associated Viral Decay

Following initiation of combined ARV therapy, several phases of viral decay are observed. After a short lag of 1-2 days after the onset of ARV treatment, a rapid phase of viral decay is ensues, usually with a half-life of 1-2 days. This first phase of viral decline is largely attributed to suppression of viremia from activated CD4+ T cells [32]. The second and more gradual phase of viral decay then follows with a half-life of 2-3 weeks and it is hypothesized that at this stage, viremia is sourced from macrophages [32, 340]. In most individuals undetectable blood viral levels can be achieved after 2 months of successful therapy, and intensification of therapy allows for a much shorter duration to attain undetectable viral loads [341, 342]. Despite this aviremic status, HIV infection still persists. This is evidenced by the use of sensitive single copy PCR assays to detect HIV RNA that show the persistent presence of low-level viremia (<50 copies/ml) [343], while intermittent viral shedding from genital mucosal secretions still persists in many individuals despite effective ARV therapy [100]. As a result, a third phase of viral infection has more recently been described, with a half-life of 38 weeks; while a fourth and long-lived viral decay phase has been demonstrated with an infinite half-life [32]. These later phases of viral decay are suggested to originate from the longer-lived latent viral reservoir in memory T cells that are present in the gut mucosa even 7-10 years after successful ARV therapy [255] (discussed in section 1.6.3.6). It is this latent reservoir that poses the last significant hurdle towards eradication of HIV infection through long-term successful ARV treatment.
1.8.3 Effect of ARV therapies on CD4 Lymphocytes

1.8.3.1 Recovery of Blood CD4+ T Cell Counts

While the immediate aim of ARV therapy is to suppress HIV replication, the restoration and maintenance of the body’s CD4+ T cell pool is just as important towards averting AIDS opportunistic infections and death. Generally, it has been shown that the lower the CD4+ T cell count at initiation of ARV treatment, the longer it takes to recover normal CD4+ T cell levels [344, 345]. As such, earlier initiation of ARV treatment is vital towards complete restoration of blood CD4+ T cell counts. Current World Health Organization (WHO) guidelines suggest that antiretroviral therapy is initiated when CD4 counts fall below 350 CD4+ counts/ul in developed countries, and a threshold of 250 CD4+ counts/ul in developing countries or where health resources are limited [2]. Despite these guidelines the debate still rages on when combination ARV therapy should be initiated in patients who have not yet had an AIDS defining event [346-348]. Studies show that initiation of therapy at CD4 counts of less than 250/ul instead of a threshold of 350 counts/ul is associated with triple the rate of death and increased adverse events [348]. Other studies have suggested that even earlier initiation of therapy with >500 CD4 counts/ul confers even better prognostic value than the standard lower CD4 counts [347].

1.8.3.2 ARV-Mediated Recovery of CD4 Lymphocytes in Mucosal Tissues

The gut mucosa is most impacted by HIV infection; it houses up to 80% of CD4+ T cells in the body, and almost 90% of these cells are depleted within two weeks of
infection [222, 225, 226]. Relatively short-term ARV therapy (<4 years) indicates that reconstitution of CD4 T cell frequencies in the gut lags behind that in blood and is often insufficient to restore these populations in the gut to levels observed in uninfected individuals [257, 258]. More recent work from our lab suggests that gut CD4 T cell immune reconstitution is possible within individuals who receiving completely suppressive ARV for prolonged durations (>7 years) [259]. Nonetheless, as HIV is known to preferentially deplete various CD4+ T cell subsets in the gut (section 1.6.3.2), the effect of ARV in restoring these highly susceptible populations in the mucosa remains unknown.

1.9 HIV VACCINES

1.9.1 Ideal properties of an effective vaccine

ARV therapy may reduce the incidence of HIV at a population level. Mathematical modelling has suggested that widespread rollout of HIV testing and immediate ARV treatment may reduce the prevalence of HIV to less than 1% within 50 years [349]. However, nearly 80% of HIV-infected adults in SSA, who account for two-thirds of the world’s population infected with HIV, remain unaware of their status [285]. Even if initiated, such a strategy would cost US $1.7 Billion per year [349], and as such would be unaffordable for most of the developing world. Therefore, vaccines may offer the only possible solution to stemming the HIV endemic globally.
A successful HIV vaccine candidate would ideally be safe, affordable and should elicit an effective and long-lasting immune response comprised of both neutralizing antibodies (NAbs) and cytotoxic t lymphocytes (CTLs) capable of recognizing diverse viral strains. Also, since most infections globally occur across the genital-rectal mucosa following sexual exposure, then vaccine-induced immune responses should also be present at the mucosal site of exposure to protect against infection or rapidly clear the infection if it is acquired. Prevention of infection (sterile protection) is the ideal goal of HIV vaccines as no evolutionary/escape pressure on the virus would be exerted by the vaccine-induced immune responses [350]. Nonetheless, a HIV vaccine can still be useful if it has a therapeutic effect on preventing/slowing disease progression and CD4+ T cell loss, as well as reducing the steady-state viral load [350]. However, vaccines designed for therapeutic use once infected may lose potency over time due to HIVs ability to continually evolve and evade host immune mechanisms.

1.9.2 Human HIV Vaccine Trials

As of 2011, 163 human HIV vaccine trials had been completed or were in process worldwide; 3 in phase III, 31 in phase II and the remainder in phase I (www.iavireport.org/trials). While it is difficult to entirely capture the breadth of human vaccines here, a brief overview of the themes and lessons learnt from phase III vaccines are highlighted below.
1.9.2.1 AIDSVAX B/E and AIDSVAX B/B HIV Vaccines.

The AIDSVAX B/B (VAX004) and AIDSVAX B/E (VAX003) were used in two parallel protein vaccine trials run in Europe and Thailand respectively and comprised of recombinant gp120 antigens [350, 351]. These vaccines demonstrated significant induction of antibody titres against HIV envelope strains included in the vaccine, and peak gp120 antibodies significantly inversely correlated with HIV incidence [352-354]. Disappointingly however, there was no difference in the study endpoint, HIV acquisition, between the vaccine and placebo groups [355, 356]. Follow up studies suggested that induced antibodies were mostly binding, and not neutralizing antibodies. Therefore, vaccine induced antibodies may have been more a marker of vaccination, rather than a true correlate of protection [354].

1.9.2.2 The MRKAd5 Vaccine.

The MRKAd5 T cell vaccine was evaluated in the STEP trial, and consisted of a trivalent vaccine with a 1:1:1 mixture of replication-defective adenovirus serotype 5 (Ad5) viral vectors expressing HIV gag, pol and nef genes [350, 351]. Phase I studies indicated that the MRKAd5 vaccine was capable of inducing positive IFNg-ELISPOT immune responses to two or more peptide pools in the majority (72%) of participants [357], and all three HIV proteins were targeted by 44% of the vaccines during the phase III trial [351]. Additionally, CD8+ and CD4+ T cells were shown by intracellular cytokine staining to produce IFNg, IL2 and TNFa cytokine immune responses to the vaccine [358]. Interim per-protocol analysis of the study vaccine-arm had 19 infections (4%), while the placebo-arm had 10 (2.12%) infections
indicating that the vaccine was in fact increasing susceptibility to HIV infection rather than preventing it [359]. Another study conducted in South Africa around the same time, the Phambili Trial [360] [358], displayed similar results and both studies were halted prematurely.

The reasons why this vaccine failed, is unknown. However, it could be that suboptimal immune responses were generated by T lymphocytes within the vaccines, where 31% had detectable immune responses that were much lower in magnitude than those observed in HIV infected individuals who are long-term non-progressors [358]. Furthermore, breakthrough infections amongst vaccines had genetically distinct HIV virions, suggesting that immune responses generated by the vaccine were not cross-reactive enough [351]. Finally, vaccine-generated CTL immune responses demonstrated poor recognition of infected cells [361], implying that immune responses generated were perhaps not a true correlate of protection.

1.9.2.3 RV144 Vaccine.

To date, the RV144 vaccine, also referred to as the ‘Thai Trail’, is the only vaccine to show modest protective efficacy against HIV infection. According to the modified intention-to-treat analysis, this study demonstrated an overall efficacy of 31.2% [362]. The vaccine consisted of the Aventis Pasteur’s ALVAC-HIV canarypox vector prime expressing HIV-1 clade B gag, protease, and gp120 linked to the transmembrane anchoring region of gp41 peptides. Following ALVAC priming, the participants then received VaxGen’s AIDSVAX B/E, a recombinant vaccine expressing HIV-1 clade B and E gp120 genes.
The Thai Trial vaccine strategy was meant to induce broadly neutralizing antibodies; however, the resultant antibodies of this trial had a very low titre of NAbs that did not have broad specificity against heterologous strains. This highlights the issues plaguing current vaccines, which have yet to identify the correct antigen(s) to induce broadly neutralizing antibodies that are believed to be key towards mediating protection. Follow up sub-studies of the Thai Trail show that having env specific IgG antibodies was associated with increased protection (estimated odds ratio 0.57), while having env specific IgA antibodies was not associated with any protective advantage [363].

Vaccine efficacy in the Thai trial ranged from 31.2% to 26.2% depending on the analysis used, and this has generated some criticism [362]. Typically, the significance threshold for a protective vaccine to be considered efficacious is 30%. In the Thai Trial, exclusion of participants who became infected with HIV before the immunization dose was complete in the analysis resulted in the vaccine showing no significant efficacy (26.2%). Furthermore, sub-analysis of the data indicated that majority of protection was observed in the first year after vaccination, and waned somewhat after that. Lastly, high-risk individuals did not seem to be protected by the virus [362]. Therefore, although this study may generate important clues on possible immune correlates of a vaccine required to mediate protection, opinions still vary on how much this trial should influence future HIV research [364].
1.9.3 Lessons Learnt

The common theme amongst all evaluated HIV vaccine candidates’ remains that the correlates necessary to induce immune protection against HIV still remain unknown [350]. Additionally, even though all phase III vaccine candidates have shown immunogenicity, this might not necessarily be a good thing, as was the case in the STEP trial (T cell IFNg production) and the RV144 trial (env IgA antibodies) [358, 363]. Therefore it is of utmost importance for the vaccine field that the ‘true’ correlates of immune protection be identified first in order to help design the appropriate protective vaccine. Individuals who are repeatedly exposed, but remain uninfected may offer some useful insights for future vaccine designs (see section 1.5.5).

Vaccine candidates are often times initially tested in non-human primates and the results generated, although useful, may have little relevance in humans [365]. This is highlighted by the MRKAd5 vaccine designed from results in rhesus macaques showing that an Ad5/SIVgag vaccine protected against the SIV/HIV hybrid SHIV [365], but this did not hold true in human trials. Vaccine efficacy between humans and non-human primates may differ perhaps owing to inherent differences in HLA/MHC antigen presentation between species. For instance, Ad5 vaccines generate much broader CD8+ lymphocyte immune responses in monkeys than were observed in the STEP trial [351], and broader responses –especially gag-are thought to mediate the best immune control over HIV [287]. In all, although animal models will remain critical in testing the safety and comparative
immunogenicity of candidate vaccines, they are unable to predict the efficacy of vaccines in humans; only human trials can do so [365].
Chapter 2: SIGMOID Th17 POPULATIONS, THE HIV LATENT RESERVOIR AND MICROBIAL TRANSLOCATION IN MEN ON LONG-TERM ANTIRETROVIRAL THERAPY.

2.1 ABSTRACT

Objective. Th17 cells play an important role in mucosal defence and repair, and are highly susceptible to infection by HIV. Antiretroviral therapy (ART) suppresses HIV viremia and can restore CD4+ numbers in the blood and gastrointestinal mucosa, but the resolution of systemic inflammation and gut microbial translocation is often incomplete. We hypothesized that this might relate to persistent dysregulation of gut CD4+ Th17 subsets.

Methods. Blood and sigmoid biopsies were collected from HIV-uninfected men; chronically HIV-infected, ART-naïve men; and men on effective ART for >4 years. Sigmoid provirus levels were assayed blind to participant status, as were CD4+ Th17 subsets, systemic markers of microbial translocation and cellular immune activation.

Results. There was minimal CD4+ Th17 dysregulation in the blood until later stage HIV infection, but gastrointestinal Th17 depletion was apparent much earlier, along with increased plasma markers of microbial translocation. Plasma LPS remained elevated despite overall normalization of sigmoid Th17 populations on long term ART, although there was considerable inter-individual variability in Th17 reconstitution. An inverse correlation was observed between plasma LPS levels and gut Th17 frequencies, and higher plasma LPS levels correlated with an increased gut HIV proviral reservoir.

Conclusions. Sigmoid Th17 populations were preferentially depleted during HIV infection. Despite overall CD4+ T cell reconstitution, sigmoid Th17 frequencies after
long-term ART were heterogeneous and higher frequencies were correlated with reduced microbial translocation.
2.2 INTRODUCTION

Human immunodeficiency virus type 1 (HIV) and simian immunodeficiency virus (SIV) infections are characterized by a dramatic depletion of CD4+ T cells in the gastrointestinal mucosa (gut) and a more gradual CD4+ T cell decline in the peripheral blood [227], culminating in the spectrum of opportunistic infections and malignancies that define the Acquired Immunodeficiency Syndrome (AIDS). Important predictors of the rate of this CD4+ decline in the peripheral blood are the plasma HIV RNA viral load (VL) and the degree of systemic immune activation [10, 366]. The early depletion of CD4+ T cells from the gut mucosa may play a role in driving systemic immune activation, since this mucosal immune damage impairs the normal barrier function of the gut and allows increased translocation of pro-inflammatory bacteria from the gut lumen into the systemic circulation [227].

IL-17 producing CD4+ T cells (Th17 cells) safeguard the integrity of mucosal surfaces by inducing proliferation of enterocytes, promoting the recruitment of neutrophils to areas of fungal/bacterial infection, and mediating their subsequent activation to produce antimicrobial defensins [230, 231, 272]. In keeping with this, autoimmune Th17 deficiency leads to chronic mucosal and cutaneous candidiasis [367]. In this context they appear to play an opposite role to CD25+FoxP3+ regulatory T cells (Tregs), which dampen potential harmful host immune responses to commensal bacteria [368]. Therefore the mucosal balance between Treg and Th17 CD4+ subpopulations may facilitate local adaptive effector T cell responses while allowing for tolerance of normal bowel flora in the gut [369-371]. However, Th17 cells are highly susceptible to HIV/SIV infection and are preferentially
depleted during HIV infection [160, 164, 233]. This is associated with disease progression in both humans [372] and pathogenic SIV non-human primate models, while mucosal Th17 numbers are maintained in non-pathogenic SIV infection of African green monkeys and sooty mangabeys [164, 373]. Therefore, HIV-associated Th17 depletion in the gut mucosa may be an important cause of increased systemic microbial translocation [164, 233, 374].

The goal of effective antiretroviral therapy (ART) is an undetectable HIV plasma VL and normalization of the peripheral blood CD4+ T cell count. Although the reconstitution of gut CD4+ T cell numbers is slower [257] these may eventually reach near-normal levels [259]. Nonetheless, a substantial proviral reservoir persists in the gut mucosa [255] and microbial translocation and systemic immune activation may remain increased despite ART [375, 376]. The latter may drive adverse health outcomes that persist despite effective ART, such as cardiovascular disease and neurocognitive dysfunction [375, 377]. We hypothesized that mucosal Th17 depletion might persist on long-term ART despite overall CD4+ T cell reconstitution, and that this might allow ongoing microbial translocation. To address this hypothesis, we examined the impact of long-term ART on Th17 populations in blood and the sigmoid colon.
2.3 MATERIALS AND METHODS

2.3.1 Ethics statement.
All participants provided written informed consent, and the study protocol was
reviewed and approved by the Research Ethics Boards at St. Michaels’ Hospital,
Toronto and the University of Toronto.

2.3.2 Participant recruitment and study groups.
Participants were enrolled through the Maple Leaf Medical Clinic in Toronto,
Canada, and consisted of three groups: (1) therapy-naïve men who had been HIV-
infected for at least 6 months (Chronic Infection; CI); (2) HIV-infected men on ART
with an undetectable HIV plasma VL for at least 4 years (Long-term Suppressed;
LTS); and (3) HIV uninfected (HU) men. To further assess the impact of HIV
infection on T cell subsets, HIV infected participants were defined as having early or
advanced HIV infection, based on a cut-off of 350 CD4 T cells/mm³, the threshold at
which ART initiation is currently recommended [378]. All subsequent immune
assays were performed by research personnel blinded to participant group.

2.3.3 Processing of peripheral blood and sigmoid biopsies.
Blood was collected by venipuncture into Acid Citrate Dextran solution A (BD
Bioscience, La Jolla, CA) and peripheral blood mononuclear cells (PBMC) were then
isolated via Ficoll-Hypaque density centrifugation as previously described [379].
Sigmoid pinch biopsies were obtained approximately 25-30cm from the anal verge,
and mononuclear cells were isolated by Collagenase type II digestion (Sigma Aldrich
St Louise, MI) as previously described [259]. Briefly, biopsies were incubated first in
a 0.5 µg/ml and then a 1.0 µg/mL collagenase solution on a shaking heating-block at
37°C for 30 minutes each.
2.3.4 Immune studies.
Isolated mononuclear cells from blood and the sigmoid colon were stimulated with staphylococcal enterotoxin B (SEB; 3µg/ml) (Toxin Technologies, Saratosa, FL) for 1 hour at 37°C and 5% CO2. 1 µg/ml of Brefeldin A was added, and cells incubated for 5h at 37°C and 5% CO2. Cells were then permeabilized and stained with combinations of fluorochrome-labeled monoclonal antibodies specific for CD3, CD8, CD4, CD69, HLA DR, CD25, FoxP3, IFNγ and IL17A, (BD BioSciences and eBioscience, San Diego, CA). Samples were acquired on a FACSCalibur flow cytometer (BD Systems) and data analysis performed using Flow Jo analytical software version 7.2.4 (Treestar, Ashland, OR). Tregs were defined as CD4+ T cells co-expressing CD25 and FoxP3; Th17 cells were defined as CD4+ T cells producing IL17A upon SEB stimulation.

2.3.5 Plasma markers of microbial translocation.
Lipopolysaccharide (LPS) levels were measured using the limulus amebocyte lysate assay kit (Cambrex; Charles City, IA). Plasma samples were diluted 20% in endotoxin-free water (Cambrex), and heated to 80°C for 15 minutes to inactivate plasma proteins, then LPS levels were assayed using manufacturer’s instructions. We also measured levels of soluble CD14 (sCD14) glycoprotein (R&D Systems; Minneapolis, MN) and Endotoxin core binding IgM antibodies (EndoCAb; Hycult Biotech) using commercially available ELISA kits. All samples were run in duplicate and background readings were subtracted from reported values. All assays were run as per manufacturer’s instructions.
2.3.6 Measurement of HIV proviral DNA.
CD8+ T-cells were depleted from gut mononuclear cells using a column-based cell separation technique (StemCell Technologies, Vancouver, BC, Canada) and proviral DNA levels were assayed as previously described [259]. Briefly, to determine the proviral HIV DNA copies per million CD8+ depleted gut T-cells, genomic DNA was isolated using the Puregene DNA isolation kit according to the manufacturer’s specifications (Gentra, Minneapolis, MN). 1µg of DNA was then used as template for real-time PCR in an iCycler (Bio-Rad, Hercules, CA). The amplification reaction was carried out in triplicate using 0.5 µM primers, 0.2µM fluorescent probe, 0.8mM dNTPs, 5mM MgCl₂, and 2.5U Platinum Taq Polymerase (Invitrogen, Carlsbad, CA) in 50µl total volume. The following primers were used:

5’GGTCTCTCTGGTTAGACCAGAT-3’ (5’ primer) and 5’-CTGCTAGAGATTTTCCACACTG-3’ (3’ primer) along with the fluorescent probe 5’-6FAM-AGTAGTGTTGGCCCCGTCTGTT-TAMRA-3’. PCR conditions consisted of a denaturation step at 95°C for 3 min followed by 45 cycles of 15 sec at 95°C and 1 min at 59°C. Standard curves were prepared as previously described [259] and the copy number of HIV DNA per 1x10⁶ CD8+ depleted T-cells was reported.

2.3.7 Statistical analysis.
Statistical analysis was performed using SPSS 17 (SPSS Chicago, IL). Non-parametric analysis (Mann-Whitney with asymptotic 2-tailed tests) was used to compare groups, and correlations were examined by Spearman’s rank regression analysis.
2.4 RESULTS

2.4.1 Study participants

Forty-one participants were recruited within three groups: chronically HIV-infected, therapy-naïve participants (CI; n=16; table 1a); participants on long-term suppressive ART (LTS; n=15; Table 1b); and HIV uninfected participants (HU; n=10). CI participants had a median absolute CD4+ T cell count of 325/mm³ (range 105-990/mm³), and a blood viral load of 48,009 (range 2,766-500,000) HIV RNA copies/mL. LTS participants had been on therapy for a median of 96 months (8 years; range 51-207 months), with no detectable plasma HIV RNA for 80 months (6.7 years; range 48-129 months). Most LTS participants had initiated therapy with a nadir CD4+ T cell count <350/mm³ (11/15) or after an AIDS-defining illness (ADI; 2/16); three participants had initiated therapy during earlier stages of HIV infection, with a mean absolute CD4+ T cell count of 590/mm³.

2.4.2 Impact of untreated HIV infection on blood Th17 subsets and plasma markers of microbial translocation.

Overall, chronic untreated HIV infection was not associated with differences in the frequency of blood Th17 cells when compared to HU participants (median, 0.28% vs. 1.01%; p=0.109). However, significantly reduced blood Th17 frequencies were seen in CI participants with a blood CD4+ T cell count below 350 /mm³, the threshold at which ART is currently recommended [378] (CD4+ T cell count <350/mm³ vs. HU; median Th17 0.12% vs. 1.01%, p=0.013; Figure 2.1a, left 3 groups). In keeping with the opposing effects of HIV infection on Th17 and Treg subsets, the blood Th17/Treg ratio was reduced almost twenty-fold in therapy-naïve
participants (median HU vs. CI 5.7 vs. 0.3, p=0.005). Again, this decrease was confined to CI participants with late stage HIV infection (median Th17/Treg ratio 0.2 vs. 2.3 during untreated early HIV, p=0.020, and vs. 5.7 in HU, p=0.003; Figure 2.1b, left 3 groups). The blood Th17/Treg ratio was not associated with HIV viral load (data not shown), and no differences in IFNγ producing CD4+ T cells (Th1 subsets) were seen across study groups (data not shown).

As has been described elsewhere [239], HIV-infected ART-naïve participants had increased plasma levels of LPS compared to uninfected controls (median, 1.29 vs. 1.18 EU/ml; p=0.007; Figure 2.2a), and soluble CD14 (sCD14) levels were also elevated (median, 1.40 vs. 0.71µg/ml in HU; p=0.002; Figure 2.2c). No differences were evident in both Endotoxin Core IgM Antibodies (EndoCAb IgM) titres and LBP levels in plasma (both p>0.15; Figure 2.2 b, d), and no associations with disease stage or Th17 subsets were seen. Plasma LPS was not correlated with these immune subsets, but was associated with an increased blood HIV viral load (Spearman’s rho=0.615, p=0.044; Figure 2.2e). T cell activation was apparent in CI participants (HLA-DR+CD4+, 1.73% vs. 0.56% in HU, p= 0.003; HLA-DR+CD8+, 7.37% vs. 0.99%, p=0.003; CD69+CD4+, 1.74% vs. 0.39%, p= 0.010; Figure 2.3).

2.4.3 Impact of antiretroviral therapy on blood Th17 subsets and markers of microbial translocation.

After long-term ART blood Th17 frequencies remained reduced compared to HU controls (median, 0.22% vs. 1.01% in HU, p=0.020). This reduction was limited to participants who had initiated ART during advanced infection (CD4 <350/mm³; median 0.17% vs. 1.01% in HU; p=0.008), and in the three participants where ART
had been initiated early the blood Th17 frequencies were similar to HU controls (median 0.80% vs. 1.01%; p=0.732; Figure 2.1a, 2 right panels). While the Th17/Treg ratio in the LTS group was comparable to HU participants overall (1.8 vs. 5.7; p=0.176), there was a trend to a lower ratio in participants who had initiated ART during late stage infection (CD4 <350/mm³: median 0.68 vs. 5.7; p=0.081; Figure 2.1b, 2 right panels) but not those where treatment was started earlier (CD4 >350/mm³; median 8.0 vs. 5.7; p=0.909).

LPS levels remained elevated in LTS compared to HU participants (median 1.45 vs. 1.18 EU/ml; p=0.002) and were comparable to ART-naive individuals (median 1.45 vs. 1.29 EU/ml in CI; p=0.106; Figure 2.2a). EndoCAb IgM titres were lower in the LTS group than either HU (median 26.25 vs. 88.5 MMU/ml; p=0.017) or CI participants (median 26.25 vs. 77.0 MMU/ml, p=0.035; Figure 2.2b). sCD14 levels were reduced in the LTS group compared to CI participants (median 1.06 vs. 1.40 µg/ml; p=0.039), but remained higher than uninfected controls (median LTS vs. HU, 1.06 vs. 0.71µg/ml; p=0.047; Figure 2.2c). Markers of microbial translocation were not directly correlated with systemic immune activation in the LTS group (all p>0.2), and CD4+ T cell expression of HLA-DR and CD69 were similar in LTS and HU participants (HLA-DR, 0.49% vs. 0.56% in HU, p=0.953; CD69, 1.03% vs. 0.39% in HU, p= 0.266; Figure 2.3).

Overall, plasma LPS levels remained elevated and blood Th17 frequencies depressed despite ART. Therefore, we hypothesized that Th17 dysregulation might persist in the gut mucosa despite ART, allowing for ongoing microbial translocation.
2.4.4 Effect of HIV infection and therapy on Th17 CD4 subsets in the sigmoid colon.

We collected sigmoid biopsies from a subset of recruited participants in whom blood samples were also obtained; HU (n=5), CI (n=7) and LTS (n=8). Sigmoid Th17 frequencies were reduced during untreated HIV infection (median LTS vs. HU, 0.25% vs. 0.74%; p=0.042; Figure 2.4a, centre group), with a dramatic reduction in the Th17/Treg ratio (median ratio 0.2 vs. 1.7 in HU, p=0.004; Figure 2.4b, centre group). In contrast to blood, Th17 dysregulation in the sigmoid colon was apparent during early as well as later HIV stages (median Th17/Treg ratio, 0.1 in CI early stage vs. 0.2 in CI late stage; p=NS). There was no direct correlation between these immune perturbations and direct or indirect markers of microbial translocation (all p>0.2).

In the long-term ART group no differences were apparent between HIV-infected and HU participants in either the mucosal Th17 frequency (Th17 median 1.40% vs. 0.74% in HU; p=0.558; Figure 2.4a, right) or the sigmoid Th17/Treg ratio (median 2.4 vs. 1.7 in HU; p=0.884; Figure 2.4b, right). However, there was substantial inter-individual heterogeneity within the Th17 subset: sigmoid Th17 frequencies in 4/8 ART-treated participants were equal to or higher than uninfected participants, while the remaining 4/8 participants had relatively reduced Th17 frequencies (Figure 2.4a). Participants with sigmoid Th17 frequencies above the median tended to have higher blood CD4+ T cell counts at the time of sampling (715 vs. 545 CD4 counts/mm³; p=0.144), and to have been on ART longer (159 months vs. 99 months; p=0.149). No differences were apparent in nadir CD4+ T cell counts, disease stage prior to ART initiation or frequency of sigmoid CD4+ T cell levels following therapy between these groups (data not shown). Sigmoid T cell subsets in
the LTS group were not associated with plasma markers of microbial translocation, although there was a weak inverse relationship between Th17 frequencies and plasma LPS levels (Spearman’s rho= -0.476, p=0.233).

2.4.5 Associations of HIV proviral DNA in the sigmoid colon after long-term therapy. The HIV proviral load was assayed in the sigmoid colon of all participants on long-term ART (n=8). Gut provirus was detectable in all participants (median 240.95 HIV DNA copies/10^6 CD8 depleted cells, range 9.1-899.0), although provirus levels tended to decrease with the duration of ART (Spearman’s rho= -0.65, p=0.058). Two major associations were apparent. First, the sigmoid HIV proviral load was directly correlated with the plasma LPS level (Spearman's rho=0.762, p=0.028; Figure 2.5a) and tended to be inversely associated with the plasma EndoCAb IgM titre (Spearman’s rho= -0.515, p=0.192). There was no association with plasma sCD14 levels (Spearman’s rho= -0.167, p=0.693). Secondly, the size of the sigmoid provirus reservoir was inversely correlated with the sigmoid Th17 frequency (Spearman’s rho= -0.762, p=0.028; Figure 2.5b). This was specific to the Th17 subset, as there was no association with the degree of overall CD4+ reconstitution (Spearman’s rho= -0.286, p=0.493; Figure 2.5c). LTS participants with sigmoid Th17 frequencies above the median had a reduced sigmoid HIV proviral load (665.1 vs. 82.0 /10^6 CD8+ depleted cells, p=0.043; Figure 2.5d). No significant associations were seen with gut Treg frequencies or markers of inflammation, or with any blood T cell subsets (data not shown).
2.5 DISCUSSION

HIV infection is associated with a marked depletion of gastrointestinal CD4+ T cells and with gut-systemic translocation of luminal bacteria [239]. The latter may be an important driver of systemic immune activation and HIV immunopathogenesis [239, 380-382], and in some studies has been shown to persist despite effective HIV therapy [383, 384]. The cause of persistent microbial translocation in participants on long term ART is not clear, and we hypothesized that this might relate to persistent dysregulation of the Th17 subset in the gut mucosa. Our studies confirm that untreated HIV infection is associated with mucosal Th17 dysregulation and gut microbial translocation [239, 381, 382], and found that there was complete overall restoration of HIV-associated defects in sigmoid Th17 and Treg subsets in our long-term ART (LTS) group. However, this overall restoration masked substantial intra-group heterogeneity, and within the LTS group incomplete restoration of gut Th17 frequencies (but not overall CD4+ T cell restoration) was directly associated with higher sigmoid provirus levels, and the latter was also correlated with plasma LPS levels.

Recent studies suggest that direct effects of the virus on gut epithelial cells and the mucosal basement membrane may be an important factor contributing to microbial translocation [385]. We only saw a weak direct association between gut Th17 frequencies and microbial translocation, but our sample size was small and this may be an important area for future research. The reasons underlying heterogeneity in gut Th17 reconstitution in our cohort were not clear, although
incomplete reconstitution tended to be associated with a shorter duration of ART and a lower blood CD4+ T cell count at the time of sampling.

Significant differences were apparent in the effects of HIV infection and therapy on T cell subsets in the blood and gut. Both Th17 frequencies and Th17/Treg ratios were normal in the blood during the early stage HIV, but were dysregulated in participants with a CD4+ T cell count below 350/mm$^3$ and remained low in the blood of such participants even after long-term ART. In contrast, a dramatic reduction in the sigmoid Th17/Treg ratio was apparent at all stages of chronic untreated HIV infection, but there was near-complete resolution of these defects on therapy (albeit with the significant inter-individual heterogeneity discussed above). The clinical implications of persistent blood Th17/Treg dysregulation in the context of delayed ART initiation are not clear, and this may represent another important area for future research. These data suggest that early ART initiation may result in more complete reconstitution of the systemic CD4+ T cell functional repertoire. Furthermore, since these blood CD4+ T cell subsets were only altered during relatively advanced HIV infection this may explain, at least in part, the discrepancy in findings of previously published studies regarding the impact of HIV infection on these CD4+ functional subsets [164, 386-388].

Our finding that there was no reduction in LPS levels, a plasma marker of microbial translocation despite effective ART was unexpected, and is at odds with some [239, 376, 389, 390] but not all [383, 384] prior studies. Elevated LPS levels in this cohort were confirmed by a second blinded ELISA and were also associated with a significant depletion of EndoCAb titres. The latter may result from saturation
and/or depletion of endotoxin-specific antibodies and is also thought to indicate increased microbial translocation [391]. In addition, the associations of plasma LPS levels with both the sigmoid provirus reservoir and mucosal Th17 frequencies strongly suggests that this was a true observation, although clearly not one that is applicable to all treated cohorts [239, 376, 389, 390].

There are several limitations to our study. Our sample size was relatively small, and so these observations need to be confirmed by larger studies. The flow cytometric studies were run on a 4-colour FACSCalibur, which limited the number of surface markers that could be examined and meant that we could not ascertain whether alterations in specific T cell memory subsets were associated with the differences in Th17 frequency and Th17/Treg ratio. Our FoxP3 staining was dimmer than has been reported elsewhere (data not shown), likely since specialized FoxP3 staining buffers were not used. However, since the same protocol was used throughout and all data were acquired and analysed blindly, our results and conclusions should not be affected. Nonetheless, for this reason Treg subset-specific data is not presented, except in the context of Th17/Treg ratios.

In summary, we found that the sigmoid provirus reservoir remained high despite long-term suppressive ART, and was associated with persistently elevated gut-systemic microbial translocation and with impaired restoration of sigmoid Th17 populations. Starting ART with a blood CD4+ T cell count above 350/mm³ was associated with improved restoration of sigmoid Th17 subsets, and therefore this might be an important clinical rationale for early initiation of therapy.
Table 2.1. Clinical characteristics of antiretroviral-naïve HIV-infected participants.

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Table 2.2. Clinical characteristics of participants on long-term antiretroviral therapy.

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Abbreviations: ART, Antiretroviral therapy. ADI, AIDS defining illness. Viral load sensitivity was >50 HIV RNA copies/ml.
Figure 2.1.
FIGURE 2.1. Blood Th17 CD4R T cells following HIV infection and antiretroviral therapy. (a) Th17 frequency and (b) Th17/Treg ratio are shown in HIV-negative participants, HIV-infected, therapy-naive participants with CD4+ T-cell counts more than 350 cells/ml (early chronic infection, CI) or less than 350 cells/ml (late CI), and HIV-infected, ART-treated participants who started therapy during early or late disease (long term suppressed on ART; LTS). Horizontal lines represent median values. (c) Representative FACS dot plots show the percentage of CD4+ T cells producing IL17a after incubation with medium alone (right) or 3 mg/ml staphylococcal enterotoxin B (SEB; left).
Figure 2.2.
FIGURE 2.2. Plasma markers of bacterial translocation in HIV infection.

Levels of (a) lipopolysaccharide (LPS), (b) IgM EndoCAb, (c) soluble CD14 (sCD14) and (d) LPS binding protein (LBP) in (i) HIV negative participants (HU), (ii) HIV-infected participants who are therapy-naïve (CI), and (iii) HIV-infected participants on long-term suppressive ART (LTS). Horizontal lines represent median values. (e) In participants with untreated chronic HIV infection, LPS levels were positively correlated with HIV plasma viral load (VL).
Figure 2.3.
FIGURE 2.3. Associations of HIV and ART with systemic immune activation.

Percentages of (a) HLA-DR+CD4+, (b) HLA-DR+CD8+ and (c) CD69+CD4+ T cells are shown in HIV negative, HIV-infected therapy-naïve participants (CI) and HIV-infected participants on long-term suppressive ART (LTS), with horizontal lines indicating median values.
Figure 2.4.
FIGURE 2.4. Sigmoid Th17 CD4+ T-cell subsets following HIV infection and antiretroviral therapy. (a) Th17 frequency and (b) Th17/Treg ratio in the sigmoid colon of HIV-negative participants, HIV-infected, therapy-naive participants (CI), and HIV-infected participants on long-term suppressive ART (LTS).
Figure 2.5.
FIGURE 2.5. Associations of HIV proviral load in the sigmoid colon of long-term suppressed participants. Sigmoid HIV provirus levels were assayed in participants on ART with an undetectable plasma viral load (VL) for at least 4 years. (a) Provirus levels were positively correlated with the plasma lipopolysaccharide (LPS) level. (b) Sigmoid provirus levels were negatively correlated with the sigmoid Th17 frequency but not (c) the overall sigmoid CD4 T-cell proportion. (d) Provirus levels were significantly increased in participants with sigmoid Th17 frequencies lower than the group median.
Chapter 3: EVALUATION OF A QUANTITATIVE REAL-TIME PCR ASSAY TO MEASURE HIV-SPECIFIC MUCOSAL CD8+ T CELL RESPONSES IN THE CERVIX.

3.1 Abstract
Several candidate HIV vaccines aim to induce virus-specific cellular immunity particularly in the genital tract, typically the initial site of HIV acquisition. However, standardized and sensitive methods for evaluating HIV-specific immune responses at the genital level are lacking. Therefore we evaluated real-time quantitative PCR (qPCR) as a potential platform to measure these responses. beta-Actin and GAPDH were identified as the most stable housekeeping reference genes in peripheral blood mononuclear cells (PBMCs) and cervical mononuclear cells (CMCs) respectively and were used for normalizing transcript mRNA expression. HIV-specific cellular T cell immune responses to a pool of optimized CD8+ HIV epitopes (HIV epitope pool) and Staphylococcal enterotoxin B (SEB) superantigen control were assayed in HIV infected PBMC by qPCR, with parallel assessment of cytokine protein production. Peak HIV-specific mRNA expression of IFN-gamma, IL-2 and TNF-alpha occurred after 3, 5 and 12 hours respectively. PBMCs were titrated to cervical appropriate cell numbers to determine minimum required assay input cell numbers; qPCR retained sensitivity with input of at least 2.5x10⁴ PBMCs. This optimized qPCR assay was then used to assess HIV-specific cellular T cell responses in cytobrush-derived cervical T cells from HIV positive individuals. SEB induced IFN-gamma mRNA transcription was detected in CMCs and correlated positively with IFN-gamma protein production. However, qPCR was unable to detect HIV-induced cytokine mRNA production in the cervix of HIV-infected women despite robust detection of gene induction in PBMCs. In conclusion, although qPCR can be used to measure ex vivo cellular immune responses to HIV in blood, HIV-specific
responses in the cervix may fall below the threshold of qPCR detection. Nonetheless, this platform may have a potential role in measuring mitogen-induced immune responses in the genital tract.
3.2 Introduction

Globally, 33 million people are now infected with the Human Immunodeficiency Virus (HIV) [392] and more than 90% of new infections occur across the mucosal lining of the genital or gastrointestinal tract [34]. Women now account for most new HIV infections [34, 393-395], with acquisition usually occurring across the cervico-vaginal mucosa during sex [34]. HIV-specific T cell responses are an important component of the host immune response against HIV, and have been described in the genital mucosa of both HIV-infected and exposed but uninfected women [191, 396-399]. However, the role of genital HIV-specific cellular immunity in HIV susceptibility and transmission remains poorly defined, in part, due to methodological limitations [395, 400]. Currently, several candidate HIV vaccines aim to induce virus-specific cellular immune cytotoxic T lymphocyte (CTL) protection [376, 401-403]. Therefore, the ability to monitor HIV-specific cellular immune responses in the female genital tract will be important in monitoring and evaluating these HIV vaccines, and may help to characterize the mucosal pathogenesis of HIV transmission and susceptibility. Unfortunately, conventional cellular immune assay platforms lack the sensitivity to measure these mucosal responses in a reliable and reproducible way [404]. This is due in large part to the low cell numbers obtained from standard sampling techniques such as the cervical cytobrush or cervico-vaginal lavage [396-399]. In vitro expansion of cervical cells to overcome these limitations is possible, despite the issue of potential fungal/bacterial contamination when cervical lymphocytes are maintained in long-term culture [398]. However, cervical cell expansion precludes the assessment of ex vivo mucosal immune response frequency, and the preferential expansion of certain memory subsets using this technique means that the repertoire and memory phenotype of cervical cells obtained via expansion may not reflect
in vivo mucosal T cell populations [400, 405]. In addition, assay platforms such as ELISPOT, chromium (Cr\textsubscript{51}) lysis and proliferation (CFSE dilution or H\textsubscript{3}-thymidine uptake) have a limited capacity to measure multiple immune functions. More recently, multi-parameter flow cytometry assays in peripheral blood mononuclear cells (PBMC) from HIV infected patients have demonstrated that the breadth and quality of immune responses appear more important than the quantity of responses in HIV control [406]. Therefore relying on measurements of narrowly defined HIV-specific immune responses might overlook the spectrum of responses needed for efficient HIV control. However, the recovery of few cervical mononuclear cells (CMCs) may still challenge the feasibility of flow cytometry in measuring HIV-specific genital tract immune responses [397].

Quantitative Real-Time PCR (qPCR) is a molecular technique that might be adapted to overcome several of these obstacles. The technique is highly sensitive [407, 408], and in theory could simultaneously assay the induction of multiple immune genes in response to antigenic stimulation, despite low sample cellular input. qPCR has previously been utilized to measure basal cytokine mRNA transcript levels in unstimulated cervical cells obtained by cytobrush sampling [409, 410], as well as in clonally expanded PBMCs [408]. In addition, qPCR has frequently been used to assay systemic antigen-specific immune responses in blood [407, 408, 411]. However, there are no published reports using this platform to evaluate genital mucosal immune responses. We therefore adapted the qPCR assay to define the optimal kinetics for HIV-specific mRNA gene induction, evaluate qPCR’s suitability to measure these immune responses using cervical-appropriate reduced cell numbers in a PBMC model, and compared our gene expression results with conventional protein measurement assays. This optimized
qPCR assay was then used to assess HIV-specific cellular T cell responses in cervical cytobrush-derived T cells from HIV positive individuals.

3.3 Methods

3.3.1 Study population and ethics statement
13 HIV positive women from the Majengo-Pumwani sex-worker cohort in Nairobi, Kenya, and 5 HIV positive men from the Maple Leaf Clinic in Toronto were recruited for this study. These participants were nested in our study as part of larger immune studies in their respective cohorts. All participants provided written informed consent, and the study protocol was reviewed and approved by the Research Ethics Boards at the University of Nairobi and the University of Toronto.

3.3.2 Sample processing and in vitro antigenic stimulation
Blood samples and cervical cytobrush specimens were obtained from HIV infected participants in Canada and Kenya who were not actively menstruating. All HIV-infected participants had a blood CD4+ T cell count above 350/mm³, and were antiretroviral therapy naïve. Sample collection and processing was performed as previously described [397]. Briefly, cervical samples were obtained by first scraping the external cervical os using a plastic cell scraper (Benzi Jinshuo Applicator Co) and a cervical histobrush (Histobrush Spectrum Lab) was then inserted into the cervical os and rotated through 360°. A mini-cervical lavage was then performed using 1ml PBS to collect loosened cells. All genital tract samples were then combined into a 50ml sample collection tube containing 10mls of PBS and transported to the laboratory on ice within 2 hours. Blood was collected by venipuncture into heparin Vacutainer tubes (BD Bioscience). Peripheral blood mononuclear cells (PBMC) and cervical mononuclear cells
(CMCs) were isolated by density gradient centrifugation. Samples were washed twice and resuspended in RPMI 1640 media with 10% heat inactivated fetal bovine serum, 1% Penicillin and 1% streptomycin (R10; FBS; Sigma). PBMCs were then used fresh for stimulation experiments or cryopreserved at -150°C in FBS containing 10% DMSO until use. All cervical T cell assays were performed using freshly obtained cytobrush samples. Both PBMC and CMC numbers were determined by light microscopy, based on trypan blue exclusion.

PBMC and CMC samples were incubated with: i) a pool of 49 immunodominant CD8+ optimized HIV epitopes (HIV epitope pool) restricted by a range of class I HLA alleles, at a concentration of 2.0ug/ml/peptide; ii) R10 tissue culture medium alone or iii) staphylococcal enterotoxin B superantigen (SEB; Sigma) at 3.0ug/ml. The use of predefined, immunodominant CD8+ HIV epitopes from multiple HIV clades and restricted by a variety of different HLA types would allow for measurement of CD8+ immune responses in most participants. Kinetics and sensitivity experiments utilized a variety of input cell numbers and incubation durations (see results) at 37°C in 5% CO₂. Cell pellets and supernatants were harvested by centrifugation at 10,000 rpm for 5 minutes and placed in RNA later solution (Ambion). Culture supernatants were stored as is at -80°C prior to qPCR or protein measurements.

3.3.3 RNA extraction
RNA was extracted from cell pellets and genomic DNA was eliminated using the Qiagen RNeasy Plus Kit (Qiagen) as per manufacturer’s instructions. RNA was eluted in 30ul of nuclease free water. The quantity of extracted RNA was evaluated using the Nanodrop ND1000 (Thermofisher Scientific). To ensure the integrity of RNA, only samples with a 260/280 OD ratio of between 1.7-2.0 were used.
3.3.4 Reverse Transcription and qPCR

Complementary DNA (cDNA) was created by adding 10ng of RNA to the reverse transcription master mix and reverse-transcribed using the Superscript III Kit (Invitrogen) as per manufacturer’s instructions. The cDNA product was then diluted 1:7 in nuclease free water and the samples stored at -20°C. Genomic DNA (gDNA) standards were isolated from purified placental tissue and used as a universal standard over a 7-log (36 ng – 0.4 ng) dilution range to calculate relative gene expression levels [412]. A broad dynamic range of standards was used to account for the varied expression levels of different targeted genes. Single intra-exon gene-specific primers were generated using Primer Express Software (Perkin Elmer Applied Biosystems). Each primer pair was evaluated for specificity by melting curve analysis to ascertain that only one product was amplified and a BLAST search was performed to confirm that primer sequences amplified only the target gene of interest. Primer pairs generating multiple peaks (indicative of primer-dimer artefacts or non-specific gene amplification), or primers with less than 90% primer-pair amplification efficiency were discarded. The final primer pairs used are listed in Table 1.

The qPCR assay was performed in a 10ul volume in a 384-well plate. ABI Prism 7900HT (Applied Biosystems) and SYBR green fluorescent dye was used to detect amplification under the following amplification conditions: i) 1 warm-up cycle for 2 min at 50°C ii) 1 pre-amplification cycle for 10 minutes at 95°C, 40 amplification cycles for 15 seconds at 95°C and for 1 minute at 60°C, iii) end-amplification cycle for 15 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 95°C. All reactions were run in triplicate with a non-template control blank) for each primer pair to control for contamination or primer-dimers. Quantitative PCR values crossing threshold (Ct) were obtained during the
exponential amplification phase using SDS 2.3 Software (Applied Biosystems) analysed for respective gene quantities and exported into Microsoft Excel for further analysis.

### 3.3.5 Cytokine Protein Quantification

Cytokine concentrations (pg/ml) from culture supernatants were determined using the flow cytometry based Cytokine Bead Array Kit (CBA; BD Biosciences) or the Searchlight Multiplex-ELISA (M-ELISA; Aushon Biosystems) as per manufacturer’s instructions. Interferon Gamma (IFNγ), Tumour Necrosis Factor alpha (TNF α ) and Interleukin 2 (IL-2), cytokines were assayed. The CBA limit of detection for IFNγ, TNF α , IL-2 was 20 pg/ml for all evaluated analytes while the M-ELISA limit of detection for IFNγ, TNF α , and IL-2 was 0.5, 0.5, 0.1 pg/ml respectively. CBA samples were run in unicate while M-ELISA samples were assayed in duplicate. Background (basal) cytokine levels were subtracted from presented antigen and superantigen specific cytokine levels.

### 3.3.6 Statistical analysis

Gene quantities were calculated from standard curves in arbitrary units and values were then analysed using the NormFinder model within a Microsoft Excel add-on (http://mdl.dk/publicationsnormfinder.htm). The most stable housekeeping gene was selected to normalize target gene expression levels as described elsewhere [407]. Briefly, to assess antigen-specific gene induction, results for each target gene were normalized by dividing the amount of the amplified gene target by the amount of the housekeeping gene for each sample. This ratio was then further divided by the respective negative control (medium alone) at the given time point, and reported as a fold gene-induction ratio [407]. Non-parametric Mann-Whitney U and Wilcoxon Signed Ranks statistical analyses were
performed using SPSS Version 17.0 software (SPSS Inc.) to analyse the relationship between gene and protein expression over different treatments.

3.4 Results
3.4.1 Selection of housekeeping genes
Reliable comparisons of gene expression between samples require the normalization of input amounts, reverse transcription and amplification reaction efficiencies. PBMCs and CMCs from 5 HIV infected individuals were incubated for 6 hours in R10 medium alone, HIV epitope pool, or SEB, and mRNA was evaluated for the expression of the commonly-used housekeeping genes including β-actin, β-2-Microglobulin (B2M), TATA box binding protein (TBP), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and human hypoxanthine phosphoribosyltransferase (HPRT) [413, 414]. Housekeeping gene expression stability across different tissues and treatments was evaluated using the NormFinder analysis algorithm which identified β-actin as the most stable housekeeping gene in PBMCs (stability value, 1622.17), while GAPDH was the most stable in CMCs (stability value, 104.92). In PBMCs, the top 4 housekeeping genes appeared to have a narrower spread in stability values (range, 254.6 stability units) suggesting their stability their expression remains constant in this cell type. However in CMCs, GAPDH housekeeping gene emerged as the most stable gene with no closely comparable housekeeping gene among the top 4 evaluated genes (range, 433.8 stability units). This finding emphasizes the need to validate candidate housekeeping genes in each sample source of interests. In both cell types, B2M gene was the least stable. Table 2 summarises the ranking of housekeeping genes from the most to least stable. GAPDH and β-actin genes were selected as our respective reference.
housekeeping gene for normalization of cytokine gene induction in CMC and PBMC cell types respectively.

3.4.2 Time course of HIV-specific mRNA induction and cytokine release

To characterize virus-specific cellular T cell cytokine induction kinetics, one million PBMCs from 4 HIV positive individuals were stimulated with the predefined HIV CD8+ peptide epitope pool for 12 hours; and ex vivo cultured cells were harvested at hourly intervals for the first six hours and then once every two hours up to 12 hours. At this point the experiment was terminated since our goal was to mimic the time course of cervical T cell assays, and in our experience these may become contaminated in the context of more prolonged incubation. mRNA and protein levels of IFNγ, TNFα and IL-2 were quantified in parallel. Cytokine mRNA and protein kinetics were defined in PBMCs because of relative abundant immune cell yields in comparison to cervical cytobrush samples.

IFNγ mRNA was the most abundantly induced cytokine in all PBMC stimulation conditions, followed by TNFα and IL-2 respectively. Median HIV-specific mRNA induction profiles differed for each cytokine (Figure 3.1): IFNγ peaked after 3 hours of stimulation (fold induction 7.8) and IL-2 peaked after 5 hours (fold induction 3.9). TNFα displayed a bimodal induction profile with peaks, with the first mRNA induction peak occurring after 5-6 hours (fold induction 3.0-3.3 fold) and the second after 12 hours (fold induction 3.7). In contrast, supernatant protein levels of both IFNγ and TNFα increased steadily throughout the 12-hour experimental period. No IL-2 protein was detected in cell supernatants despite early mRNA induction.
Based on the above optimal cytokine kinetics, we selected to incubate PBMCs and CMCs with HIV epitope pool for 6 hours. This secondary optimal time point would allow for maximal measurement of all cytokine gene induction levels and protein production amounts simultaneously.

3.4.3 Input cell numbers and measurement of cellular T cell responses by qPCR

We next examined the ability of the qPCR assay platform to detect HIV-specific cellular T cell responses in the context of limited input cell numbers. Five chronically HIV-infected, antiretroviral therapy-naïve participants with >350 CD4+ T cell counts/mm³ were randomly selected for these assays. All participants demonstrated robust HIV-specific immune responses with an input cell number of 1x10⁶ PBMC/well. PBMCs were plated at various concentrations (1x10⁶, 1x10⁵, 7.5x10⁴, 5.0x10⁴, 2.5x10⁴ and 1x10⁴ input cells/experiment) to model expected ranges of cervical cell yields from cytobrush sampling, incubated for 6 hours with R10 medium alone, SEB or the predefined HIV epitope pool, and IFN-γ mRNA induction was quantified by qPCR. Robust detection of HIV-specific IFNγ mRNA was seen across the full range of input cell numbers (Figure 3.2a), with median HIV-specific mRNA induction levels ranging from 4.2 fold (at 1x10⁶ input PBMCs) to 3.2 fold (with 1x10⁴ input PBMCs). Likewise, SEB-induced IFNγ mRNA induction levels remained constant across higher input cell numbers, from 91.6 fold (at 1x10⁶ input PBMCs) to 73.7 fold (at 2.5x10⁴ input PBMCs). However, SEB-induced IFNγ induction dropped off rapidly when less than 2.5x10⁴ input cells/experiment were utilized, to just 6.0 fold above background (Figure 3.2b), but was still higher than HIV antigen specific induction levels at the same cell concentration.
These findings suggest that even at low numbers of input cells, modeled after cervical cytobrush cell yields, qPCR is capable of detecting both antigen and superantigen induced cytokine mRNA.

3.4.4 Evaluation of HIV-specific cervical cell immune responses using qPCR

Having established optimal cytokine kinetics and cell number sensitivity in PBMC, we next evaluated the ability of the qPCR platform to measure ex vivo cervical HIV-specific immune responses. Cervical cytobrushes and PBMCs were collected from 13 HIV-infected women in whom robust HIV-specific IFNγ T cell responses had been detected in blood with an input number of 1x10^6 PBMC/well. Cytokine gene induction was assayed by qPCR after 6 hours of incubation with media alone, SEB or HIV epitope pool, and cytokine protein levels in supernatant were assayed in parallel by M-ELISA.

Following stimulation with HIV epitope pool, induction of IL-2 (p=0.042) but not IFNγ or TNFα (IFNγ, p=0.138; TNFα, p=0.144; Wilcoxon Signed Ranks test) was observed compared to medium alone in CMCs. Overall however, median HIV-specific induction in CMCs for all genes remained at 1.0 fold for all cytokines (Figure 3.3) suggesting that qPCR was unable to detect any HIV-specific immune responses in the genital tract. Nonetheless, robust detection of HIV-specific cellular T cell responses was observed in PBMCs from the same participants (median fold induction; IFNγ, 6.3; TNFα, 2.33; IL-2, 1.3; see Figure 3.3). CMC incubation with SEB significantly induced mRNA expression of IFNγ above background (median 5.0 fold, p=0.012), TNFα (median 1.0, p=0.043) and IL-2 (median 1.9 fold, p=0.017) as evaluated using the Wilcoxon Signed Ranks test (Figure 3.4). In addition, SEB-induced cervical IFNγ mRNA expression was
strongly associated with supernatant cytokine levels measured by multiplex ELISA (Spearman’s rho = 0.780, p=0.005; Figure 3.5) but this was not the case for IL-2 or TNFα levels. In PBMCs, the qPCR platform readily detected SEB-induced cytokine expression above background (IFNγ median, 148.8 fold, p=0.001; IL-2 median, 130.9 fold, p=0.008; TNFα median, 7.4 fold, p=0.001; Wilcoxon Signed Ranks test; Figure 3.4).

After dividing the total cytobrush CMC yield into 3 wells for each treatment, median CMC cell counts after Ficoll separation were 1.93 x10^5 (range 5.33x10^4 -1.33x10^6 cells) per experiment. There was a positive association between CMC numbers and the induction of IL-2 gene expression by the HIV epitope pool (Spearman’s rho = 0.879; p<0.001) and by SEB (Spearman’s rho = 0.611; p=0.035). However, no association was observed between CMC numbers and the induction of either IFNγ or TNFα expression by the HIV epitope pool or SEB (all p≥0.1). In addition, input CMC numbers did not correlate with cervical HIV-specific or SEB-induced supernatant cytokine levels by multiplex ELISA (all p>0.4). Therefore, observed IFNγ and TNFα cytokine levels appear to be independent of input cervical cell numbers.
3.5 Discussion

Assay platforms that can reliably quantify HIV-specific cellular immune responses in the genital tract are urgently needed to monitor the immunogenicity of candidate HIV vaccines and elucidate the mucosal immune correlates of HIV transmission and susceptibility. Inherent limitations in sensitivity, ability to measure polyfunctional immune responses and low cervical cell yields often precludes the use of conventional immune assays in this context. Therefore, we designed a qPCR assay with the goal of measuring \textit{ex vivo} HIV-specific cellular T cell responses from cervical cytobrush specimens. The evaluated qPCR assay was robustly able to quantify SEB mediated cytokine induction in both CMCs and PBMCs despite low input cell numbers. However, HIV-specific cellular T cell responses could only be measured in PBMCs.

It is unclear why the qPCR assay was unable to detect HIV-induced cellular T cell responses in cervical cells, particularly in light of the sensitivity demonstrated at low PBMC input numbers. Cervical HIV-specific T cell responses have been found to mirror those present in the blood [199, 397-399], with a frequency similar to or greater than those in blood [397] and a common ontogeny [199]. While this is not a universal finding [396], it seems unlikely that a true discordance in blood-cervical T cell responses would account for the failure of this QPCR-based technique to detect responses in all 13 participants studied, particularly given the robust T cell responses that were seen in PBMC. Instead, an insufficient sensitivity of the assay to detect responses with this input number of cervical cells seems more plausible. The lack of measurable HIV-specific mRNA immune responses in the genital mucosa may also be due to the lower relative proportion of cervical mononuclear cells that are CD8+ T cells, compared to blood. Less than 5% of cervical mononuclear cells collected via cytobrush sampling are actually T
cells [398], while blood CD8+ T cell populations range from 12-23% in HIV infected individuals [415]. The reduced CD8+ T cell proportion may have resulted in diminished qPCR assay sensitivity regardless of high overall cytobrush cervical cell yields. In our study total input cell numbers were counted by trypan blue exclusion on a haemocytometer, and so we were not able to determine the precise proportion of mononuclear cells that were CD8+ T cells in either compartment. This limitation would be better addressed by enumerating T cell subsets via flow cytometry assays, such as the Guava automated cell counter [416]. In addition, the use of invasive or pooled samples from multiple genital sampling techniques, including cervical biopsies, cervicovaginal lavage or the collection of two or more cytobrushes may increase yields of cervical mononuclear cells [397, 417]. Lastly, PCR amplification inhibitors in cervical derived cells have been reported elsewhere [391, 418, 419], and this may also have diminished the ability to detect cellular immune responses in the genital tract via qPCR. However, the identification and impact of mucosal qPCR inhibitors on measuring cervical cellular immune responses were not evaluated here and require further investigation to ascertain their impact.

Despite the lack of HIV-specific immune responses, SEB induced responses in CMCs were detected by qPCR in most (but not all) participants, indicating that the assay was functional in cervical cells. SEB cross-links T cell receptors (TCRs) and MHC complexes activating both CD4 and CD8 T cells regardless of antigen specificity [420], unlike the 8-mer HIV epitope pool that only stimulates HIV-specific CD8+ T cells [192]. The broader immune activation profile afforded by SEB may therefore facilitate enhanced detection of mRNA induction at the cervical mucosa. In addition, the higher
affinity constant for SEB-TCR binding and the mechanism of SEB cross-linking and stabilization of the TCR-MHC during activation may result in higher avidity for SEB superantigen binding compared to individual peptide-mediated MHC-TCR interactions [177]. Consequently, greater T cell activation may have induced higher cytokine mRNA levels following stimulation with SEB superantigen relative to HIV epitope pool stimulation allowing for mRNA detection. However, the evaluation of superantigen and antigen specific interactions with TCR is beyond the scope of this report. In a minority of participants, cervical SEB-induced immune responses were not always detectable by qPCR. A lack of viable cervical cells may be one possible explanation, and cell viability was not ascertained in these experiments. However, SEB-treated cervical cells from both HIV-infected and uninfected women may sometimes be unresponsive in flow cytometry-based assays despite the demonstration of cell viability via a live/dead gating strategy (unpublished observation; McKinnon L and Kaul R), and so a lack of cell viability is unlikely to explain these results.

The expression of target genes must be corrected to account for variation in the quantity and quality of extracted RNA, reverse transcription and PCR amplification efficiencies and to measure the relative quantities of target genes[421]. However, the expression of housekeeping genes also varies under different experimental conditions [413, 421, 422]. Therefore we screened a panel of five commonly used housekeeping genes in CMC and PBMC to evaluate their stability under different treatment conditions, and found GAPDH and β-actin genes to be the most stable housekeeping genes, respectively. Our findings agree with one [423] but not another [413] of only two prior studies evaluating qPCR to measure gene expression ex vivo in cervical tissue. However,
these studies were not using qPCR to assay antigen-specific responses where short-term stimulation might alter the stability of potential housekeeping genes. For this reason we used the NormFinder software to directly evaluate intergroup stability of candidate housekeeping genes in our immunostimulated samples [422]. In addition, our selected housekeeping genes may have varied from other reports as our samples were derived from HIV infected participants. HIV has been demonstrated to exert substantial effects on the transcription pathways of several classes of genes [424], including commonly used “housekeeping genes” selected in the above mentioned studies [425]. This study therefore highlights the importance of selecting a stable reference gene for normalization of target gene expression based on empirical evaluation for a given assay condition, cell type and infection status.

No IL-2 protein was detected in cell culture supernatants using the cytometric bead array, despite the detection of low-level IL-2 mRNA levels by qPCR. In other studies describing discordance between mRNA detection and protein synthesis it has been postulated that a few highly transcriptionally active cells may permit detection of cytokine mRNA, but that these highly active cells do not secrete enough protein to be quantified using protein assays[426]; in this scenario IL-2 protein would be secreted into the cell supernatant but fall below the lower limit of detection of the CBA assay (20pg/ml). Discordant kinetics between mRNA induction and protein production is another possible explanation. Finally, since our culture experiments did not block the IL-2-receptor, any IL-2 produced may have been consumed by activated immune cells [411, 427].
In summary, we describe the process of development and testing of a real-time quantitative PCR assay to measure *ex vivo* cervical HIV-specific cellular T cell responses. We demonstrate an appropriate algorithm for the selection of housekeeping genes, and determine the optimal cytokine induction kinetics and cell input numbers for measuring such responses in the blood. However, the resulting optimized qPCR assay was not able to detect HIV-specific cellular T cell responses in the cervix of HIV-infected women despite similar input cell numbers, and despite the demonstration of a robust immune response in the PBMCs from the same participant. The reasons for this result are not clear, but we believe that the description of the assay development process will be of use to those in the mucosal vaccine field who are endeavouring to develop robust platforms to evaluate vaccine-induced T cell responses in the female genital tract.
Table 3.1. Primer sequences for SYBR green Real-Time PCR.

<table>
<thead>
<tr>
<th>mRNA Target</th>
<th>Forward Primer Sequences 5’→3’</th>
<th>Reverse Primer Sequences 3’→5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>AGGGAAGCGAAAAAGGAGTCA</td>
<td>GGACAACCATTACTGGGATGCT</td>
</tr>
<tr>
<td>TNFα</td>
<td>GCCAGAATGCTGAGGACTT</td>
<td>GGCCTAAGGTCCACTTTGTGTCA</td>
</tr>
<tr>
<td>IL2</td>
<td>ATGAGACAGCAACCATTGTGAATTTT</td>
<td>CACTTAAATTATCAAGTCAGTGTGAGATGA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TCCCTTGCCATCCTAAAAGCCACCC</td>
<td>CTGGGCCATTTCTCCTAGAGAGAGAG</td>
</tr>
<tr>
<td>TBP</td>
<td>GGGCATTATTGTGCACTGAGA</td>
<td>TAGCAGCACGGTATGAGCAACT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGGACCTGACCTGCGTCTCA</td>
<td>CCCTGTGGCTGTGAGCACAATTC</td>
</tr>
<tr>
<td>B2M</td>
<td>GAGTGCTGTCTCCATGTGATGT</td>
<td>AAGTTGCGACGCCCCCTCCTAGAG</td>
</tr>
<tr>
<td>HPRT</td>
<td>GCCTATAGACTATCAGTCTCCCTTTGG</td>
<td>TGCTGTGGTTAAGAGAATTAAAAA</td>
</tr>
</tbody>
</table>

Interferon Gamma (IFNg); Tumour Necrosis Factor alpha (TNFa); Interleukin 2 (IL2); β-2-Microglobulin (B2M); TATA box binding protein (TBP); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Ribosomal Protein L32 (RPL32); human hypoxanthine phosphoribosyltransferase (HPRT).
Table 3.2. NormFinder rankings of candidate reference housekeeping genes

<table>
<thead>
<tr>
<th>Rank</th>
<th>CMC</th>
<th>Stability value</th>
<th>PBMC</th>
<th>Stability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>104.9</td>
<td>β-Actin</td>
<td>1622.2</td>
</tr>
<tr>
<td>2</td>
<td>β-Actin</td>
<td>538.7</td>
<td>HPRT</td>
<td>1833.8</td>
</tr>
<tr>
<td>3</td>
<td>HPRT</td>
<td>574.6</td>
<td>TBP</td>
<td>1859.2</td>
</tr>
<tr>
<td>4</td>
<td>TBP</td>
<td>588.1</td>
<td>GAPDH</td>
<td>1876.8</td>
</tr>
<tr>
<td>5</td>
<td>B2M</td>
<td>3563.8</td>
<td>B2M</td>
<td>6997.3</td>
</tr>
</tbody>
</table>

β-2-Microglobulin (B2M); TATA box binding protein (TBP); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); human hypoxanthine phosphoribosyltransferase (HPRT).

Genes are ranked from the most stable (1) to the least stable (5) according to NormFinder gene-stability analysis algorithms. Housekeeping gene stability was evaluated by comparing between 5 different HIV+ PBMC or CMC samples and within an individual treated with either Negative control (R10 media,) HIV antigen (HIV epitope pool) or positive control (SEB).
Figure 3.1

a  

**IFNγ**

b  

**TNFα**

c  

**IL2**
Figure 3.1. Comparison of the cytokine kinetics of HIV-specific mRNA and protein induction. 1X10^6 peripheral blood mononuclear cell samples from 4 HIV infected individuals were incubated with HIV CD8+ T cell HIV optimized epitope antigen e (HIV epitope pool) and mRNA and protein induction assayed via qPCR and CBA respectively. Bars represent median values with lines showing interquartile ranges measuring HIV-specific IFNγ (a), TNFα (b) and IL-2 (c) mRNA and protein induction kinetics are shown. Background levels of protein have been subtracted from reported values above.
Figure 3.2

(a) HIV epitope pool

Fold gene induction

Cell numbers/experiment

(b) SEB

$\frac{p = 0.016}{p = 0.016} \quad \frac{p = 0.016}{p = 0.009}$

Fold gene induction

Cell numbers/experiment
Figure 3.2. Effect of blood mononuclear cell input number on qPCR assay. A box and whiskers plot showing peripheral blood mononuclear cell samples from 5 HIV infected subjects which were plated at varying concentrations ranging from $1 \times 10^6$ to $1 \times 10^4$ cells/experiment and treated with R10 media, HIV epitope pool antigen or SEB positive control for 6 hours and IFNγ mRNA fold expression assayed using qPCR. Horizontal lines represent median values. (a) HIV-specific response did not significantly decrease when cell numbers decreased from $1 \times 10^6$ to $1 \times 10^4$ cells/experiment. (b) However, the magnitude of SEB induced IFNγ mRNA induction significantly decreased when less than $2.5 \times 10^4$ cells/experiment were used.
Figure 3.3

(a) IFNγ

(b) TNFα

(c) IL2

Fold gene induction

CMC  PBMC

CMC  PBMC

CMC  PBMC
Figure 3.3. HIV-specific cytokine gene induction in cervical and blood mononuclear cells from HIV infected participants. (a) IFNγ, (b) TNFα and (c) IL-2 mRNA induction in cervical mononuclear cells (CMCs) and peripheral blood mononuclear cells (PBMCs) from 13 HIV infected women following incubation with HIV pool. Horizontal lines represent median values. The qPCR assay readily detected HIV-specific cytokine immune responses in PBMCs but was unable to do so in CMCs.
Figure 3.4

(a) IFNγ

(b) TNFα

(c) IL2
Figure 3.4. SEB superantigen mRNA cytokine induction in cervical and blood mononuclear cells from HIV infected participants: (a) IFNγ, (b) TNFα and (c) IL-2 mRNA induction in cervical mononuclear cells (CMCs) and peripheral blood mononuclear cells (PBMCs) from 13 HIV infected women following incubation with superantigen. Horizontal lines represent median values. The qPCR assay detected IFNγ and IL-2 mRNA in CMCs but not TNFα, while all cytokines were detected in PBMCs.
Figure 3.5

\[ r^2 = 0.780 \]
\[ p = 0.005 \]
Figure 3.5. Correlation of SEB induced IFN$\gamma$ mRNA and protein induction in cervical cells from HIV infected participants: Horizontal lines represent median values. IFN$\gamma$ mRNA induction was associated to IFN$\gamma$ protein production in SEB treated samples.
Chapter 4: BLUNTED IL17/IL22 AND PRO-INFLAMMATORY CYTOKINE RESPONSES IN THE GENITAL TRACT AND BLOOD OF HIV-EXPOSED, SERONEGATIVE FEMALE SEX WORKERS IN KENYA.

4.1 ABSTRACT

Background. Identifying the immune correlates of reduced susceptibility to HIV remains a key goal for the HIV vaccine field, and individuals who are HIV-exposed, seronegative (HESN) may offer important clues. Reduced systemic immune activation has been described in HESNs individuals. Conversely, pro-inflammatory T cell subsets, particularly CD4+ T cells producing the cytokine IL17 (Th17 cells), may represent a highly susceptible target for HIV infection after sexual exposure. Therefore, we characterized the cellular pro-inflammatory and IL17/IL22 cytokine immune milieu in the genital mucosa and blood of HESN female sex workers (FSWs).

Methods and Results. Blinded lab personnel characterized basal and mitogen-induced gene and cytokine immune responses in the cervix and blood of HESN FSWs (n=116) and non-FSW controls (n=17) using qPCR and ELISA. IL17 and IL22 production was significantly reduced in both the cervix and blood of HESNs, both in resting cells and after mitogen stimulation. In addition, HESN participants demonstrated blunted production of both pro-inflammatory cytokines and β-chemokines.

Discussion and Conclusions. We conclude that HIV exposure without infection was associated with blunted IL17/IL22 and pro-inflammatory responses, both systemically and at the site of mucosal HIV exposure. It will be important for further studies to examine the causal nature of the association and to define the cell subsets responsible for these differences.
4.2 INTRODUCTION

Human immunodeficiency virus (HIV) currently infects over 33 million people globally [392], and women are disproportionally affected [34, 392]. While most infected women acquire HIV across the cervical or vaginal mucosa during sex [34], HIV transmission is surprisingly inefficient with an overall risk per coital act of much less than 1% [428]. Nonetheless, there is heterogeneity in susceptibility, and the immune milieu of the female genital tract may be an important determinant of exposure outcome [42]. Individuals who have been HIV exposed but remain seronegative (HESN) have been described in multiple settings and include female sex workers (FSWs) from HIV endemic countries, partners within HIV serodiscordant couples and some men who have sex with men [429]. Understanding the immune correlates of HIV protection in HESN individuals may inform protective vaccine and microbicide design.

Previous studies have begun to elucidate the immune correlates of protection against HIV in HESN individuals. Some individuals manifest virus-specific CD4+ and CD8+ T cell responses, although it is not clear whether these responses are causally protective (reviewed in [430]). More recently, HESN individuals have been shown to demonstrate reduced basal T cell activation in the blood compartment [172-174, 213] while dampened immune activation in vivo has been correlated with reduced cellular susceptibility to HIV infection in culture [172]. Since HIV preferentially replicates in activated CD4+ lymphocytes [57], this relative immune quiescence may decrease the mucosal availability of activated CD4+ T cell targets, making it less likely that sexual HIV exposure will result in productive infection [153, 173, 213]. Indeed, macaque models show that blocking early mucosal inflammation and the recruitment of activated target cells to the genital mucosa was associated with protection from repeated SIV
challenge in vivo [43]. These findings suggest that a ‘critical mass’ of activated HIV target cells and/or inflammation at the genital mucosa may be required for productive HIV infection [217]. However, it is not known whether immune quiescence extends to the genital mucosa, the site of initial HIV-target cell interaction.

The specific CD4+ T cell subsets present at the mucosal site of HIV exposure may also play a role in susceptibility [431]. IL17 producing CD4+ T cells (Th17) cells are important in coordinating mucosal anti-microbial immune responses [432]. However, many [24, 167, 374] (but not all [164]) studies have found that Th17 cells may be preferentially infected by HIV, perhaps because they are activated and terminally differentiated cells that express high levels of CCR5 and α4β7 [21, 24, 161]. IL22 producing CD4+ T cells (Th22 cells) are involved in the maintenance of mucosal integrity and may assist Th17 cells in maintaining antimicrobial immune function [232], but also express high levels of HIV co-receptors and may be preferentially depleted from mucosal sites during HIV infection [24, 433].

It is possible that mucosal HIV acquisition after sexual exposure may be enhanced by an increased number/proportion of mucosal Th17 and Th22 cells and/or mucosal T cell immune activation [24, 153, 167, 433]. Conversely, their decrease may serve as a correlate of immune protection against HIV infection. To evaluate this hypothesis, we examined the genital mucosal immune responses in cervical and blood mononuclear cells within a HESN FSWs cohort from Nairobi, Kenya. Here, we report that HESN FSWs have decreased functional pro-inflammatory, IL17 and IL22 cellular immune responses in the blood, compared to lower risk women; and for the first time extend this functional immune quiescence to the genital mucosa.
4.3 METHODS

Ethics statement.

All participants provided written informed consent, and study protocols were reviewed and approved by the Research Ethics Boards at the Kenyatta National Hospital, Kenya and the Universities of Manitoba and Toronto, Canada.

4.3.1 Study population and diagnostics.

HIV negative FSWs were enrolled from a dedicated sex worker cohort in the Majengo Nairobi area of Kenya. Non-pregnant women with no history of commercial sex work (low-risk controls; LRC), were recruited from the nearby Pumwani Mother-Child Maternity Clinic, where their children obtained routine care. All women were confirmed to be HIV-uninfected using both serology and an optimized sensitive PCR assay specific for env, nef and vif HIV-1 provirus genes adapted to detect African clades [180, 434]. Herpes simplex type 2 serology was performed using the Kalon IgG enzyme linked immunosorbent assay (ELISA) (Kalon Biological). T vaginalis culture was performed (In Pouch TV (Biomed Diagnostics), a gram stain was performed, and blood was tested for syphilis serology (rapid plasma reagin; RPR). Bacterial vaginosis was defined as a Nugent score of 7–10 on the Gram stain [435], and candidiasis was defined as the presence of fungal hyphae. No diagnostics were performed for N. gonorrhoeae or C. trachomatis. Blood samples and cervical cytobrush specimens were obtained from women who were not actively menstruating, and a behavioural questionnaire was completed.

4.3.2 Specimen collection and in vitro stimulation.

Specimen collection and processing was performed as previously described [116, 192]. Briefly, cervical samples were obtained by first scraping the external cervical os
using a plastic cell scraper (Benzi Jinshuo Applicator Co) and a cervical histobrush (Histobrush Spectrum Lab) was then inserted into the cervical os and rotated through 360°. A mini-cervical lavage was then performed using 1ml PBS to collect loosened cells. Then the scraper, swab and mini-lavage cervical samples were combined into a 50ml sample collection tube containing 10mls of PBS and transported to the laboratory on ice within 3 hours. Blood was collected by venipuncture into heparin Vacutainer tubes (BD Bioscience). Peripheral blood mononuclear cells (PBMC) and cervical mononuclear cells (CMCs) were isolated by density gradient centrifugation and resuspended in RPMI 1640 media with 10% heat inactivated fetal bovine serum, 1% Penicillin and 1% streptomycin (R10; FBS; Sigma). CMC and PBMC enumeration and viability were assessed by trypan blue exclusion.

PBMCs (1x10^6) or all available CMCs were incubated for 6 hours left in culture R10 medium alone (negative control) or stimulated with staphylococcal enterotoxin B superantigen (SEB; Sigma) at 3.0ug/ml at 37°C in 5% CO₂. SEB was used as a positive mitogen control to induce IL17, IL22 and other pro-inflammatory cytokine expression and production, similar to other studies [436-439]. A 6-hour incubation was selected to maximize both protein and RNA yield for targeted immune outputs [440]. After incubation, cell pellets and supernatants were harvested by centrifugation at 10,000 rpm for 5 minutes and cell pellets placed in RNALater solution (Ambion). Both cell pellets and culture supernatants were cryopreserved at -80°C, and were later thawed for quantification of mRNA and protein.
4.3.3 qPCR and mRNA quantification.

An optimized quantitative real-time PCR (qPCR) assay was used to quantify mRNA as previously described [440]. Briefly, RNA was extracted from cell pellets and genomic DNA was eliminated using the Qiagen RNAeasy Plus Kit (Qiagen) as per manufacturer’s instructions. Complementary DNA (cDNA) was created by adding 10ng of RNA to the reverse transcription master mix and reverse-transcribed using the Superscript III Kit (Invitrogen) as per manufacturer’s instructions. Genomic DNA (gDNA) standards were isolated from purified placental tissue and used as a universal standard over a 7-log (36 ng – 0.4 ng) dilution range to calculate relative gene expression levels [412, 440]. Single intra-exon gene-specific primers were generated using Primer Express Software™ (Perkin Elmer Applied Biosystems) or OligoPerfect™ (Invitrogen).

Due to recovery of a limited amount of lymphocytes, within cervical cytobrush samples [440], we limited the amount of genes examined via our qPCR assay to only IL17a (IL17), IL22, IL6, and IFNγ mRNA; a list of primer sequences used to detect immune and housekeeping gene mRNA transcription are listed in Table 4.1.

SYBR green fluorescent dye was used to detect amplification under the following amplification conditions: i) 1 warm-up cycle for 2 min at 50°C ii) 1 pre-amplification cycle for 10 minutes at 95°C, 40 amplification cycles for 15 seconds at 95°C and for 1 minute at 60°C, iii) end-amplification cycle for 15 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 95°C. All reactions were run in triplicate. Quantitative PCR values crossing threshold (Ct) were obtained during the exponential amplification phase using SDS 2.3 Software (Applied Biosystems) and analysed for respective gene quantities and exported into Microsoft Excel for further analysis. Prior validated housekeeping genes, GAPDH and β-Actin [440], were used as the respective reference genes to assess target
gene expression in CMC and PBMCs respectively. Gene quantities were calculated from standard curves in arbitrary units; and to assess antigen-specific gene induction, results for each target gene were normalized by dividing the amount of the amplified gene target by the amount of the housekeeping gene for each sample and reported as the normalized gene induction ratio. Since qPCR lacks sensitivity in the context of low CMC counts [440], this assay was only performed in samples having more than 2x10^5 CMC counts as enumerated using trypan blue exclusion.

### 4.3.4 Cytokine quantification.

Cytokine concentrations (pg/ml) in culture supernatants were determined using Searchlight™ chemiluminescent Multiplex-ELISA assay (M-ELISA; Aushon Biosystems, IL17a (IL17), IL6, IL10, MCP-1, IFNγ, RANTES, MIP1α, MIP1β, SDF-1β, IL8) or using a stand-alone ELISA (Platinum™ ELISA, e-Biosciences, IL22) as per manufacturer’s instructions. ELISA assays required relatively little sample volume input and were multiplexed by design, which afforded us the ability to examine a relatively broader range of immune parameters using this output. All samples were run in duplicate. Unknown values were analysed using the 4PL standard curve fit model through Searchlight Array Analyst software™ (Aushon Biosystems). Cytokine levels in the media control (background) from each participant were subtracted from all presented HIV pool and SEB specific cytokine levels. In our ELISA standard curves, the following criterion was used to report all unknown value data; software reported readings falling below the lowest standard were reported as is, and if reported as ‘undetectable’, a ‘zero’ value was inputted, while no extrapolated values were reported beyond the top known standard. For data analysis purposes, values exceeding the upper standard were reported as the upper limit of detection plus an arbitrary 1 pg/ml.
4.3.5 Statistical analysis.
Non-parametric Mann-Whitney U, Pearson $\chi^2$ test and Wilcoxon Signed Ranks statistical analyses were performed using SPSS Version 18.0 software (SPSS Inc.). The number of unprotected sexual HIV exposures over the past year was estimated based on the number and reported condom use with each client group (casual clients, repeat clients and regular partners), assuming an HIV prevalence in male clients of 20% and a condom failure rate of 2%.

4.4 RESULTS
4.4.1 Study participants.
A total of 116 HIV-uninfected FSWs (HESN FSWs) were recruited from a dedicated female sex worker clinic in Majengo, Nairobi, and 17 lower risk women with no prior history of commercial sex work were recruited from a paediatrics clinic affiliated with the Pumwani Maternity Hospital, Nairobi (low-risk controls; LRC). Age, menopause and the prevalence of genital infections were similar between controls and HESN FSWs (Table 4.2). Median cervical mononuclear cell (CMC) number was 1.2x10$^5$ (range 0 - 4.38 x10$^6$) in the HESN FSW group and 2.5 x10$^5$ (range 0 - 2.22x10$^6$) in the control group. FSW participants had been engaged in commercial sex work for a median of 9 years (range 1-38), and reported a median of 21 casual clients per week (range 3-160). Self reported condom use by FSWs was high with casual clients (98%) and repeat clients (98%), but much lower with boyfriends/regular partners (21%; Table 4.3). FSW had a median of 30 (range 4-168) estimated unprotected sexual HIV exposures over the past year. The HIV status of FSW male clients was not available.
4.4.2 Cytokine production by blood lymphocytes in HESN female sex workers.

In unstimulated (media incubated) PBMC, the most striking difference between HESN and controls was a reduced production of IL17 (p=0.009) and IL22 (p<0.001) in the HESN group (Figure 4.1a). In addition, HESNs also manifested a substantial and consistent reduction in the basal production of pro-inflammatory cytokines, including IFNγ (p=0.002), IL8 (p=0.006), SDF-1b (p=0.043), MIP-1b (p<0.001) and RANTES (p<0.001) (Figure 4.1a). After short-term incubation with the mitogen SEB, HESN participants still demonstrated a much lower production of the cytokines IL17 and IL22 in blood lymphocytes (IL17, p<0.001; IL22, <0.001, Figure 4.1b), but no difference was now apparent for other pro-inflammatory cytokines or chemokines (Table 4.4). Overall, the production of IL17 and IL22 was substantially reduced in HESN FSW, both in unstimulated PBMC and after incubation with the mitogen SEB, and basal production of other pro-inflammatory cytokines was also blunted. There was no association between cytokine responses in blood and either the duration of prior sex work, the current number of weekly clients or the calculated number of HIV exposures over the past year (data not shown).

Since HIV acquisition generally occurs in high-risk FSWs after sexual exposure at the level of the genital mucosa, similar assays were attempted using cervical mononuclear cells. However, cytokine and chemokine protein levels in CMC supernatants consistently fell below the level of ELISA detection (~1 pg/ml), likely due to both the low recovery of mucosal cell numbers obtained from cervical cytobrush sampling [440], as well as the relatively low enrichment of mononuclear lymphocytes within such samples [40].
**4.4.3 Immune gene expression in the blood and cervix of HESN female sex workers.**

Generally, qPCR assays offer high sensitivity and in theory could amplify low signals resultant from low cellular input to levels sufficient for relative quantitation [407, 441]. Therefore, we also used a qPCR platform to examine Th17/Th22 associated cytokine responses in the cervix (IL17 and IL22), as well as to confirm our prior ELISA findings regarding reduced pro-inflammatory cytokine production in blood lymphocytes (two cytokines; IL6, IFNγ). In unstimulated CMC there was no difference in IL17 or IL22 gene expression between HESN and control women (Table 4.4), although IL6 expression was slightly reduced in the former group (p=0.049; Figure 2a). However, after incubation with the SEB, HESN cervical cells demonstrated substantial and consistently reduced induction of IL17 (p=0.008) and IL22 (p=0.002), as well as of the other pro-inflammatory genes (both IFNγ and IL6; each p≤0.028; Figure 4.2b).

Gene expression in the blood followed a similar pattern, with significantly reduced expression of IL22 (p=0.046) and IL6 (p=0.004) in unstimulated PBMC from HESN FSWs, but no difference in IL17 (p=0.681) expression (Figure 4.2a). Following SEB (mitogen) stimulation there was again a relatively reduced expression of IL22 in HESN PBMC (p=0.021), and a weak trend to reduced IL17 expression (p=0.138; Figure 4.2b). No association was seen between cervical immune gene responses and either the duration of prior sex work, the number of weekly clients or the calculated number of unprotected HIV sexual exposures over the past year (data not shown). Overall, the qPCR platform confirmed the initial ELISA results, and for the first time expanded these observations to demonstrate blunted IL22 responses and a weak trend towards reduced IL17 production in the cervix of HESN FSWs. In contrast to blood, the reduction in
cervical cytokine responses was most apparent in HESNs following mitogen stimulation (rather than in resting cells).

4.5 DISCUSSION.

Defining the immune correlates of reduced HIV susceptibility is a key goal for the HIV vaccine field. In this relatively large cohort study, HESN FSWs demonstrated a substantially reduced production of pro-inflammatory and Th17/Th22-type cytokines in both the genital tract and blood compartments, with the blunting of these immune responses most apparent in the cervix after mitogen stimulation. While previous reports have described reduced immune activation in unstimulated blood lymphocytes from HESN individuals [172-174, 213, 214] and more recently in cervical secretions [442], our current study extends these observations in two important ways. First, we broaden the range of immune responses involved in the ‘immune quiescence’ model of HIV immune protection to include the induction of the classical Th17/Th22 cytokines. Secondly, we are the first to demonstrate that functional immune responses are also quiescent in the genital mucosa, which is the putative site of most HIV acquisition in female sex workers. This is important since increased levels of genital pro-inflammatory cytokines [443] and innate immune factors [444] have been associated with increased HIV acquisition in women from sub-Saharan Africa.

The reduced production/induction of IL17 and IL22 in both the blood and genital tract of HESN participants might be causally related to HIV exposure without infection, since both Th17 and Th22 cell subsets are enriched at mucosal sites and may be particularly HIV susceptible [24, 153, 160, 169, 233] (although not all studies have found this [164]). Th17 (CCR4+CCR6+) cells have been shown to harbour higher HIV proviral
levels than other CD4 subsets *ex vivo* [166], and blocking of MIP3a, the natural CCR6 ligand, can protect macaques from repeated SIV challenge *in situ* [43]. Additionally, Th17 cells express elevated levels of the surface marker α4β7 and preferentially bind HIV gp120 envelope [24], express increased levels of the HIV entry co-receptor CCR5 [24, 167], and produce lower levels of CCR5 binding β-chemokines [167] potentially increasing their susceptibility to HIV infection. Interestingly, the pro-inflammatory cytokine IL6 is a critical cofactor in the development of Th17 cells [445], and was also reduced in both the blood and genital tract of HESN participants.

In addition to relative immune quiescence, HESN status has been associated with HIV-specific T cells with a pro-inflammatory functional profile (reviewed in [430]). This seems at odds with the findings of immune quiescence in our study, as well as in others [172-174, 213, 442]. While these two observations appear paradoxical, this need not be the case [173, 213]. Cellular immune quiescence in the genital mucosa may result in a reduced pool of susceptible CD4+ T cells after initial sexual HIV exposure, reducing HIV acquisition risk, and repeated exposure may then generate the mucosal HIV-specific T cell responses that have been observed. Whether these responses are a paraphenomenon of exposure or contribute to protection is not known [98, 207, 446], but their low frequency may mean that they can contribute to protection without greatly increasing the number of mucosal activated cells [173, 212, 213]. Therefore, these two immune correlates may work in tandem to protect against HIV.

Despite these interesting results, it is important to acknowledge potential limitations to our study. Our immune assays do not permit identification of the exact cell subpopulations responsible for measured immune responses. While IL17 and IL22 fall
under the broad category of “Th17” type cytokines, several cell types other than classical CD4+ αβ T cells that are isolated by density centrifugation can also produce them, including γδ T cells, and NKT cells [447]. Future cytometric studies will be needed to further identify these cells. Additionally, although we were readily able to measure gene induction in the genital mucosa, the inability to measure protein production here using a sensitive ELISA platform might suggest that these cells could be anergic and this would need to be evaluated in further immunologic studies. While the two platforms used (qPCR and ELISA) were generally in agreement, subtle differences in results were apparent. Discordant kinetics of mRNA expression and protein production levels may explain this observation. Peak mRNA levels and their subsequent decline may have preceded the peak protein production levels attained at the end of the 6-hour incubation period [440]. Alternatively, transcript and protein concordance has been reported to vary within various settings, and could correlate anywhere from 17%-100% [448-450] such that measured mRNA levels may only be partially indicative of protein secretion. Nevertheless, despite some inter-platform variability, our results consistently show that HESN FSWs have an immuno-quiescent phenotype in blood (ELISA and qPCR) and cervix (qPCR), expanding several other studies identifying immune quiescence in blood as a correlate of relative HIV protection [172-174, 213, 214], as well as a distinct pattern of soluble immune factors in genital secretions [442].

We estimated the number of unprotected HIV exposures over the past year, based on current condom use and numbers within various client groups, and assuming that the HIV prevalence in all male partners was 20%. However, our immune studies did not find any association between this calculated annual HIV exposure rate, or other sex work
parameters with the magnitude of immune responses in sex workers. Some studies have defined relative HIV ‘resistance’ in female sex workers based on criteria such as a threshold duration of prior follow up [180]. However, in this study we defined all HIV-uninfected sex workers as potentially HIV-exposed but seronegative (HESN), since all women had been engaged in commercial sex work for at least one year prior to cohort enrolment. The HIV prevention program that is offered to all cohort participants results in a very rapid increase in condom use and reduction in client numbers after enrolment [451], so that past HIV exposure may have been considerable despite low current sexual risk. In addition, despite high condom use with casual clients, condoms are infrequently used with regular partners and this represents a poorly-understood source for ongoing HIV exposure. These potential confounders mean that it is difficult to precisely correlate immune parameters with a quantitative measure of past HIV infection pressure.

The cross-sectional design of our study means that we cannot prove that the association between the HESN phenotype and immune quiescence is causal. Our eventual goal is to address this issue using prospective studies with HIV acquisition as an outcome. Frequent sexual activity amongst sex workers might conceivably confound our genital immunology results due to repeated exposure to semen, known to have both immunoregulatory and pro-inflammatory effects [98]. However, since immune quiescence was also found in blood, it is unlikely that frequent cervical semen exposure explains the immune differences seen in HESN sex workers. Finally, Herpes simplex type 2 (HSV-2) infection is associated with genital mucosal inflammation and recruitment of T cell and innate immune cells [116, 200]. Most participants in the study were HSV-2 infected, and screening for asymptomatic reactivation was not performed.
However, since HSV-2 was more common in the HESN women, and prior studies have shown that the pro-inflammatory mucosal immune impact of HSV-2 in HESN women is similar to controls [182], this is not likely to lead to our finding of HESN immunooquiescence.

In summary, we find that HESN female sex workers demonstrated substantial and consistent reduction in the basal and mitogen-stimulated production of Th17/Th22-associated and pro-inflammatory cytokines in both the genital and blood compartments. These immune correlates of reduced HIV susceptibility may provide important clues for future vaccine and microbicide research.
### Table 4.1. Primer sequences for SYBR green real-time PCR.

<table>
<thead>
<tr>
<th>mRNA Target</th>
<th>Forward Primer Sequences 5’→3’</th>
<th>Reverse Primer Sequences 3’→5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>TCCCTTGCCATCCTAAAAAGCCACCC</td>
<td>CTGGGCCATTCTCTTTAGAGAGAAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGGACCTGACCTGCCGTCTA</td>
<td>CCCTGTTGCTGTAGCCAAATTC</td>
</tr>
<tr>
<td>IFNγ</td>
<td>AGGGAAGCGAAAAAGGAGTCA</td>
<td>GGACAACCATTACTGGGATGCT</td>
</tr>
<tr>
<td>IL6</td>
<td>CTGTCCACTGGGCACAGAACT</td>
<td>AAAATAATTTGAGGTGCCTAAACG</td>
</tr>
<tr>
<td>IL17</td>
<td>CATGAACCTCTGTCCCCCATCC</td>
<td>CCCACGGACACAGTATCTT</td>
</tr>
<tr>
<td>IL22</td>
<td>TGCATTTGACCAGAGCAAG</td>
<td>AGTTTGGCTTCCATCTCC</td>
</tr>
</tbody>
</table>

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Interferon Gamma (IFNγ); Interleukin 6 (IL6); Interleukin 17a (IL17); Interleukin 22 (IL22).
Table 4.2. Summary of enrolled study participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>HESN sex workers</th>
<th>Low-risk controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>39 (23-64)</td>
<td>43 (32-51)</td>
<td>0.227</td>
</tr>
<tr>
<td>Menopause (%)</td>
<td>9/101 (8%)</td>
<td>2/16 (11%)</td>
<td>0.681</td>
</tr>
<tr>
<td><strong>Clinical variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genital infections/abnormalities:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genital herpes (%)</td>
<td>80/103 (78%)</td>
<td>12/17 (70%)</td>
<td>0.313</td>
</tr>
<tr>
<td>Syphilis (%)</td>
<td>3/104 (3%)</td>
<td>0/7 (0%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Trichomonas (%)</td>
<td>0/114 (0%)</td>
<td>0/17 (0%)</td>
<td>1</td>
</tr>
<tr>
<td>Bacterial vaginosis (%)</td>
<td>21/116 (18%)</td>
<td>1/17 (6%)</td>
<td>0.181</td>
</tr>
<tr>
<td>Candidiasis infection (%)</td>
<td>27/116 (23%)</td>
<td>3/17 (18%)</td>
<td>0.531</td>
</tr>
</tbody>
</table>
Table 4.3: Summary of condom use in enrolled female sex workers

<table>
<thead>
<tr>
<th>Self-reported condom use</th>
<th>Casual clients*</th>
<th>Repeat clients*</th>
<th>Regular client/boyfriend*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never (0%)</td>
<td>0/100 (0%)</td>
<td>0/88 (0%)</td>
<td>46/67 (68%)</td>
</tr>
<tr>
<td>Some (&lt;50%)</td>
<td>1/111 (&lt;1%)</td>
<td>1/88 (&lt;1%)</td>
<td>2/67 (3%)</td>
</tr>
<tr>
<td>Most (&gt;50%)</td>
<td>1/111 (&lt;1%)</td>
<td>1/88 (&lt;1%)</td>
<td>5/67 (8%)</td>
</tr>
<tr>
<td>Always (100%)</td>
<td>98/111 (98%)</td>
<td>86/88 (98%)</td>
<td>14/67 (21%)</td>
</tr>
</tbody>
</table>

* Data was not available for all 116 enrolled sex workers. Respondent numbers are shown for each variable.
Table 4.4: Summary of immune gene and protein levels in enrolled participants

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sample</th>
<th>Immune Readout</th>
<th>R10 media alone</th>
<th>SEB stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HESN median</td>
<td>LRC median</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR</td>
<td>Blood</td>
<td>IL17 (ratio)</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL22 (ratio)</td>
<td>0.002</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFNg (ratio)</td>
<td>0.022</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL6 (ratio)</td>
<td>1.789</td>
<td>4.077</td>
</tr>
<tr>
<td></td>
<td>Cervix</td>
<td>IL17 (ratio)</td>
<td>0.011</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL22 (ratio)</td>
<td>0.005</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFNg (ratio)</td>
<td>0.010</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL6 (ratio)</td>
<td>0.404</td>
<td>1.189</td>
</tr>
<tr>
<td>ELISA</td>
<td>Blood</td>
<td>IL6 (pg/ml)</td>
<td>1.386 x 10^4</td>
<td>1.722 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL8 (pg/ml)</td>
<td>5.768 x 10^3</td>
<td>6.853 x 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL10 (pg/ml)</td>
<td>9.860 x 10^1</td>
<td>1.037 x 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL17 (pg/ml)</td>
<td>8.600 x 10^8</td>
<td>8.610 x 10^1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL22 (pg/ml)</td>
<td>2.800 x 10^1</td>
<td>1.312 x 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFNg (pg/ml)</td>
<td>4.000 x 10^4</td>
<td>7.200 x 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDF-1b (pg/ml)</td>
<td>2.780 x 10^1</td>
<td>2.091 x 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIP-1a (pg/ml)</td>
<td>3.653 x 10^4</td>
<td>5.434 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIP-1b (pg/ml)</td>
<td>4.425 x 10^4</td>
<td>2.394 x 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RANTES (pg/ml)</td>
<td>2.514 x 10^4</td>
<td>5.994 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCP-1 (pg/ml)</td>
<td>1.076 x 10^4</td>
<td>8.763 x 10^3</td>
</tr>
</tbody>
</table>

A Mann-Whitney U test was performed to compare differences between groups and the ‘p’ statistic reported. qPCR data is presented as a ratio of target gene expression over the housekeeping gene. Staphylococcus enterotoxin B (SEB); HIV exposed seronegative (HESN), Low risk control (LRC), qPCR (real time PCR); Enzyme linked immunoabsorbent assay (ELISA).
Figure 4.1

a) Unstimulated

PBMC

IL17  IL22  IFNγ

Protein (pg/mL)

Low-risk controls  HESN

p<0.009  p<0.001  p<0.002

IL8  SDF1b  MIP1b  RANTES

Protein (pg/mL)

Low-risk controls  HESN

p<0.006  p<0.043  p<0.001  p<0.001

b) Mitogen (SEB) stimulated

PBMC

IL17  IL22

Protein (pg/mL)

Low-risk controls  HESN

p<0.001  p<0.001
Figure 4.1. Decreased Th17 and pro-inflammatory cytokine production in HESN blood lymphocytes.

Figure shows cytokine levels in supernatants of blood lymphocytes from study participants blood mononuclear cells (a) incubated in culture medium alone (unstimulated), and (b) after SEB mitogen stimulation. Cytokine concentrations (pg/ml; IL17, IL22, IFNg, IL8, SDF-1b, MIP1b, RANTES, IL10, MCP-1, MIP1a, and IL6) in culture supernatants were determined using an ELISA platform, and significant cytokine differences between groups are illustrated. Box and whisker plots are plotted with whiskers covering the 95-5 percentiles, dots representing outliers and horizontal lines representing the median.
Figure 4.2

Unstimulated

CMC

PBMC

Mliogen (SEB) stimulated

a)

b)
Figure 4.2. Decreased Th17 and pro-inflammatory cytokine gene expression in the HESN cervix and blood.

Figure shows immune gene expression in mononuclear cells from both blood (PBMC) and cervix (CMC) in study participants that were either (a) incubated in culture medium alone (unstimulated), or (b) after SEB mitogen stimulation. A qPCR assay was used to determine mRNA induction of IL17, IL22, IFNg, and IL6 genes. Box and whisker plots with whiskers covering the 95-5 percentiles, with horizontal lines representing the median.
Chapter 5: *PLASMODIUM CHABAUDI* MALARIA IN MICE INCREASES MUCOSAL IMMUNE ACTIVATION AND EXPRESSION OF PUTATIVE HIV SUSCEPTIBILITY MARKERS.

Unpublished work.
5.1 ABSTRACT

Background: Malaria and HIV have a high degree of geographical overlap in sub-Saharan Africa, and there is evidence from HIV-malaria co-infected individuals that each may enhance the transmission of the other. We hypothesized that malaria might also enhance HIV susceptibility within an HIV-uninfected individual, through malaria-induced alterations in mucosal immunology at the mucosal sites of HIV sexual exposure. This hypothesis was tested in a murine model of malaria.

Methods: Female C57/BL6 mice were infected with *P. chabaudi* malaria using a standardized protocol. Blood, gastrointestinal tissues, upper and lower genital tract tissues and iliac lymph nodes were sampled 10 days post-infection, and the expression of HIV susceptibility and immune activation markers by T cells was assessed using multiparameter flow cytometry.

Results: *P. chabaudi* infection increased the expression of the mucosal homing marker α4β7 on both CD4+ and CD8+ T cells in blood. Expression of the HIV co-receptor CCR5 substantially increased on CD4+ T cells from both blood and mucosal tissues, and malaria-infected mice also demonstrated increased expression of the immune activation markers CD38, MHC-II and CD69 on T cells from both the blood, gut and the genital mucosa.

Conclusion: Systemic malaria infection in this murine model increased T cell immune activation and expression of the mucosal homing marker α4β7 in blood. Malaria also induced mucosal immune activation and increased mucosal expression of the HIV co-receptor CCR5. If malaria induces similar mucosal immune changes in humans, this would be expected to enhance HIV susceptibility in both the genital tract and gut mucosa.
5.2 INTRODUCTION

Over 33 million people are currently infected by Human Immunodeficiency Virus type-1 (HIV), with an estimated 1.8 million AIDS-related deaths in 2010 [1]. Sub-Saharan Africa (SSA) has been most affected by HIV, and the per-contact risk of HIV transmission is increased three-fold in studies from sub-Saharan Africa (SSA) compared to high-income countries [7]. Malaria is a parasitic infection with a reported 300-500 million infections a year [452] that caused an estimated 1.2 million deaths in 2010 [138]. HIV and malaria overlap in many regions of the world, particularly in sub-Saharan Africa. Here these infections are halo-endemic, particularly in Zambia, Zimbabwe, Mozambique, Malawi and the Central African Republic where the HIV prevalence is over 10%, and 90% of the population is exposed to malaria [140]. A similar spatial overlap is also evident in Kenya where both infections are endemic[4, 141-143]

Mounting evidence suggests that HIV and malaria interact at a molecular level. HIV infection causes a gradual, steady decline in blood CD4+ T cells and eventual immune dysfunction. In studies from SSA, lower blood CD4+ levels and more advanced HIV disease stage are both associated with an increased risk of malaria infection and with an increased risk of developing severe malaria disease [453, 454], such that HIV has been estimated to cause an additional 3 million malaria cases and 65,000 malaria deaths [144]. Conversely, individuals living in areas of high malaria endemicity are twice as likely to be infected with HIV [145]. Malaria increases the HIV blood viral load in advanced disease by almost one-log [146], and since this is a strong predictor of sexual transmission risk [97, 99], malaria would be expected to substantially enhance HIV transmission at a community level. Based on these data, mathematical models suggest
that interaction of HIV and malaria within an adult population of roughly 200,000 in Kisumu, a western Kenya town, may have been responsible for 8,500 excess HIV infections and 980,000 excess malaria episodes over the last 3 decades [141].

While HIV and malaria are synergistic within co-infected individuals, less is known about the possible effect of malaria infection on HIV susceptibility within a malaria mono-infected person. Malaria infection results in systemic (blood) activation of the innate and adaptive immune systems [132]. In addition, and this malaria-induced immune activation has also been associated with increased rates of HIV replication [455], which returns to normal 8-9 weeks post-malaria treatment [146]. Non-human primate models suggest that activated immune cells are critical for productive HIV infection after a mucosal exposure, as HIV preferentially replicates in these cells [57, 217]. Therefore, if malaria were to be associated with immune activation at mucosal sites of HIV exposure, as well as in the blood, then this might be expected to increase HIV susceptibility. After T cell priming and activation by mucosal-derived dendritic cells [162], activated T cells can be targeted to mucosal sites through trafficking receptors such as α4β7, CCR6 and CCR9 [456]. In addition, α4β7 has been shown to bind directly to HIV gp120, and to enhance virus infection in vitro [21, 161]. Therefore, increased CD4+ T cell expression of α4β7 might enhance HIV susceptibility through increased homing of activated target cells to mucosal sites of virus exposure, as well as through increased HIV susceptibility of individual CD4+ T cells.

The effect of malaria on the immunology of the genital mucosa, the putative site of most HIV acquisition in SSA, is unknown. We recently found increased levels of T cell immune activation in cervical T cells from young women in Kisumu, Kenya,
independent of common genital infections [457]. Blood samples were not available for malaria diagnostics or immunology studies, but malaria is holo-endemic in Kisumu, and we hypothesized that it might cause increased genital T cell immune activation [6]. To investigate this hypothesis, we have examined the impact of malaria on mucosal T cell immunology in a well-established mouse malaria model.

5.3 METHODS

5.3.1 Plasmodium chabaudi chabaudi AS murine infection.
The Animal Care Committee of the University of Toronto approved the animal experimental protocol, and all experiments involving animals were performed in accordance to university institutional guidelines. C57BL/6 mice (Charles River) were allowed to acclimatize at the University of Toronto animal facilities for at least 7 days before experiments, and kept under pathogen-free conditions with a 12-hour light cycle. Female mice of 8-12 weeks of age were used in all experiments. 5 days prior to animal infection, all mice were treated once with 2mg of long-lasting Depo-Provera (Depo; dihydroxyprogesterone acetate) by subcutaneous injection, to maintain mice in the diestrous stage and minimize hormone-induced cyclical alterations in the female genital mucosa [458]. Blood stage Plasmodium chabaudi chabaudi AS (PCCAS) parasites were cultured in the laboratory as previously described [459], and were used to inoculate experimental animals by intraperitoneal injection of 1x10^6 parasites. PCCAS parasitemia is known to peak between 8-10 days followed by immune clearance in C57BL/6 mice [460]. Therefore, infection was allowed to progress for 10 days. Prior to animals sacrifice, a thick blood smear was collected via tail vein and giemsa stained to confirm and count parasitemia using protocol R HEMA Stain Set (Fisher Scientific).
5.3.2 Sample collection and tissue processing
Immediately following sacrifice (with CO₂) blood was sampled through cardiac puncture into 2ml microtubes containing 200ul of acid citrate dextrose (ACD) to prevent coagulation. Collected blood was then treated with non-fixing lysing solution (BD Pharm Lyse™; BD) as per manufacturer’s instructions to lyse red blood cells, and samples were then stored on ice. Mouse cecal (gut), vaginal and cervical (genital) tissue were then excised and digested to obtain tissue lymphocytes using an established protocol from our previous work [461]. Here tissue sections were sliced open longitudinally, cleaned in 1% FBS (Gibco) in PBS (Gibco), then cut into ~1-2 cm segments. Mucosal tissues were then washed for 5 minutes in pre-stripping buffer containing: 1mM DTT, 5% FBS, 1mM EDTA in PBS. Tissues were then transferred into a 50ml tube containing stripping buffer (PBS, 1% FBS, 5mM EDTA, 1mM DTT) and were incubated in a heat-shaker with agitation at 37 C for 30 minutes. The tissue was then allowed to sediment before the supernatant containing extracted intraepithelial lymphocytes (IEL) cells was removed and then washed twice in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 20% FBS and stored on ice. Remaining tissue was further minced into ~4mm segments using scissors before being digested in digestion medium containing: DMEM, 20% FBS, 2mg/ml collagenase D (Roche) and 20ug/ml DNase (Sigma) in a heat shaker incubator with agitation for 30 minutes at 37C. The supernatant containing lamina propria lymphocytes (LPL) was then extracted after allowing the tissue to sediment, before fresh digestion medium was used and the digestion repeated for another 30 minutes and the LPL harvested. Both IEL and LPL cells were pooled and washed twice in DMEM and then sequentially passed through a 100um, then a 40um cell strainer before being placed on ice [461]. Iliac lymph nodes (iLN) that drain the genital tract were also excised and
placed into culture dishes containing enough 1%FBS in PBS solution to prevent them from drying; a sterile plunger used to disrupt the lymph node sacs and release viable lymphocytes. Lymph nodes were then collected and rinsed twice in DMEM before being placed on ice. With the exception of blood lymphocytes, all other tissues were pooled into groups of 3 mice each to allow for sufficient cells for flow cytometric analysis.

5.3.4 Flow cytometry assays
Cells were stained with aqua live/dead fixable stain (Invitrogen) to establish viability and with the following surface immune marker antibodies for 30 minutes in 1%FBS in PBS; TCRβ, CD4, CD8, α4β7, CCR9, CCR6, CCR5, CD69, CD38, and MHC-II (ebiosciences). Cells were then washed in DMEM before being placed in 2% paraformaldehyde solution in PBS. FACS analysis was then performed on the samples using an LSR II flow cytometer (BD) and analyzed using FlowJo software (TreeStar).

5.3.5 Statistical analysis
Mann-Whitney non-parametric tests were performed using Graphpad Prism (Prism), and P values of <0.05 were considered significant. Tissues were weighed after excision and the absolute number of cells was reported as cell counts per 1x10^6 cells/100 grams of tissue.
5.4 RESULTS

5.4.1 Effect of murine malaria infection on CCR5 expression and T cell activation in blood.
15 mice were infected with PCCAS for 10 days and matched with uninfected controls before they were sacrificed and the frequency of T cells expressing CCR5 and immune markers of activation was examined in blood. In blood, PCCAS infection was associated with a substantial increase in the frequency of CD4+ T cells expressing CCR5 (Figure 5.1a; representative example from 4 experimental replicates). Similarly, there was a significant increase in the CD4+ and CD8+ T cell expression of CD38, MHC-II and CD69 (the latter on CD8+ T cells only) in the malaria-infected group compared to malaria-uninfected controls (Figure 5.1 b-e). Therefore, PCCAS infection induced substantial and consistent systemic T cell immune activation. These experiments were subsequently repeated three times with similar results (data not shown).

5.4.2 Effect of PCCAS malaria infection on T cell mucosal trafficking markers.
Within the same experimental system we then examined the expression of mucosal T cell trafficking receptors in blood lymphocytes, specifically the expression of α4β7, CCR9 and CCR6. Compared to the uninfected controls, PCCAS-infected mice demonstrated significantly increased levels of α4β7 expression on blood CD4+ and CD8+ T lymphocytes (Figure 5.2 a-b). However, the expression of CCR9 and CCR6 T cell trafficking markers on T lymphocytes was similar between both study groups, and CCR9+ expression was actually decreased on blood CD8+ T cells in malaria-infected animals (Figure 5.2 c-d).
5.4.3 Effect of PCCAS malaria infection on T cell numbers in the gut and genital mucosa.

The increased expression of α4β7 by both CD4+ and CD8+ blood T lymphocytes during PCCAS infection might be expected to traffic activated T cells to mucosal sites. Therefore, we next quantified T cell numbers in the gut and genital mucosa. Malaria was not associated with any overall difference in the number of CD3+, CD4+ or CD8+ T lymphocytes in the gut or genital mucosa (Figure 5.3 a-c). Furthermore, α4β7 expression by mucosal T cells was similar in the malaria-infected and uninfected groups. The findings were similar when examining α4β7 expression frequency, rather than absolute cell numbers (data not shown). Therefore, PCCAS infection does not alter either the overall number of mucosal T lymphocytes or the expression of α4β7+ by T lymphocytes in mucosal tissues.

5.4.4 Malaria impact on mucosal T cell activation and CCR5 expression.

PCCAS had a profound impact on the expression of CCR5 and immune activation markers by blood T cells. Therefore, we next examined these parameters within the gut (cecum) tissues and genital mucosa (vagina and cervix), as well as in the iliac lymph nodes that drain the genital tract (iLN). In the gut, PCCAS infected animals’ demonstrated significantly increased T cell expression of the HIV co-receptor CCR5, as well as the activation markers CD38 and CD69, although MHC-II expression was similar to uninfected controls (Figure 5.4 a-d, left column). In the genital mucosa, PCCAS infection was associated with increased T cell expression of CD38, as well as increased CCR5 and MHC-II on genital CD4+ T cells, while CD69 expression was unaffected (Figure 5.4 a-e, middle column). Finally, increased expression of MHC-II and CD69 was
apparent on T lymphocytes from the iLN of malaria-infected animals, as well as increased CCR5 and CD38 expression on lymph node CD4+ T cells (Figure 5.4 a-d, right column). Overall, we observed significant increases in immune activation and CCR5 expression within both the gut and genital mucosal tissues of PCCAS-infected animals.

5.5 DISCUSSION

The per-contact risk of HIV transmission is three-fold higher in sub-Saharan Africa compared to high-income countries [7], and genital immunology plays a key role in susceptibility to HIV [6]. We previously showed that, in the absence of genital infections, young women from western Kenya had a similar overall number of cervical CD4+ T cells compared to women in the United States, but that many more cervical CD4+ T cells were activated and expressed the HIV co-receptor CCR5 [457]. Given the strong geographical overlap between HIV and malaria in Kenya [4, 141-143], we hypothesized that malaria might increase cervical T cell activation and enhance mucosal susceptibility to HIV. We tested this hypothesis in a murine model by examining the effects of *P. chabaudi* infection on putative markers of HIV susceptibility in the blood and mucosal tissues. First, we demonstrated that malaria infection increased immune activation in blood, confirming prior *in vivo* [148] and *in vitro* studies [462, 463]. In addition, while overall T cell numbers remained the same or decreased in mucosal tissues, malaria infection dramatically increased immune activation and the expression of CCR5 on mucosal (gut and genital) CD4+ T cells, closely modelling our prior findings in Kenyan women [457].

To the best of our knowledge, this is the first demonstration that malaria, a pure systemic infection, can alter mucosal immunology. However, placental malaria infection
in HIV-infected pregnant women has been associated with a three-fold increase in CCR5 expression on placental cells within the chorionic villous [151], and in some studies [152, 464], but not all [465], associate malaria in pregnancy with up to a three-fold increase in the risk of vertical HIV transmission. In our study, malaria induced a dramatic increase in α4β7 T cell expression in blood. Similar increases in α4β7 expression in blood have been reported following vaccination against yellow fever, another mosquito-transmitted systemic infection [466]. Increases in blood α4β7 expression would be expected to traffic T cells to the mucosa; however, we did not observe increases in T cell numbers or α4β7 expression on lymphocytes within examined tissues. These findings are not surprising given that α4β7 expression is known to switch in favour of αEβ7 in mucosal tissues to facilitate better anchoring of cells [467, 468].

Malaria is an important public health issue causing up to 500 million infections and an estimated 1.2 million deaths annually each year [138, 452]. Evidence indicates this parasitic infection may also enhance HIV sexual transmission at a population level, as malaria increases HIV viral levels, which is a known independent risk factor for sexual HIV transmission [97, 99, 146]. Our study suggests that malaria may also cause an increase in HIV susceptibility in HIV-uninfected individuals. However, it will be critical to confirm these murine findings in the human context, as well as to examine whether malaria therapy is able to reduce HIV target cell numbers at mucosal sites and hence HIV susceptibility. If this were the case, it would provide a rationale for future clinical trials to evaluate malaria treatment and/or prevention as an HIV prevention tool, and underscores the need for a global push to eradicate malaria [469, 470]. Nonetheless, our findings in
this murine model are provocative and closely parallel our observations of cervical T cell alterations in young women from western Kenya [457].

Other caveats should also be noted. In order to ensure sufficient T cell numbers for our assays, mucosal samples were pooled from mice, masking potential inter-individual variation. In addition, our genital processing protocol pooled T cells from the upper and lower genital tract, and so we cannot comment on whether malaria had different immune effects in these anatomical regions. Whether HIV acquisition actually takes place in the upper or lower genital tissues (or both) is unknown, but separate exploration of these genital mucosal sites will be important in future studies. The duration of malaria-induced mucosal alterations is unknown, and this may affect the evaluation of malaria prevention as a way to reduce HIV susceptibility in clinical trials. Indeed, the longevity of genital immune alterations after HSV-2 reactivation has been implicated as a reason for the failure of genital herpes suppression therapy to reduce HIV acquisition [124], although the life-long nature of genital herpes infection makes this quite distinct to malaria. Finally, it is important to note that HIV transmission rates are extremely high in southern Africa despite low rates of malaria transmission [471, 472], and so this infection alone cannot explain the enhanced sexual transmission of HIV in SSA.

In summary, we have demonstrated in a murine model that malaria infection significantly increases the mucosal expression of immune parameters expected to enhance HIV susceptibility, closely paralleling genital immune findings from young women in a malaria-endemic area. While our findings suggest a novel mechanism by which malaria may increase HIV transmission in endemic areas, confirmatory human studies are needed.
Figure 5.1

a) CCR5

b) CD38

c) MHC-II

d) CD69

e) Isotype control

Uninfected control

Malaria infected
Figure 5.1 Malarial PCCAS infection increases expression of immune activation markers in blood. The frequencies of CD4+ and CD8+ T cells expressing (a) CCR5, (b) CD38, (c) MHC-II, and CD69 in blood, and representative flow cytometry density plots from a single experiment (e) are shown. Results are representative of four repeat experiments, and each dot represents a single mouse sample. Horizontal lines in the graphs represent the median. *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001.
Figure 5.2

(a) \( \alpha 4\beta 7 \)

(b) Uninfected control vs. Malaria infected

(c) CCR9

(d) CCR6
Figure 5.2 Malarial PCCAS infection increases expression of α4β7, but not other mucosal trafficking markers in blood. A graph summarizing the expression of α4β7 (a) in blood T lymphocytes from a single experiment, and representative flow cytometry density plots (b) from CD4+ T cells are illustrated. The frequencies of CCR9 (c) and CCR6 (d) expression on blood T lymphocytes from a single experiment are also shown. Results are representative of four repeat experiments, and each dot represents a single mouse sample. Horizontal lines in the graphs represent the median. *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001.
Figure 5.3

(a) Gut mucosa

(b) Genital mucosa

CD3 Counts

CD4 Counts

CD8 Counts
Figure 5.3. Mucosal T cell numbers are not altered by Malarial PCCAS infection.

The number of cells expressing CD3 (a), CD4 (b), and CD8 (c) are shown in graphs from a single experiment. Results are representative of four repeat experiments, and each dot represents data from tissue samples pooled from three mice. Horizontal lines in the graphs represent the median. *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001.
Figure 5.4

a) Gut mucosa

b) CD38

c) MHC-II

d) CD69

e) Isotype control

Uninfected control

Malaria infected

Genital mucosa
Figure 5.4. Malarial PCCAS infection increases expression of some immune activation markers in the gut and genital mucosa, as well as in the iliac lymph nodes (iLN). The frequencies of CD4+ and CD8+ T cells expressing (a) CCR5, (b) CD38, (c) MHC-II, and CD69 (d) in the gut (left column), genital mucosa (center column) and iLN (right column) from a single experiment is shown. Representative density flow cytometry plots from genital mucosal samples (e) are shown. Results are representative of four repeat experiments, and each dot represents a single mouse sample. Horizontal lines in the graphs represent the median. *P < 0.05, **P < 0.01, ***P < 0.001, **** P <0.0001.
Chapter 6 : DISCUSSION AND FUTURE DIRECTIONS
HIV is a serious chronic infection that has a significant impact on public health globally, where it infects over 33 million people and results in 1.8 million deaths per year [1]. The majority of new HIV infections are acquired across the genital or gastrointestinal mucosa, and once infection is established, it leads to the profound depletion of CD4+ mucosal cells may drive subsequent pathogenesis. Therefore, for my PhD doctoral studies I characterized the interaction between HIV and mucosal immune cells to evaluate (i) whether the failure of HIV treatment to completely reverse HIV immunopathogenesis is related to persistent depletion of Th17 cells from the gut, (ii) the functional genital mucosal correlates of reduced susceptibility to HIV infection and lastly (iii) whether a purely systemic infection that is common in Africa (malaria) might alter mucosal markers of HIV susceptibility in a mouse model. In this chapter, I briefly introduce and discuss my findings, and offer useful insights on possible future research directions of my work.

6.1 SIGMOID Th17 POPULATIONS, THE HIV LATENT RESERVOIR AND MICROBIAL TRANSLOCATION IN MEN ON LONG-TERM ANTIRETROVIRAL THERAPY.

HIV infection results in the early and profound depletion of gut CD4+ T cells, particularly the mucosal Th17 CD4+ T cell subsets that play an important role in the mucosal defence and repair of the gut wall. HIV infection is associated with increased microbial translocation, which is a leading cause of increased systemic immune activation – and immune activation is in turn a key driver of HIV disease progression. Non-human primate models of HIV suggest there is a link between Th17 depletion, immune activation and disease progression. Here, infection of the
natural primate hosts of SIV (African green monkeys and sooty mangabeys) is associated with the maintenance of normal Th17 levels, the absence of immune activation and infection which does not progress to AIDS [164, 373]. Conversely, SIV infection of non-natural hosts (rhesus macaques) is fatal and is associated with preferential depletion of Th17 cells, immune activation and progression to AIDS [160, 164, 233]. Therefore, maintaining or restoring Th17 populations in the gut may be key towards containing HIV disease pathogenesis.

Antiretroviral therapy can restore overall CD4+ T cell numbers in the gut [259]. However, microbial translocation and immune activation still persist [376, 473]. I hypothesized that the latter observations could be due to incomplete restoration of gut Th17 cells. As part of my thesis research I investigated the frequency of Th17 cells and the ratio of Th17/Treg cells in the gut of individuals receiving successful long-term antiretroviral therapy.

My data confirmed that untreated HIV infection was associated with significant depletion of gut Th17 cells, which increased translocation of microbial products into the blood, and increased systemic immune activation. Antiretroviral therapy was associated with overall restoration of gut Th17 cell frequencies, but the degree of restoration was quite heterogeneous, and microbial translocation still persisted. I found no direct link between gut Th17 levels and markers of microbial translocation in plasma. However, individuals who had successful restoration of gut Th17 levels on therapy demonstrated a reduced gut HIV proviral reservoir, and the size of the HIV proviral reservoir was significantly smaller in those participants with better gut Th17 restoration. Nonetheless, my overall results disproved my original
hypothesis that incomplete gut Th17 reconstitution was responsible for the persistent microbial translocation and immune activation observed during HIV infection despite effective antiretroviral therapy.

Persistence of microbial translocation despite overall gut Th17 restoration was unexpected and was at odds with some [239, 376], but not all studies [376, 473]. In a blinded fashion to participant study group, I again repeated three different readouts evaluating markers of microbial translocation in plasma, and confirmed that microbial translocation did indeed remain elevated in individuals receiving antiretroviral therapy. Furthermore, the association of my microbial translocation data with both the gut HIV proviral DNA levels and mucosal Th17 frequencies suggests that my observations were real.

The persistence of microbial translocation despite overall restoration of gut Th17 CD4+ T cell frequencies following prolonged effective antiretroviral therapy was surprising. Reasons underpinning this observation are unclear, but could be attributed to several factors. Firstly, it is plausible that the absolute numbers of gut Th17 cells remained depleted despite overall restoration of Th17 frequency (proportion), if there was an overall reduction in the former. However, since gut tissues were not weighed before my cytometric assays were performed, I am unable to test this hypothesis, which should be examined in future studies. Alternatively, it is possible that the reconstituted gut Th17 cells have reduced functionality, leading to persistence of microbial translocation despite numeric restoration. A parallel body of evidence shows that normal frequencies of HIV-specific CD8+ T cells are maintained throughout infection, but these cells lose their cytolytic functionality.
during advanced disease/AIDS [290]. This loss in functionality is attributed in part to T-cell exhaustion and is demonstrated by the expression of PD-1 [294] and Tim-3 [295], in addition to continued expression of T cell central memory (CD27) but not later stage effector T cell markers of maturation [290, 296]. Therefore, it will be important for future studies to examine the functionality of these antimicrobial Th17 cells in the gut, as well as their exhaustion and maturation phenotypes. Lastly, other gut innate and adaptive immune cells also play a role in maintaining the gut wall antimicrobial immune barrier such as the innate enteric T helper like Th17 cells (iTh17) [461] and adaptive Th22 CD4+ T cells [168, 474, 475]. Therefore, future studies should also examine the proportions, numbers, and function of these cells in the gut, as well as the gut wall barrier integrity itself to see if they are defective.

The clinical implications of the persistent microbial translocation and immune activation despite Th17 restoration by antiretroviral therapy are uncertain. However, my data may fall in line with the overall observation that immune activation persists throughout HIV infection and this elevated activation status has been linked to increased adverse health outcomes such as cardiovascular disease and neurocognitive dysfunction despite antiretroviral therapy [473, 476].

Additional data generated by my research provided some interesting insights on Th17 cells and their role in microbial translocation in treated HIV infection. However, these results also raise important questions that need to be answered by future studies. First, although depletion of depletion of Th17 cells in the gut was apparent during early (CD4 T cell counts >350/ul) untreated infection, in blood the
depletion of these cells was delayed until advanced HIV disease (CD4 T cell counts <350/ul), and antiretroviral therapy only restored blood Th17 cell populations if therapy was initiated early (nadir CD4 T cell counts >350/ul). This data suggests that early initiation of therapy may allow maintenance of the systemic CD4+ T cell repertoire. The long-term clinical implications of this observation are unknown, and this will be an important avenue to pursue in future studies.

6.2 EVALUATION OF A QUANTITATIVE REAL-TIME PCR ASSAY TO MEASURE HIV-SPECIFIC MUCOSAL CD8+ T CELL RESPONSES IN THE CERVIX.

Several candidate HIV vaccines inducing virus-specific protective cellular immune responses have been designed [376, 401-403]. However, so far, no standardized immune assay exists to monitor and evaluate the efficacy of these vaccines at the genital mucosal level, where one is most likely to get exposed to HIV. Additionally, the development of genital mucosal assays will help characterize the mucosal pathogenesis of HIV transmission and susceptibility. Standard cytobrush and genital lavage genital sampling recover a limited number of immune cells, which precludes the use of most existing immune assays. Furthermore, most standard immune assays such as ELISPOT, chromium (Cr51) lysis and proliferation (CFSE dilution or H3-thymidine uptake) have a limited capacity to measure multiple immune functions. More recently, multi-parameter flow cytometry assays in peripheral blood mononuclear cells (PBMC) from HIV infected patients demonstrated that the breadth and quality of immune responses may be more important than the quantity of responses in HIV control [406]. Therefore relying on measurements of narrowly defined HIV-specific
immune responses might overlook the spectrum of responses needed for efficient HIV control. However, the recovery of few cervical mononuclear cells (CMCs) makes polyfunctional assessment of genital responses by flow cytometry very challenging [397]. Therefore, as part of my research I optimized and then evaluated real-time PCR (qPCR) as a potential platform to measure multiple genital immune responses.

I evaluated several constitutively expressed (housekeeping) genes and selected the most stably expressed gene in cervical tissues and blood to normalize immune transcript expression. Within HIV-infected individuals, I then evaluated the sensitivity of the qPCR assay in blood cells, using similar cell numbers to those that would be expected from cervical cytobrush specimens, and found that qPCR remained sensitive with input cells of at least 2.5 x10^5 blood cells. However, in a subsequent large-scale field study assessing genital responses in HIV-exposed and HESN women, I unexpectedly found that the platform was not able to detect HIV-specific T cell responses in HIV-infected women. Although robust cytokine responses to mitogen could be detected, my overall results mean that the assay platform (in its current form) will not be suitable for assessing vaccine-induced cellular responses in the female genital tract.

It is unclear why the optimized qPCR assay failed to detect HIV-specific immune responses in cervical-derived cells, despite success in PBMC-based assays with ‘cytobrush appropriate’ input cell numbers. I was able to detect virus-specific immune responses in the blood of the same women where no immune responses were detected in the cervix, suggesting that our assays were functional. It is also unlikely that there was a true absence of HIV specific cells from the cervix. Cervical
HIV-specific T cell responses have been found to mirror those present in the blood [199, 397-399], and occur with a similar/greater frequency to those present in blood [397]. Indeed both genital mucosal and systemic immune compartments show a common ontogeny of these HIV-specific immune responses [199]. Therefore, it is highly likely that HIV-specific immune responses were present in the genital tract, but my qPCR platform was unable to detect them. Another hypothesis is that there were insufficient lymphocyte cells obtained from cytobrush sampling. In accordance, less than 5% of cervical mononuclear cells collected via cytobrush sampling are actually T cells [398], while in blood lymphocyte frequencies are approximately 20% or more in HIV infected individuals (personal observation). Unfortunately, although we enumerated overall cervical cell counts after sampling, there may have been significantly less lymphocyte numbers here. Larger studies done on cytobrush derived samples in our lab demonstrate that on average, only ~600 cells are CD3+ (2 % of cytobrush derived cells), 230 are CD4+ (0.8 % of cytobrush derived cells) and 180 are CD8+ (0.7 % of cytobrush derived cells). Therefore, T cells are very low in number and frequency in the female genital mucosa (personal observation from 30 HIV-uninfected women). Finally, an alternate hypothesis explaining our data is that cervical samples had PCR amplification inhibitors, as have been reported elsewhere [391, 418, 419], that may have significantly decreased our assays sensitivity. This hypothesis will need to be tested in future studies.

If the major problem was low T cell numbers, then in vitro expansion of cervical cells is one option that can be used in future studies to overcome the
limitation of cell numbers using standard immune assays such as flow cytometry. However, potential contamination issues with commensal genital microbes (fungal/bacterial) during long-term cervical cultures might curtail the use of this technique. Furthermore, expanded cervical cell populations may preferentially expand some memory T cell subsets skewing the observations made from expanded cultures. Alternatively, future studies may look into using more invasive techniques to yield sufficient cervical cell numbers such as cervical punch biopsies, or the use of two or more cervical cytobrushes. At present, Dr. Kristina Broliden and Dr. Taha Hirbod at the Karolinska Institutet in Sweden in collaboration with our lab are piloting the use of cervical punch biopsies to see if they yield sufficient cervical lymphocytes to allow for use of standard multiparameter flow cytometry assays. However, emerging data from Dr. Florian Hladik’s group suggests that both methods have comparable cervical cell yields [NIH Mucosal Group workshop 2012]. Additionally, cutting-edge single-cell assays capable of simultaneously characterizing multiple immune responses from an individual cell are currently in the final stages of development at The Douglas Kwon labs at the Ragon Institute in Boston Massachusetts. If released for public/commercial use, this platform will negate the need for invasive genital sampling and standard mucosal cytobrush or lavage techniques will yield adequate cells for immune evaluation. Future studies using either of these sampling techniques or technologies may offer a better insight of the genital immune responses during infection, and will help in characterizing the mucosal immunogenicity of candidate vaccines.
HIV can infect both resting and activated CD4+ T cells in the genital mucosa [57]. However, it is thought that activated CD4+ T cells may be required at the mucosal site of HIV exposure for local virus propagation and eventual systemic dissemination [57, 217]. HIV preferentially replicates within activated CD4+ T cells [217], and blood lymphocytes from individuals with chronic immune activation exhibit higher susceptibility to HIV infection *ex vivo* [477]. Identifying the immune correlates of reduced susceptibility remains a key goal for the HIV vaccine field, and individuals who are HIV-exposed but seronegative (HESN) may offer some useful insight. In these individuals, reduced immune activation in blood has been identified as a possible correlate of immune protection [172, 174, 213, 214]. It is possible that mucosal HIV acquisition after sexual exposure may be enhanced by an increased number/proportion of mucosal Th17 and Th22 cells and/or mucosal T cell immune activation [24, 153, 167, 433]. My earlier research in the gastrointestinal tissue of HIV-infected individuals indicated that Th17 cells are preferentially depleted during chronic untreated HIV infection, implying enhanced susceptibility of this cell subset. However, it has yet to be determined whether genital Th17 cells and/or mucosal immune quiescence play a role in ‘real world’ HIV susceptibility. I therefore characterized the Th17-type cytokine and pro-inflammatory cytokine production by cervical mononuclear cells from the genital mucosa in a well-defined cohort of HIV-exposed but seronegative (HESN) sex workers in Nairobi.
Using the optimized qPCR assay and a multiplex-ELISA platform optimized in chapter 3, I measured ex vivo and mitogen-stimulated gene and protein induction in the cervical mucosa. I show data here illustrating that HIV exposure without infection was associated with blunted IL17 and reduced pro-inflammatory immune responses in the cervix. My data expands the immune correlates of immune protection from HIV to include blunted mucosal IL17 responses and confirms for the first time that functional immune-quiescence extends to the genital mucosa, the putative site of most HIV acquisition in Nairobi female sex workers.

There is a growing body of evidence from systemic studies suggesting that immune quiescence is associated with protection from HIV acquisition in HESN individuals [172, 174, 213, 214]. At the same time, other studies demonstrate that HESN individuals also mount HIV-specific T cell responses [191, 197, 200, 207, 478, 479], which may appear contradictory to my findings. However, a recent hypothesis suggests that both of these phenotypes may co-exist in HESNs [173, 212, 213]. Here, cellular immune quiescence in the genital mucosa may result in a reduced pool of susceptible CD4+ T cells after initial sexual HIV exposure, reducing the risk of HIV acquisition. Nonetheless, this non-productive initial HIV exposure might also be sufficient to generate the HIV-specific immune responses previously reported in HESNs. Virus-specific immune responses in the genital mucosa of HESN are reported to be about 10-fold lower than those observed in HIV infected individuals [192], and I hypothesize that this low frequency response may provide some subsequent protection against HIV without inducing substantial mucosal
inflammation. As such, my findings would be one component of several interdependent mechanisms mediating immune protection from HIV in HESNs.

My findings though preliminary, may be informative towards the vaccine and microbicide field. Specifically, perhaps my findings imply that both of these prevention interventions should strive towards inducing minimal genital pro-inflammatory immune responses, and reducing the recruitment of highly susceptible HIV target cells to the genital mucosa. Indeed, preliminary studies in non-human primates show that topical use of glycerol monolaurate microbicide gel could prevent repeated high-dose SIV challenge by blocking early pro-inflammatory events that lead to recruitment of target cells to the genital mucosa [43]. Interestingly, this gel blocked MIP3a, the natural ligand of the CCR6 receptor found in Th17 cells (CCR4+CCR6+). Conversely, the topical vaginal application of nonoxyl-9 (N-9) microbicide gel in women resulted in increased vaginal inflammation and HIV target cell recruitment in vivo and corresponded with increased HIV acquisition ex-vivo [480]. Therefore, my findings may identify a very important correlate of protection.

My study had potential limitations that will need to be addressed in future studies. First, assays used here (qPCR and ELISA) did not permit identification of the exact cell subpopulations responsible for IL17 production. IL17 production was used as a surrogate marker identifying the presence of Th17 cells in the blood and genital mucosa. However, several cell types other than classical CD4+ αβ T cells can also produce IL17, including γδ T cells, and NKT cells [447]. Future cytometric studies will be needed to identify and confirm the nature of the IL17 producing cells.
In addition, my study was cross-sectional by design and therefore I cannot establish whether the described mucosal immune quiescence and decreased Th17-type cytokines in HIV exposed, but uninfected commercial sex workers were a ‘true’ correlate of protection. Commercial sex worker may have been a confounder in my study that could just as likely influenced my observations. In this scenario, female sex workers are more likely to have more sexual partners than low-risk non-sex worker controls, and are therefore more likely to be frequently exposed to semen. Semen is capable of inducing an immunoregulatory mucosal environment that assists with fertilization and implantation [481], and therefore may have influenced the observed levels of immune quiescence. Therefore, to better identify ‘true’ correlates of immune protection participants in my study will have to be prospectively observed over time and the acquisition of HIV used as a primary outcome. Alternatively, a randomized controlled study evaluating the use of a compound capable of inducing mucosal quiescence, and that uses HIV infection as an endpoint may better test the validity of my observations.

6.4 Plasmodium Chabaudi Malaria in Mice Increases Mucosal Immune Activation and Expression of Putative HIV Susceptibility Markers.

The per-contact risk of HIV transmission is 3-fold higher in sub-Saharan Africa compared to other high-income countries [7] and it is likely that genital immunology may play a key role in susceptibility to HIV. A recent baseline study for a microbicide trial from our lab found that in the absence of any sexually transmitted infections, women from Kisumu Kenya, had higher levels of cervical immune activation than those from
San Francisco, USA [457]. Kisumu is an area holo-endemic for malaria and HIV, and several studies show that these each of these two infections interact with each other at a molecular level to fuel their prevalence [141, 145, 146]. Indeed, mathematical models based on malaria-HIV co-infected individuals suggest that the interaction of HIV and malaria within an adult population of roughly 200,000 in Kisumu may have been responsible for 8,500 excess HIV infections and 980,000 excess malaria episodes over the last 3 decades than in a scenario where there is no interaction [141]. Malaria infection results in activation of the innate and adaptive immune system to control infection [132]. Given the geographical and molecular interaction of these two epidemics and the observed holo-endemicity of these two infections in Western Kenya, I hypothesized that malaria may increase susceptibility to HIV in the genital mucosa and tested this hypothesis in a murine model by examining the effects of *P. chabaudi* malaria infection on putative markers of HIV susceptibility in the gut and genital mucosa.

*P. chabaudi* systemic malaria infection increased the expression of the HIV entry co-receptor CCR5 on lymphocytes as well as T cell immune activation markers (CD38, CD69, HLA-DR) in the blood, gut and genital mucosa.

Observed changes in mucosal tissues following malaria infection are quite interesting given that malaria is purely a systemic infection. If the same holds true in humans, then malaria infection may indeed drive susceptibility to HIV. CCR5 is both a marker of immune activation and also functions as a HIV co-receptor. Non-human primate studies show that infection of activated CD4+ T cells is required for dissemination of infection and seroconversion [57, 217]. Thus the increase in CCR5 as well as other T cell immune activation markers may result in an increased risk of
productive HIV infection upon exposure. Furthermore, studies have shown that the CCR5 receptor on CD4+ T cells is critical for R5 viral infection. Individuals with a homozygous CCR5-Δ32 mutation show near absolute protection from HIV infection [154, 482]. Malaria might also have a direct impact on HIV susceptibility through CCR5 expression. Placental malaria infection in HIV-infected pregnant women has been associated with a three-fold increase in CCR5 expression on placental cells within the chorionic villous [151]. Increased CCR5 expression in pregnancy has in turn been associated with an up to three-fold increase in the risk of vertical malaria transmission in some [150, 152, 464], but not all studies [465]. Therefore, increased mucosal CCR5 expression and immune activation following malaria infection may increase susceptibility to HIV in humans.

My findings in an animal model are preliminary but they may have important implications on public health policy given the significant amount of infections and deaths that both malaria and HIV cause in sub-Saharan Africa every year. The data that I generated arguably provides a key rationale for future observational clinical studies and eventually randomized control studies to assess the impact of anti-malarial treatment or eradication to evaluate if this will help stem the spread of HIV in regions where both infections are endemic. However, it is critical to note that our findings were observed within a murine model and will need to be confirmed in humans first. These studies are currently under preparation in our lab. Second, my preliminary study only demonstrated that malaria increases putative markers of HIV susceptibility in common mucosal sites of HIV acquisition. *In vitro* human tissue culture experiments or *in vivo* primate models are also required to link observed
malaria-induced immune changes with increased susceptibility to HIV acquisition. Lastly, HIV transmission rates remain extremely high in some parts of sub-Saharan Africa that have no malaria, such as South Africa. Therefore, malaria is not the only environmental infection that enhances acquisition of HIV in this region. Other than the typical sexually transmitted infections, several other infections, such as tuberculosis, geohelminths, schistosomiasis and filariasis, are geographically prevalent in sub-Saharan Africa and are also known to interact with HIV. Here, treatment of these infections, including malaria, has been noted to reduce HIV viremia [135], and it is also possible that these infections may potentially alter transmission and/or susceptibility to HIV. Therefore, future studies evaluating the genital and rectal mucosal impact of other prevalent infections in this region will be of much interest.
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APPENDIX: EFFECT OF RALTEGRAVIR INTENSIFICATION ON HIV PROVIRAL DNA IN THE BLOOD AND GUT MUCOSA OF MEN ON LONG-TERM THERAPY: A RANDOMIZED CONTROLLED TRIAL

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A1. ABSTRACT

Background: Highly active antiretroviral therapy (HAART) dramatically reduces plasma HIV-1 viremia. However, despite completely suppressive HAART, it has been suggested that low-levels of viral replication may persist in the gut mucosa and elsewhere in individuals on long-term HAART.

Objective: We conducted a double-blind randomized, placebo-controlled trial (RCT) evaluating whether intensification of HAART in long-term virologically suppressed individuals with raltegravir is associated with a reduction in the level of proviral HIV-1 DNA in CD4+ T cells in blood and the sigmoid colon (gut).

Methods: Long-term (>4 years) virologically suppressed HIV-infected individuals on standard HAART were randomized 1:1 in a double-blind fashion to receive raltegravir (400mg twice/day) or placebo for 48 weeks. After week 48, all participants were treated with raltegravir to week 96. Blood and sigmoid biopsies were sampled and the frequency of CD4+ T cells carrying HIV-1 proviral DNA was determined.

Results: 24 study patients were recruited. At 48 weeks, no difference was apparent between participants receiving raltegravir or placebo in blood HIV-1 proviral levels (p=0.62), CD4+ T cell counts (p=0.25) and gut proviral loads (p=0.74). Similarly, prolonged raltegravir intensification up to week 96 had no further effect on both blood and gut HIV-1 proviral loads and blood CD4+ T cell counts.

Conclusion: In long-term virologically suppressed patients on standard HAART, intensification with raltegravir did not result in further decay of CD4+ T cells carrying HIV-1 proviral DNA in either the blood or gut after 48 or 96 weeks of therapy, or in any increase in CD4+ T cell counts.
Effective, highly active antiretroviral therapy (HAART) suppresses viremia to levels below the limit of detection of commercial assays (<50 copies/ml) in individuals infected with human immunodeficiency virus-1 (HIV) [1, 2]. However, several studies demonstrate that a significant proportion of infected individuals receiving apparently suppressive HAART still maintain residual plasma viremia (1-49 copies/ml) [3-5]. This residual viremia is also detectable in HIV infected individuals who have received effective HAART for almost a decade [6, 7]. The source of residual viremia in individuals receiving HAART is unclear, but it may originate from infected cells supporting ongoing viral replication, release of virus from stable reservoirs upon sporadic activation, cell-to-cell spread, and/or a combination of these [4, 8-12]. If so, then the addition of potent new therapeutics may further reduce low-level viremia in individuals taking standard HAART and constitute a key step towards clearing HIV infection [9, 13].

The emergence of new HIV antiviral agents such as raltegravir, an integrase inhibitor, presents a novel way to potentially target residual virus. When added to a standard HAART regimen, raltegravir intensification has shown promise in reducing the time to attainment of an undetectable blood viral load [14-16]. In addition, mathematical models predict that raltegravir treatment intensification may result in reduction of residual low-level viremia [16]. Despite these theoretical benefits, recent studies have not shown any impact of raltegravir intensification on residual plasma viremia or the proviral load in blood [17-20]. However little has been done to characterize of the impact of raltegravir intensification on HIV proviral levels in the gut mucosa, which is believed to be a potentially important reservoir of latent virus [6, 21-23]. In this regard, a recent
promising study by Yukl and colleagues demonstrated that raltegravir intensification could reduce the amount of unspliced HIV RNA in the terminal ilium mucosa [24]. However, this was a short term, open label pilot study that had enrolled participants receiving a variety of other antiretroviral agents in addition to raltegravir [24].

Therefore, to better ascertain the impact of raltegravir-only intensification on the viral reservoir in both the blood and gastrointestinal tissues there is a need for a long-term randomized, placebo-controlled trial (RCT). We hypothesized that prolonged raltegravir intensification in this context would be associated with a decrease in HIV proviral DNA in both blood and gut CD4+ T cells, and conducted a prospective, double-blind, RCT to assess this.

**A3. MATERIALS AND METHODS**

**A3.1 Ethics statement.** All participants provided written informed consent, and the study protocol was reviewed and approved by the Research Ethics Boards at St. Michael’s Hospital and the University of Toronto, Toronto, Ontario, Canada.

**A.3.2 Participant recruitment and study design.** Twenty-four participants were enrolled through the Maple Leaf Medical Clinic in Toronto, Canada, and consisted of HIV-infected men on HAART with an undetectable HIV plasma viral loads (<50 copies/ml) for at least 4 years. Enrolled participants were required to be on their first standard HAART regimen with 2-3 nucleoside reverse transcriptase inhibitors (NRTIs), and 1-2 protease inhibitors (PIs) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) for at least four years. Participants were excluded if they had an AIDS-defining illness in the
6 months preceding recruitment or had taken mono or dual HAART in the past. An a priori sample size calculation identified that 12 participants were required per group to detect a 0.4 log$_{10}$ difference in the change from baseline to 48 weeks in plasma proviral HIV DNA levels between treatment groups with 80% power and p-value < 0.05.

Blocked randomization was carried out using variable block sizes. Randomization and concealment allocation was organized by the study statistician (JR). Once enrolled, each participant was required to complete a baseline questionnaire, which included demographic, clinical and laboratory characteristics.

In this double-blind RCT, enrolled participants were randomly assigned in a 1:1 fashion to receive either raltegravir (400mg twice a day) or placebo for 48 weeks. At week 48 all participants were unblinded and those receiving the placebo were rolled over to the intervention arm and all patients were treated with raltegravir to week 96.

The primary objective of this study was to evaluate the change of proviral HIV-1 DNA in total CD4$^+$ T cells from baseline to week 48 in participants randomized to the raltegravir arm (400mg raltegravir) for 48 weeks in addition to their current standard combination antiretroviral regimen versus the control arm, who remained on their current standard combination antiretroviral regimen. As a secondary objective we also evaluated the effect of raltegravir intensification on blood CD4$^+$ T cell populations. At week 48, a post-hoc phase II analysis of our study was conducted where raltegravir therapy was added to the previous placebo group and independently analysed to confirm our previous week 0-48 results in our raltegravir-only group. In additional in participants who received raltegravir for 96 weeks (raltegravir-arm) were independently evaluated to
determine if prolonged raltegravir intensification had any long-term effects on proviral HIV DNA and CD4+ T cell populations in the blood and gut.

To evaluate clinical and laboratory parameters, blood draws were performed on all participants at approximately 4-week intervals from baseline to week 96, and sigmoid biopsies were obtained at baseline, week 48 and at week 96 for all participants. This study summarizes results for the MK-0518 (raltegravir) viral decay study registered at ClinicalTrials.gov (NCT#:NCT00520897).

**A.3.3 Processing of peripheral blood and sigmoid biopsies.** Blood was collected by venipuncture into Ethylenediaminetetraacetic acid (EDTA) tubes (BD Bioscience, La Jolla, CA) and peripheral blood mononuclear cells (PBMC) were then isolated via ficoll-hypaque density centrifugation as previously described [25]. Sigmoidoscopy samples were obtained approximately 25-30cm from the anal verge, and were incubated first in a 0.5 µg/ml and then a 1.0 µg/mL collagenase solution on a shaking heating-block at 37°C for 30 minutes each. After obtaining single cell suspension, CD8+ T cells were depleted using anti-CD8 magnetic beads (StemCell Technologies, Vancouver, BC, Canada).

**A.3.4 Measurement of HIV proviral DNA.** CD4+ T cells were enriched from PBMC using a column-based cell separation technique (StemCell Technologies, Vancouver, BC, Canada). Realtime polymerase chain reaction (PCR) was carried out on genomic DNA isolated from 1–2 x10^6 purified resting or activated CD4+ T cells using the Puregene DNA isolation kit (Gentra, Minneapolis, MN) in accordance with the manufacturer’s specifications. 1µg of DNA was then used as a template for PCR in an iCycler (Bio-Rad,
Hercules, CA). The amplification reaction was carried out in triplicate using 0.5 µmol/L primers, 0.2 µmol/L fluorescent probe, 0.8 mmol/L dNTPs, 5 µmol/L MgCl2, and 2.5 U Platinum Taq Polymerase (Invitrogen) in 50 µL total volume. The following primers were used: 5' GGTCTCTCTGGTTAGACCAGAT-3' (5' primer) and 5'-CTGCTAGAGATTTTCACACTG-3' (3' primer) along with the fluorescent probe 5'-6FAM-AGTAGTGTGTGCCGGCTCTGTT-TAMRA-3'. PCR conditions consisted of a denaturation step at 95°C for 3 min, followed by 45 cycles of 15 sec at 95°C and 1 min at 59°C. Serially diluted ACH-2DNA (40,000, 8000, 1600, 320, 64, 12.8, 2.56, and 0.56 cell equivalents per well in triplicates) was also subjected to the PCR conditions above to obtain standard curves. The detection limit of the assay was 2.56 copies of HIV DNA.

After endoscopic terminal ileum biopsies, tissue samples were incubated with 0.5 mg/mL collagenase (Type II-S; Sigma Aldrich, St Louise, MI) in RPMI containing 5% fetal bovine serum, HEPES, and pen-strep at 37°C for 30 min. After frequent pipetting and vortexing, cells were washed and stored on ice, and the remaining undigested tissue was treated with 1.0 mg/mL collagenase for an additional 30 min. The frequency of CD3+CD4+ T cells was determined by fluorescence-activated cell sorter analysis (FACS) (FACSCanto; BD Biosciences La Jolla, CA). A fraction of the cells were subjected to CD8 depletion (Invitrogen-Dynal, Carlsbad, CA) and the percentage of CD3+CD4+ T cells was determined by FACS. Approximately 200,000 CD8-depleted cells were lysed in 10 mmol/L Tris-HCl at pH 8 that contained 100 µg/mL proteinase K (Roche Applied Science) for 1 hour at 56°C, followed by heat inactivation of the enzyme. PCR specific for human β-actin DNA (Applied Biosystems, Foster City, CA) was carried out on the cell lysates described above to determine the exact copy number of cells per
µL of cell lysate. Serially diluted ACH-2cell lysates were prepared to obtain standard curves. Finally, PCR specific for HIV DNA was carried out as described above and the number of copies of HIV DNA per \(1 \times 10^6\) CD4+ T cells was calculated on the basis of results obtained from the FACS and PCR experiments.

**A.3.5 Antiretroviral drug concentration measurement.** To assess adherence, plasma drug concentrations were analysed in plasma samples from a randomly selected subset of participants (3 in the raltegravir group at week 48, 4 in the raltegravir group at week 96, and 3 in the placebo group that had previously received placebo then later received 48 weeks of raltegravir therapy and sampled at study week 96). Plasma drug concentrations of raltegravir were determined by using a validated liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) method. A 200 µL plasma sample was spiked with 50 µL 6,7-dimethyl-2,3-di(2-pyridyl)-quinoxaline (Aldrich, Milwaukee, WI, USA) as internal standard (IS) and subjected to protein precipitation with acetonitrile (1:3) followed by centrifugation at 2000 X g for 5 minutes. The LC-MS/MS system consisted of a HP1100 LC system (Agilent Technologies, Wilmington, DE, USA) with a Supelcosil™ ABZ+ (15 cm x 4.6mm, 3 µm) C18 column (Supelco, Bellefonte, PA, USA) coupled to an API-2000 mass spectrometer (AB/MDS/Sciex, Concord, ON, Canada) with a turbo ion spray source. LC was performed at 40 °C with a gradient elution of acetonitrile-0.1% (v/v) formic acid in water at a flow rate of 1ml/min. Mass was quantified using electrospray multiple reaction monitoring (MRM) in positive mode and the MRM transitions were m/z 445 to 109 and m/z 313 to 246.4 for raltegravir and the IS, respectively. The absolute recoveries were 93-100% and raltegravir was stable for 24
hours at 4 °C after sample preparation and during 3 freeze-thaw cycles. The effective linear range was 22.5-4500ng/mL. Interbatch precision varied between 6.1 and 12.7% (CV%) and intrabatch accuracy varied between 98.8 and 102.8 %. In addition, since all enrolled study participants were receiving standard HAART, the concentrations of PIs and NNRTIs in the plasma samples were also tested in those patients where raltegravir levels were also assayed. These drug concentrations were determined using a validated LC-MS/MS assay as previously published [26].

**A.3.6 Statistical methods.** Baseline demographic and clinical characteristics were summarized within treatment groups using median and interquartile ranges (IQR) for continuous variables, and frequencies and percentages for categorical variables, and were compared using Wilcoxon rank sum tests for continuous variables and chi square tests or Fisher’s exact test for categorical variables. Wilcoxon signed-rank tests were used to compare HIV proviral DNA or CD4+ counts between visits within groups. Analysis of covariance (ANCOVA) was used to assess the effect of treatment between groups at week 48 by adjusting for baseline values.

**A.4 RESULTS**

**A.4.1 Study Participants**

Twenty-four HIV-infected men who had maintained undetectable HIV viral loads (<50 copies/ml) for at least 4 years were recruited into the study. Enrolled participants were randomized to receive raltegravir intensification (n=12) or placebo (n=12) in addition to their standard HAART regimen.
At baseline, the two study groups had comparable demographic and clinical variables including age, CD4+ and nadir T cell counts, blood and sigmoid proviral levels (Table A.1). Participants in the placebo and raltegravir-intensified groups were all receiving standard HAART and had a comparable distribution of those taking NNRTI, PI or boosted PI-based therapies (Table A.1). One participant in the placebo group dropped out at week 6 before the study completion due to mild self-reported adverse events (insomnia, dizziness, nausea).

**A.4.2 Effect of HAART Intensification with raltegravir on blood HIV DNA proviral load.**

To observe the effect of raltegravir-intensified therapy over time blood HIV DNA proviral levels in CD4+ T cells were assayed in all enrolled participants (Figure A.1). Compared to baseline, a minor increase in blood DNA proviral level was observed in the raltegravir-intensified group (median +0.06 log_{10}/1x10^6 CD4+ T cells; Wilcoxon signed-rank test p=0.04) and a similar but non-significant trend to increased proviral levels was also observed in the placebo group (median +0.06; p=0.11). However, there was no difference in HIV DNA proviral levels between the two groups following 48 weeks of raltegravir intensification (ANCOVA p=0.62).

**A.4.3 Effect of raltegravir therapy on CD4+ T cell counts.**

After 48 weeks of raltegravir intensification there was a slight but non-significant decrease in CD4+ T cell counts in the raltegravir-intensified group (median -20 cells/mm^3; Wilcoxon signed-rank test p=0.26) and in the placebo group (median -10
cells/mm², p=0.68). In addition, blood CD4+ T cell counts did not differ at week 48 between groups (ANCOVA p=0.25; Figure A.2).

**A.4.4 Effect of raltegravir therapy on HIV proviral loads in the sigmoid colon.**

Proviral HIV DNA loads in the sigmoid colon were assayed and evaluated at baseline and week 48. The raltegravir-intensified group had a slight, and not statistically significant, decrease in sigmoid proviral levels/1x10⁶ CD4+ T cells (median -0.06 Log₁₀ copies, Wilcoxon signed-rank test p=0.08). However, despite an even greater decrease in proviral levels in the placebo group relative to the raltegravir group, this change did not attain significance in the placebo group (median -0.13 Log₁₀; p=0.13). Nonetheless, raltegravir intensification did not decrease sigmoid proviral levels more than the placebo group (ANCOVA p=0.74; Figure A.3).

**A.4.5 Effect of prolonged raltegravir therapy intensification in the blood and sigmoid.**

It is possible that the 48-week duration of raltegravir intensification was insufficient to establish the efficacy of raltegravir intensification. To assess this point, enrolled study participants were followed for another 48 weeks out to week 96 and a post-hoc analysis of blood and sigmoid DNA proviral levels and blood CD4+ T cell counts was then performed. Similar to week 48, there was a slight but significant increase in blood HIV DNA proviral loads in participants intensified with raltegravir for 96 weeks compared to baseline (median +0.10 log₁₀ copies/1x10⁶ CD4+ T cells, Wilcoxon signed-rank test p=0.01). This increase in blood proviral levels at week 96 was concurrent with a significant decrease in blood CD4+ T cell counts compared to baseline (median -115
cells/mm³; Wilcoxon signed-rank test p=0.05). However, no significant changes in the gut mucosa proviral levels were apparent at week 96 compared to baseline in the raltegravir-intensified group (median $+0.03 \log_{10}$ copies/1x$10^6$ CD4+ T cells; Wilcoxon signed-rank test p=0.58). For those participants at week 48 who were switched from placebo to raltegravir-intensified therapy and followed up to week 96, a similar analysis was carried out using pre-intensification values at week 48 as the new baseline. After 48 weeks of raltegravir intensification, there was a slight increase in the HIV proviral DNA load (median $+0.06 \log_{10}$ copies/1x$10^6$ CD4+ T cells, Wilcoxon signed-rank test p=0.01), while no change was observed in the blood CD4+ T cell counts within this group compared to baseline (median $-20$ CD4+ T cells/mm³, Wilcoxon signed-rank test p=0.41). In the sigmoid mucosa, proviral HIV DNA loads remained unaltered compared to week-48 baseline ($+0.04 \log_{10}$ copies/1x$10^6$ CD4+ T cells; Wilcoxon signed-rank test p=0.48).

### A.4.6 Plasma levels of raltegravir and other antiretrovirals

To ascertain treatment adherence of study participants, raltegravir concentrations were analysed in plasma samples collected from a randomly selected subset (~25%-33%) of participants in each study arm (see methods for breakdown). All available raltegravir levels were higher than the 95% inhibitory concentration of 33 nmol/L ($\approx 0.0146$ mg/L) [18], and are summarized in Table A.2. In addition, the concentrations of PIs and NNRTIs in the plasma samples of this subset of patients were consistent with good treatment adherence since all levels were in the therapeutic range (data not shown).
A.5 DISCUSSION

HIV eradication may be hindered by the inability of standard HAART to abrogate low-level residual virus replication [3, 4, 6, 7, 27]. We hypothesized that prolonged therapy intensification with raltegravir would further reduce the HIV proviral DNA load in CD4+ T cells. However, we found no evidence that 48 weeks of raltegravir intensification in the context of long-term HIV suppression on standard HAART regimens reduced blood or gut HIV CD4+ T cells carrying HIV proviral DNA, or improved blood CD4+ T cell counts. Furthermore, unblinded continuation of raltegravir intensification for an additional 48 weeks (out to 96 weeks in total) had no impact on these parameters, despite attainment of therapeutic plasma levels of raltegravir and other antiretroviral agents.

Following HAART initiation, the kinetics of viral decay are typically defined by a significant decrease in plasma virus levels and the subsequent clearance of HIV-infected cells in three distinct phases characterized by short (1 day), intermediate (14 days) and extended (39 weeks) half-lives [1, 2, 27, 28]. However, other studies suggest that an even longer-lived stable reservoir of T cells is maintained for at least 7-10 years after the initiation of therapy characterizing a fourth phase of viral decay [6, 29]. These latent reservoirs are thought to have a potential ability to replenish their levels despite effective HAART [6, 29]. Thus, the absence of decreased HIV DNA in raltegravir-treated participants suggests that this may represent an already pre-established long-lived viral reservoir, which is not significantly impacted by new infectious events that may be prevented while on intensified effective HAART [24].
Our findings are consistent with other recent studies that have found no effect of raltegravir intensification on residual HIV RNA and proviral DNA loads in the blood and also the gut [17-20]. However, not all mucosal reservoirs of HIV appear recalcitrant to raltegravir therapy. A recent report by Yukl and colleagues demonstrated that the basal cell-associated unspliced HIV RNA loads in the terminal ileum were higher than that in other gut sites, and that raltegravir intensification significantly reduced the unspliced HIV RNA burden in the ileum but not in the rest of the gut [24]. However, in our study we only sampled the sigmoid mucosa, and we measured proviral HIV DNA rather than cell-associated unspliced HIV RNA. While we are unable to comment on the possible effects of raltegravir intensification on this parameter, it should be noted that Yukl and colleagues also found no decrease in HIV proviral DNA in the ileum or elsewhere in the gut [24], and in the blood [24], which is consistent with our findings.

There are some potential limitations to our study. First, low-level residual viremia (<50 copies/ml) was not assessed, although other studies have reported that raltegravir intensification did not affect residual viremia [18-20, 24]. Furthermore, the clinical significance of this residual viremia remains to be clearly understood. In addition, we measured the HIV DNA proviral load, which also serves as a useful estimation of the persistence of HIV in viral reservoirs [6]. Second, our sample size was small and our findings may need to be confirmed in larger studies. Lastly, raltegravir-driven integrase mutations may occur in individuals receiving combination therapy [30] and this may be an important aspect to explore in future studies. Nevertheless, raltegravir-induced mutations in the integrase gene have mainly been reported in individuals with a history of
treatment failure [31-33] and are rare in individuals with successful viral suppression [34].

In summary, we found that raltegravir intensification in long-term suppressed individuals on standard HAART had no impact on HIV DNA proviral levels in the blood or the sigmoid colon. Therefore, potent additional therapeutics will need to be developed, either to prevent repletion of the residual HIV-latent reservoir or to reach the ultimate goal of HIV eradication.
### Table A.1. Summary of baseline characteristics of study participants.

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Raltegravir (IQR)/Count, n=12</th>
<th>Placebo (IQR)/Count (%), n=12</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>48.2 (45.0-54.0)</td>
<td>49.5 (42.6-55.9)</td>
<td>0.89</td>
</tr>
<tr>
<td>Years since HIV diagnosis</td>
<td>11 (8.5-15.5)</td>
<td>10.5 (6.5-18.5)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>CD4+ T cell counts (cells/mm$^3$)</td>
<td>665 (555-865)</td>
<td>610 (505-820)</td>
<td>0.50</td>
</tr>
<tr>
<td>Nadir CD4+ T cell counts (cells/mm$^3$)</td>
<td>287(40-308)</td>
<td>190(110-515)</td>
<td>0.57</td>
</tr>
<tr>
<td>Baseline blood viral load &lt;50 copies/ml</td>
<td>12 (100%)</td>
<td>12 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Blood proviral load (per 1x10$^6$ CD4+ cells)</td>
<td>1127(182-1870)</td>
<td>726 (440-1401)</td>
<td>0.77</td>
</tr>
<tr>
<td>Sigmoid proviral load (per 1x10$^6$ CD4+ cells)</td>
<td>1263 (280-3601)</td>
<td>1567 (1352-2023)</td>
<td>0.92</td>
</tr>
<tr>
<td>Current ARV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNRTI-based (%)</td>
<td>7 (58%)</td>
<td>5 (42%)</td>
<td>0.68</td>
</tr>
<tr>
<td>PI-based (%)</td>
<td>5 (42%)</td>
<td>7 (58%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Boosted PI-based (%)</td>
<td>4 (33%)</td>
<td>7 (58%)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Abbreviations: IQR, inter-quartile range. ARV, antiretroviral. NNRTI, non-nucleoside reverse transcriptase inhibitor. PI, protease inhibitor.
Table A.2. Raltegravir concentrations in plasma samples from a subset of study participants.

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient ID</th>
<th>Raltegravir concentration (mg/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raltegravir group (at week 48)</td>
<td>MKVD 11</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>MKVD 12</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>MKVD 15</td>
<td>0.29</td>
</tr>
<tr>
<td>Raltegravir group (at week 96)</td>
<td>MKVD 8</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>MKVD 11</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>MKVD 18</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>MKVD 24</td>
<td>0.62</td>
</tr>
<tr>
<td>Placebo group (48 placebo + 48 weeks raltegravir – sampled at study week 96)</td>
<td>MKVD 2</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>MKVD 14</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>MKVD 16</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*Plasma concentrations of raltegravir were determined by using validated liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS) method. All available raltegravir levels were higher than the 95% inhibitory concentration of 33 nmol/L (≈0.0146 mg/L) [483]
Figure A.1.

HIV DNA in blood CD4+ T cells

Blood HIV DNA proviral load

\(1 \times 10^{02}\) to \(1 \times 10^{04}\)

Week

0 4 8 16 24 32 40 48

Raltegravir

Placebo
Figure A.1. Blood HIV proviral levels over time. Following 48 weeks of raltegravir intensification, the median change in the $\log_{10}$ proviral HIV DNA/1x10$^6$ CD4+ T cells in the raltegravir-intensified group was +0.06 while that in the placebo treated group was +0.06, and there was no significant difference between the groups (p=0.62). The dotted lines indicate individual plots for each participant receiving raltegravir (dotted red line) or placebo (dotted blue lines) and the median levels are indicated in bold red and bold blue for the raltegravir-intensified and placebo groups respectively.
Figure A.2.

Blood CD4+ T cell counts

Week

Blood CD4+ T cell counts/mm²

- Raltegravir
- Placebo
**Figure A.2: Blood CD4+ T cell counts levels over time.**

Median CD4+ T cell levels decreased slightly in both groups from baseline to week 48 in both the raltegravir-intensified group (-20 counts/mm$^3$) and placebo group (-10 counts/mm$^3$). However, there was no significant difference in blood CD4+ T cell counts after 48 weeks of raltegravir therapy compared to placebo alone (p=0.25).
Figure A.3.
Figure A.3. Sigmoid HIV DNA proviral levels in CD4+ T cell samples over time.
After 48 weeks of follow up, there was no difference in the median change in sigmoid HIV DNA Log_{10} levels/1x10^6 CD4+ T cells from baseline between the raltegravir-intensified group (-0.06) and the placebo group (-0.13; p=0.74). The dotted lines indicate individual plots for each participant receiving raltegravir (dotted red line) or placebo (dotted blue lines) and the median levels are indicated in bold red and bold blue for the raltegravir-intensified and placebo groups respectively.
A.6 APPENDIX REFERENCES:


11. Finzi, D., Blankson, J., Siliciano, J.D., Margolick, J.B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., Quinn, T.C., Chaisson, R.E.,


patients on antiretroviral therapy: a randomized controlled trial. PLoS medicine, 2010. 7(8).


