Development and clinical application of a novel ex vivo assay to assess genital HIV susceptibility

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

A better understanding of the biological factors that promote heterosexual HIV acquisition in the female genital tract (FGT) may inform HIV prevention efforts. The mucosal cellular targets of HIV are not well defined and proposed cellular and molecular correlates include the HIV coreceptor CCR5 and markers of immune activation, including CD69. I established a novel ex vivo HIV entry assay for mucosal studies to enable assessment of ex vivo HIV infection in freshly isolated and unstimulated endocervical CD4+ T cells. HIV susceptibility was enhanced in cervix-derived CD4+ T cells compared to blood. Cervical CCR5+, CD69+, α4β7+ or α4β1+ CD4+ T cells were preferential HIV targets, however, the homing integrins α4β7 or α4β1 did not mediate direct binding to the HIV env or HIV entry into CD4+ T cells, suggesting that α4β7 and α4β1 are unlikely to be HIV entry receptors.

Next, I tested whether ex vivo HIV entry predicts subsequent heterosexual HIV acquisition. Ideally, this would be done using fresh genital samples, but these are usually not available. Hence, I performed a nested, retrospective, blinded, case-control study in blood CD4+ T cells obtained from HIV-uninfected South African female participants enrolled in the CAPRISA 004
clinical trial. Virus entry into blood CD4+ T cell subsets did not predict subsequent HIV acquisition, possibly due to the compartmentalization of immune factors in the genital mucosa. One such compartmentalized factor in the FGT is the bacterial microbiota, which is dominated in the majority of women by *lactobacilli*, while a shift towards diverse gram-negative anaerobic species, defined (based on Gram stain) as bacterial vaginosis (BV), is associated with increased HIV acquisition in women. To investigate underlying biological mechanisms that may increase HIV acquisition risk in women with BV, I assessed the impact of BV treatment using the current standard of care, oral metronidazole, on genital immunology and HIV susceptibility. BV treatment reduced endocervical CD4+ T cell susceptibility to HIV and genital IL-1 levels; however, an unexpected increase in several chemokines previously associated with increased HIV risk suggests that more studies are needed before the treatment and/or prevention of asymptomatic BV can be recommended for HIV prevention.
Acknowledgments

First and foremost I would like to thank my family whose support and openness was critical in allowing me to pursue any career of my choice. While it isn’t usually easy to explain what I do and why I do it to them, they trusted me and gave me the freedom to explore various offbeat career paths until I finally decided to do a PhD with Rupert. I cannot thank my family enough, and also my friends who supported me during this difficult time. It is due to this process of self-discovery that I was able to fully enjoy and dedicate myself to the PhD.

My supervisor Rupert has always been inspiring and supportive, and he was always open to listening to my daily impromptu discussions about science, especially when he was on his way out of his office to go to a meeting or home. He always makes time for his students and ensures that all of our projects move forward in a timely fashion and I’m sure we’re all very grateful for that. Rupert offered a good balance between guidance, structure, and freedom to pursue various projects and I think it worked out very well for the both of us. Thanks to Rupert, I was able to build great networks and collaborations with leading HIV scientists and trainees around the world. The chance to travel to Kenya and South Africa to do my projects was wonderful and unique, and I found my 2nd and 3rd homes there. Rupert is well respected by the scientific community and I am fortunate to be associated with him and I hope that we continue to collaborate productively for a long time. Rupert is extremely funny, and his management skills are exceptional, and this keeps our spirits up even if our p values are not significant. I am also very envious of Rupert’s written communication skills and I hope I have picked up a thing or two from him after all these years! I can say without a doubt that he is an amazing boss and it will not be easy after the PhD to be away from him.

Sanja’s support in the lab carried me through my PhD, and she was always my best friend in the lab. Her calm demeanour and warmth make the lab welcoming for us all. I would also like to thank my graduate school friends and mentors: Lyle, Sim, Ronald, Sergey, Nur, Jordan, Justen, Catia, Jasty, Angela, Lindi, Genevieve and many others for their help, discussions and being my friends. I’d also like to thank my present and past labmates and collaborators including Connie, Brett, Shubhendu, Hajra, Segen, Rodney, Avid, Yoojin, Jess, TJ, Onyango, Jun, and Shariq. I want to especially thank people who were critical of me, as I learned a lot from them, and I took all of their criticisms very seriously and tried to improve myself.
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List of Abbreviations

AIDS - acquired immunodeficiency syndrome
ADI - AIDS-defining opportunistic illness
AMP - anti-microbial peptide
ART - anti-retroviral therapy
ATRA - all-trans retinoic acid
AVF - altered vaginal flora
BD - Becton Dickinson
BV - bacterial vaginosis
BlaM - beta lactamase
CAPRISA - Centre for the AIDS Programme of Research in South Africa
CCR - C-C chemokine receptor type
CD - cluster of differentiation
CDC - Centre of disease control
cDNA - complementary DNA
CI - confidence interval
CIB - A calcium- and integrin-binding-A
CIB - B calcium- and integrin-binding-B
Cpz - chimpanzee
CST - community state type
CT - *Chlamydia trachomatis*
CV - coefficient of variation
CVS - cervicovaginal secretions
DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM - Dulbecco’s modified Eagle medium
DMPA - depometroxy progesterone acetate
DNA - deoxyribonucleic acid
ECD - extracellular domain
ECM - extracellular matrix
ELISA - enzyme linked immunosorbent assay
FBS - fetal bovine serum
FGT - female genital tract
FITC - fluorescein isothiocyanate
FRET - fluorescence resonance energy transfer
GM-CSF - granulocyte macrophage colony stimulating factor
hCG - human chorionic gonadotropin
HSV-2 - herpes simplex virus -2
HIV-1 - Human immunodeficiency virus-1
HIV-2 - Human immunodeficiency virus-2
IFN-γ - interferon- γ
IL - interleukin
IP-10 - interferon gamma induced protein 10
IQR - inter-quartile range
GALT - gut-associated lymphoid tissue
gp120 - glycoprotein 120
gp41 - glycoprotein 120
gp160 - glycoprotein 120
GM-CSF - granulocyte macrophage colony stimulating factor
GML - glycerol monolaurate
KAVI ICR - Kenya AIDS Vaccine Initiative – Institute of Clinical Research
LLOD - lower limit of detection
LPS - lipopolysaccharide
LTR - long-term repeat
MADCAM-1 - mucosal vascular addressin cell adhesion molecule 1
MC - male circumcision
MCC - Medicines Control Council
MCP-1 - monocyte chemotactic protein 1
MIG - monokine induced by interferon γ
MIP - macrophage inflammatory protein
MLC - mucosal lymphoid clusters
MMP - matrix metalloprotease
mRNA – messenger RNA
MSD - meso-scale discovery
NG – Neisseria gonorrhea
NIH - National Institutes of Health
NLR - NOD-like receptor
OR - odds ratio
PCR - polymerase chain reaction
pDC - plasmacytoid dendritic cell
PE - phycoerythrin
PEI - polyethyl-imine
PrEP - pre-exposure prophylaxis
PRR - pattern-recognition receptor
RANTES - regulated on activation, normal T-cell expressed and secreted
PBMC - peripheral blood mononuclear cell
PBS - phosphate buffered saline
PIC - pre-integration complex
PR - protease
PRR - pattern recognition receptor
QIM – quantitative immunofluorescence microscopy
RT - reverse transcriptase
RNA - ribonucleic acid
SIV - simian immunodeficiency virus
Sm - sooty mangabey
SSA - sub-Saharan Africa
ssRNA - single stranded RNA
STI - sexually transmitted infection
TCM - central memory T cell
TEM - effector memory T cell
TER - trans-epithelial electrical resistance
TFG-β – transforming growth factor - β
TLR - Toll-like receptor
TNF - tumor necrosis factor
TV - Trichomonas vaginalis
UN - United Nations
VCAM-1 - vascular cell adhesion molecule -1
VM - vaginal microbiota
Vpr - viral protein R
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In preparation


Chapter 1

1 Introduction

1.1 Discovery of HIV

Human Immunodeficiency Virus (HIV) is a member of the retroviridae family within the *Lentiviruses* genus and is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) [1]. Scientists at the Centre for Disease Control (CDC) discovered HIV in 1981 in gay men in the United States. Subsequently, two independent research groups isolated the virus: led by Robert Gallo in the United States and Luc Montagnier and Francoise Barre-Sinoussi in France, although, only the French scientists received the Nobel prize in Physiology and Medicine for this discovery. Shortly after the discovery of HIV-1, another genetically distinct virus (HIV-2) was discovered from infected patients in western Africa [2]. Humans do not represent the natural hosts of either of these retroviruses, and infection in humans occurred due to cross-species transmission of simian immunodeficiency virus (SIV) from chimpanzees (SIV cpz) or sooty mangabeys (SIVsm) in the case of HIV-1 [3] and HIV-2 [4,5] respectively. HIV-2 is less virulent and transmissible than HIV-1 [6-8], which is the dominant global strain of the virus worldwide and thus will be the focus of this thesis and simply be referred to as HIV.

1.1.1 Subtypes of HIV

HIV is divided into several groups, which are each thought to represent an independent transmission event of the virus from SIV into humans. The groups are termed major (M), outlier (O), non-major and non-outlier (N) and putative (P), with group M contributing the most to the pandemic [9]. Within each group, HIV is divided into clades based on sequence diversity and group M has 9 clades, also called subtypes. These include subtype A, B, C, D, E, F, G, H, and K. Subtypes A, B and C are the most prevalent [10] with subtype C infections representing just under half of all HIV-infected individuals worldwide, and predominating in southern Africa, India, and in the horn of Africa (Figure 1-1). The dominant subtype in the Americas, Western Europe and Australasia is subtype B [11]. In Kenya, where I conducted the majority of my thesis work, subtype A is the predominant strain (discussed in section 1.5.1). There is considerable genetic variability between HIV strains, and this hyper-variability results in approximately 20% variation in envelope protein within a clade, and 30% variation in envelope protein between
clades [10].

Figure 1-1 Global distribution of HIV subtypes

1.1.2 Global HIV epidemic

The 2016 UNAIDS Report on the Global AIDS Epidemic reported that there were currently 36.7 million people living with HIV and 2.1 million new infections per year at the end of 2015 [12]. Anti-retroviral therapy (ART) has dramatically extended the lifespan in HIV-infected individuals, and there have been enormous gains in scale-up: 17 million people living with HIV were on treatment by the end of 2015 compared to 12.9 and 7.5 million in 2013 and 2010 respectively [12]. This has translated to a reduction in AIDS-related mortality, from 1.5 million in 2010 to 1.1 million in 2015. Despite these gains, by the end of 2015, approximately 20 million people worldwide were without access to ARTs. In addition to addressing this unmet need, maintaining ART coverage for those that are accessing it is also challenging, which highlights the need for the development of new prevention strategies to reduce new HIV infections.
1.2 Biology of HIV

1.2.1 HIV virion structure and genome

HIV is an approximately 120 µm enveloped virus with a characteristic cone shaped core that contains two copies of a single stranded RNA molecule (ssRNA). The genome of HIV is 9kb in length and contains a long-terminal repeat (LTR) at either end with nine open reading frames in the middle which are transcribed into 15 proteins [13]. These include gag, pol, env, nef, tat, rev, vif, vpr, and vpu. However, HIV-2/SIVmac encodes vpx instead of vpu [14]. The gag, pol and env genes comprise the structural core of the virus, while the 6 remaining genes code for regulatory proteins (Figure 1-2).

![HIV structure and genome](image)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gag</td>
<td>Group-specific antigen</td>
</tr>
<tr>
<td></td>
<td>Core proteins and matrix proteins</td>
</tr>
<tr>
<td>pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td></td>
<td>Reverse transcriptase, protease, and integrase enzymes</td>
</tr>
<tr>
<td>env</td>
<td>Envelope</td>
</tr>
<tr>
<td></td>
<td>Transmembrane glycoproteins. gp120 binds CD4 and CCR5; gp41 is required for virus fusion and internalization</td>
</tr>
<tr>
<td>tat</td>
<td>Transactivator</td>
</tr>
<tr>
<td></td>
<td>Positive regulator of transcription</td>
</tr>
<tr>
<td>rev</td>
<td>Regulator of viral expression</td>
</tr>
<tr>
<td></td>
<td>Allows export of unspliced and partially spliced transcripts from nucleus</td>
</tr>
<tr>
<td>vif</td>
<td>Viral infectivity</td>
</tr>
<tr>
<td></td>
<td>Affects particle infectivity</td>
</tr>
<tr>
<td>vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td></td>
<td>Transport of DNA to nucleus. Augments virion production. Cell-cycle arrest</td>
</tr>
<tr>
<td>vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td></td>
<td>Promotes intracellular degradation of CD4 and enhances release of virus from cell membrane</td>
</tr>
<tr>
<td>nef</td>
<td>Negative-regulation factor</td>
</tr>
<tr>
<td></td>
<td>Augments viral replication in vivo and in vitro. Decreases CD4, MHC class I and II expression</td>
</tr>
</tbody>
</table>

Janeway’s Immunobiology by Garland

Figure 1-2. HIV structure (top), genome (middle), and genes (bottom) and their function
1.2.2 HIV life cycle

The first step of the HIV life cycle is virus entry into target cells (Figure 1-3). The HIV surface envelope protein complex is key to this process as it determines viral tropism and virus fusion with cell surface receptors. The HIV env protein is a heavily glycosylated surface hetero-trimer composed of non-covalently linked glycoprotein 120 (gp120) and gp41 subunits. Gp41 anchors the complex within the viral membrane while gp120 binds to cell surface receptors on HIV target cells. Approximately 15 such heavily glycosylated trimers are found on the surface of the mature virion [15]. Similar to other lentiviruses, which require binding to a primary receptor followed by a co-receptor, HIV first binds to CD4 and then to either one of two chemokine receptors - C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4). Though not necessary, virion binding to the host cell may initially be aided by non-specific attachment factors including heparin sulfate [16] and DC-SIGN [17]. The first necessary step however, is gp120 env binding to CD4, which triggers a conformational change in the env protein, thereby, exposing a co-receptor binding site, which allows the binding of HIV env to CCR5 or CXCR4. The gp41 subunit contains a stretch of hydrophobic amino acids in its ectodomain termed the fusion peptide that inserts into the target cell membrane, forms a pore in the lipid bilayer, and facilitates fusion of viral and host membranes [15] (Figure 1-3).

![Molecular mechanism of HIV entry](image)

Figure 1-3. Molecular mechanism of HIV entry

During viral entry, the nef protein acts to disrupt the actin cytoskeleton of the host cell, which allows the pre-integration complex (PIC), comprising of the core and genetic material, to be released into the cytoplasm [18,19] (Figure 1-3). Reverse transcriptase (RT) begins to reverse transcribe viral ssRNA into linear double stranded complementary deoxyribonucleic acid
(cDNA). However, this enzyme lacks proofreading activity, causing point mutations at a very high rate of $10^{-5}$ mutations/base pair/replication cycle [20,21]. Subsequently, the PIC comprised of DNA, viral proteins (RT, integrase, matrix protein, nucleocapsid, vpr), and host proteins migrate by means of a microtubule network to the nuclear pore. This process is facilitated by vpr (in HIV-1) or vpx (in HIV-2) [22-24]. Once inside the nucleus, the viral enzyme integrase cleaves the two ends of viral DNA and facilitates integration into the host genome and at this stage, the virus is referred to as a provirus and the infection is termed ‘productive’ as the provirus can serve as a template for transcription. Alternatively, the virus can remain dormant for long periods of time (despite ART) in a stage termed latency [25].

During provirus expression, the integrated DNA is transcribed into RNA by the host RNA polymerase II. HIV rev exports various forms of transcripts into the cytoplasm including unspliced (precursor poly-proteins), partially spliced (env, tat, (p14), vif, vpr, vpu), and multiply spliced mRNA (rev, tat (p16), or nef) [26]. The unspliced mRNA (also the viral genomic RNA) is translated into structural gag and gag-pol poly-protein precursors, which organize at the cell membrane and assemble with the viral genomic RNA to form virions [27]. The HIV envelope complex is produced from a gp160 precursor protein, which folds in the ER and forms extensively glycosylated trimers that are trafficked to the golgi where the host protein furin cleaves gp160 into two functional subunits [28,29], gp120 and gp41. These subunits remain non-covalently bound and form a trimer on the surface of the virion. Immature, non-infectious virions bud from the cell membrane with envelope spikes on their surface and only mature and become infectious after cleavage of the gag-pol poly-protein by viral protease (PR) [30,31] (Figure 1-3).
1.3 HIV clinical course

1.3.1 Acute/Early HIV infection

The first signal of an immune response to HIV infection is the presence of host-derived acute phase proteins in plasma including alpha-antitrypsin and serum amyloid A, approximately 3-5 days after infection [33]. Acute phase proteins are released as part of the acute phase reaction that occurs within the initial hours of an inflammatory stimulus and is characterized by systemic and metabolic changes that occur as a consequence of the body’s defense and adaptation mechanisms [34]. In acute HIV infection, a sharp rise in plasma viral load occurs concurrently with a surge in inflammatory cytokines including IFN-α and IL-15, and microparticles in plasma that are coated by phosphotidylserine, a marker of cellular apoptosis derived from HIV-infected and/or activated CD4+ T cells [35]. Initially after HIV infection, dendritic cells (DCs) are
activated, but later, monocytes, macrophages, T cells, and natural kill (NK) cells are also activated, which triggers the release of a broad range of pro-inflammatory cytokines that in turn activate these cells and create a positive feedback loop called the cytokine storm. While this early immune response has antiviral properties, it causes widespread immune activation and the mobilization of CD4+ T cells, which contributes to their early loss [36]. A rapid peak in plasma HIV viremia is reached approximately 3-4 weeks after initial infection, which can be as high as $10^6$ copies of RNA/ml [37]. Subsequently, the plasma viral load starts to decline as CD8+ T cell responses evolve and CD4+ T cell numbers fall, most dramatically in the gut-associated lymphoid tissue (GALT) and some other lymphoid organs [38].

During primary or acute HIV infection, the level of viral replication is high due to limited anti-HIV immune responses. Neutralizing antibody responses are not detected until 3 months or more after infection and the initial non-neutralizing antibody response is often directed towards the gp41 envelope stalk, rather than the gp120 component of HIV [39,40]. Initial virus-specific CD8+ T cell responses appear a few days before peak viremia, which occurs at approximately 21 days post-HIV infection, and are directed towards 1-3 epitopes commonly found in HIV gag or nef [41]. This T cell response leads to the creation of viral escape mutants that evade recognition by CD8+ T cells, and is followed by new T cell responses that cause further escape [42]. Viral escape is favored by a combination of T cell responses associated with the production of the pro-inflammatory and chemotactic factor CCL4 (also known as MIP-1β), and those that target epitopes with high variability [43]. As the CD8+ T cell response evolves, some responses focus on viral epitopes that can mutate, but result in reduced viral fitness, which likely contributes to the control of plasma viremia [42]. A combination of the evolution of the CD8+ T cell response and depletion of substrate (CD4+ T cells) causes the virus to reach a steady state called the viral set point, and CD4+ T cell numbers are restored (albeit at levels lower than in HIV-uninfected individuals) (Figure 1-5) [36,44].

Approximately 50% of acutely infected individuals display symptoms such as lymphadenopathy, fever, headache, sore throat, malaise, joint or muscle pain, or rash [45]. However, these symptoms are non-specific and are similar to the symptoms of influenza or mononucleosis, which makes the diagnosis of HIV difficult. Moreover, approximately half of all individuals with acute infection do not display any symptoms adding to the complexity of diagnosis [45].
1.3.2 Chronic infection

During the chronic stage of infection and in the absence of ART, HIV-positive individuals are divided into three groups based on clinical criteria and duration to AIDS. AIDS is defined by a CD4 count below 200 cells/µl or the development of an AIDS-defining opportunistic illness (ADI, see below). The three categories are rapid progressors (~3 years to AIDS), intermediate progressors (3-10 years to AIDS), and long-term non-progressors, who do not progress to AIDS. While 10-15% of HIV-infected individuals are rapid progressors, the majority are intermediate progressors, and a small fraction (~5%) of non-progressors remain asymptomatic with a CD4 count above 500 cells/ml for at least 8 years after HIV infection. Another group of HIV-positive individuals called elite controllers also do not progress to AIDS and are defined by having an undetectable plasma HIV viremia (<50 copies HIV RNA/ml). These individuals maintain high CD4+ T cell counts and have a high quality of life comparable to HIV-uninfected individuals [47].

In general, due to the partial control of viral replication by the immune response at the initial phase of chronic infection, a plasma viral load set-point is achieved. The CD4+ T cell count returns to near normal values of approximately 500-700 cells/µl [48], but declines steadily over time in the absence of ART at approximately 25-60 cells/µl [49], while the plasma viral load rises until an HIV-infected individual has AIDS (Figure 1-5 and 1-6). The plasma viral load is the best predictor of time to progression to AIDS, followed by the CD4+ T cell count [50].
1.3.3 AIDS

AIDS is defined by a CD4 count below 200 cells/µl or development of an AIDS-defining opportunistic illness (ADI). ADIs include infections such as tuberculosis, oral candidiasis, and pneumococcus [52] and individuals with advanced HIV disease also have an increased risk of Kaposi’s sarcoma, non-Hodgkin lymphoma, cervical cancer, and non-infectious cancers of the lung, colon and ovarian cancers [53]. AIDS is accompanied by a substantial rise in HIV viremia and without ART treatment, the average survival time for an individual with AIDS is 1-3 years [54].

1.4 Heterosexual HIV transmission

Heterosexual transmission is the most common mode of HIV transmission worldwide and women account for approximately half of all HIV-infected individuals [12]. Despite the size of the epidemic, the probability of HIV transmission for a single episode of vaginal sex between a
sero-discordant couple (either male-female or female-male) ranges from 1/250 to 1/2500 [55]. This low probability is largely in part due to mucosal defenses in the genital tract (discussed below). However, this probability is increased in certain contexts due to viral and host factors. A key viral factor that raises HIV transmission risk is a higher set-point plasma viral load or an increase in the blood viral load; however, the best defined risk factor is the genital viral load which raises HIV transmission risk independently of the blood viral load [56,57].

In addition to viral factors, factors in the uninfected sexual partner such as intra-vaginal practices [58], bacterial vaginosis [58], sexually transmitted infections (STIs) [59] and the use of the hormonal contraceptive depo-medroxy progesterone acetate (DMPA) [60,61] can also increase HIV transmission risk. In addition, a recent meta-analysis showed that the risk of HIV acquisition after a single sexual exposure is increased approximately 6-fold in lower-income countries (0.3% vs 0.05% in higher-income countries) [55], and most of the lower-income country studies included in this analysis were in sub-Saharan Africa (SSA), suggesting that residence in SSA and participation in sexual networks that characterize efficient transmission, may in and of itself, amplify HIV acquisition risk. Women may be particularly at risk as illustrated by the fact that women in Kisumu (western Kenya) between 15-19 years or 20-24 years of age were at least 3 fold more likely than men to acquire HIV infection [62]. Moreover, in the recent CAPRISA 004 microbicide study, the estimated per-exposure HIV transmission risk was 1% or more, giving an annual HIV incidence above 10% [63]. Although cultural and behavioural factors including sexual practices play a role, biological factors also play a prominent role in increasing HIV transmission risk. These include but are not limited to sexually transmitted infections (STIs), genital inflammation, bacterial vaginosis, and use of the hormonal contraceptive, DMPA [64]. I will describe the impact of several of these factors that are directly pertinent to my thesis in more detail (below).

1.5 The biology of HIV acquisition

1.5.1 Viral tropism and the selection bottleneck

While both CCR5 and CXCR4 are HIV entry co-receptors in vivo, HIV env variants can either be R5, X4 or R5/X4 (dual tropic). Although all of these viral variants are present in bodily fluids (semen, genital secretions and blood), HIV transmission through both mucosal and intravenous
routes is mediated almost exclusively by CCR5-tropic strains, which also dominate in the early stage of HIV infection. The underlying mechanisms behind this selection bias are not well understood, although viral tropism switches to X4-tropic strains in approximately half of HIV-infected patients in the chronic phase of infection and these patients have a more rapid CD4+ T cell decline and accelerated progression to AIDS [65]. In addition to the selection of CCR5-tropic strains, mucosal HIV acquisition is usually caused by a single viral variant within a swarm of viruses in genital fluids [66,67], which suggests that there are additional selection factors at the portal of entry. On a population level, specific characteristics of founder viruses which may confer a selective advantage include shorter V1/V2 loops with fewer predicted glycosylation sites in the HIV env glycoprotein [68,69], an increase in the frequency of amino acids that are associated with viral fitness, amino acids which confer greater stability of a viral protein, and features associated with immune escape [67]. Due to these considerations, the HIV pseudovirus that I produced and applied for my work was chosen to match the clade that predominated in the geographical region in which biological samples were obtained. I also chose early-transmitted, CCR5-tropic envelopes to produce HIV pseudovirions as this was most relevant to heterosexual acquisition of HIV, which was the focus on my PhD.

1.5.2 Anatomical considerations

The female genital tract (FGT) can be conceptualized into two regions based on anatomical differences: the upper and lower genital tract. The lower FGT refers to the vagina, ectocervix, and endocervix while the upper FGT includes the uterus, fallopian tubes and the ovaries (Figure 1-7). Within the lower FGT, the ectocervix and the vagina have a multilayered stratified squamous epithelium while the endocervix has a single layer of epithelial cells known as the columnar epithelium. At the border of these two types of epithelia is the transformation zone, where a transition occurs between the multilayered stratified epithelium into the single-layered squamous epithelium.
Due to obvious challenges in studying the initial events in HIV infection in humans, our understanding of this process comes primarily from studies conducted in animal models, particularly SIV infection in macaques. Using this model, a high titre SIV challenge is given intra-vaginally to macaques to ensure that productive SIV infection occurs, thereby enabling the examination of early cellular and molecular events in SIV/HIV acquisition. Studies from different investigators suggest that SIV infection may occur in different parts of the female genital tract [71,72], however, a recent report suggests that the entire genital tract may be susceptible to SIV. In comparison to the cervix, the vagina may be a preferred site for viral infection due to its larger surface area; however, the presence of the multilayered squamous epithelium is thought to reduce penetration of virions into the submucosa [73]. The transformation zone between the ectocervix and the endocervix was found to be an area where more virions had penetrated after SIV challenge [73], and this may be because of a transition of epithelial cell organization from the multi-layered squamous epithelium to a single layered columnar epithelium, resulting in more frequent gaps in the epithelial barrier [74]. Perhaps due to the presence of a single layered protective epithelial barrier, preferential HIV infection was observed in the endocervix compared to the ectocervix or vagina [73], though another group observed modestly greater HIV penetration across the squamous epithelium compared to columnar epithelium [75]. Clearly, although a considerable amount of research has been done to identify the preferred anatomical site for HIV acquisition, there is no consensus in the field; however, it appears that the entire female genital mucosa may be susceptible to HIV.

Figure 1-7. Anatomy of the female genital tract

Hladik and McElrath, Nat Rev Immunol, 2008 [70]
1.5.3 Mucosal defenses

Most sexual exposures to HIV do not cause infection because the virus is repelled by a multitude of mucosal immune defenses. In the female genital tract, these include the genital epithelium, low vaginal pH, cervical mucus, innate antimicrobial peptides (AMPs), immune cells (neutrophils, αβ and γδ T cells, dendritic cell subsets, and others) and inflammatory responses.

Epithelial integrity at all tissue sites is mediated by protein complexes that enable cells to adhere to one another through tight and adherens junctions or by the extracellular matrix (ECM) [76]. When established in culture, epithelial cells form a polarized monolayer with distinct apical and basolateral sides, and also establish a trans-epithelial electrical resistance (TER), which illustrates the tightness of the monolayer. In vivo, the epithelium of the lower FGT is lined by commensal bacteria [77], which are prevented from passing to the submucosa by the epithelial barrier. Indeed, epithelial disruption has been associated with increased translocation of bacteria across a genital epithelial monolayer in vitro [78] and this highlight the importance of an effective epithelial barrier in preventing the passage of HIV and bacteria to underlying HIV target cells in the submucosa.

On the surface of the genital epithelium rests the cervico-vaginal mucus, which is composed of water, together with a network of mucins that form a gel-like material containing immunoglobulins, soluble proteins, anti-microbial peptides, electrolytes, proteases and anti-proteases [76]. The mucus presents a physical impediment for pathogens including HIV, as it can trap the virus at acidic pH, potentially through electrostatic interactions between the negatively charged mucins and carboxylic acid groups of HIV [79]. Acidification of the vaginal milieu is caused by lactic acid production by commensal lactobacilli (discussed in more detail in Section 1.7.3 on bacterial vaginosis) and enhanced virus trapping may inhibit access of HIV to the epithelium [80].

1.5.4 Genital inflammation

In contrast to aforementioned host defenses, genital inflammation appears to be a double-edged sword as it provides antiviral defenses but may also enhance HIV acquisition risk in many contexts. A prototypical pro-inflammatory response involves the upregulation of cytokines and chemokines, which are released by various cell types including epithelial cells, dendritic cells,
macrophages, T cells, and NK cells. Prototypical pro-inflammatory cytokines include IL-1, TNF-α, and IFN-γ while chemokines include CCL-2, CCL-3, CCL-4, CCL-5, and CXCL8 and CXCL10 [64]. These factors cause the extravasation of immune cells from the blood to the site of infection and the activation of immune cells. Activated APCs, T cells and NK cells produce and respond to these signals and have improved effector functions such as increased antigen processing and presentation by APCs, release of cytolytic molecules perforin and granzyme, which enable the killing of virus-infected cells by cytotoxic T cells and NK cells. The inflammatory response is a combination of systemic and local factors including the more recently characterized tissue-resident immune cells [81-84]. The inflammatory response enables the clearance of the insult and a wound healing response follows.

While a regulated inflammatory response is critical for the clearance of a pathogen at mucosal surfaces, excessive and/or chronic genital inflammation may actually increase HIV acquisition risk through various mechanisms. Firstly, genital inflammation may increase epithelial barrier permeability by the disruption of cell-cell junctions [78] and this may increase the access of bacteria or HIV to underlying target cells in the submucosa. Indeed, elevated genital inflammation (measured by a genital pro-inflammatory cytokine score) is associated with proteomic markers of epithelial disruption [85]. Secondly, STIs such as gonorrhea, chlamydia, and HSV-2 are associated with increased HIV acquisition, and although the nuances in particular inflammatory cytokines and chemokines that are elevated differ for each STI, each STI is associated with an increase in some pro-inflammatory cytokines [86]. Perhaps the clearest example of the impact of immune activation triggered by an STI is the case of HSV-2. Asymptomatic HSV-2 infected individuals have an increased number and proportion of various genital HIV target cells (including activated CD4+ T cells) [87], and HSV-2 therapy is unable to reverse immune alterations [88], which may explain why current HSV-2 therapy does not reduce HIV transmission [89].

In addition to the role of STIs in increasing HIV risk and genital inflammation, several studies in both men and women have also shown that the genital milieu of individuals who are HIV exposed sero-negative (HESN), have an immune quiescent phenotype compared to HIV-uninfected controls [90], once again linking elevated genital inflammation to HIV risk. Perhaps most importantly, nested cohort studies have shown that pre-existing levels of genital
inflammation (assessed by elevated genital cytokines) and immune activation in both blood
[91,92] and the genital tract [93,94] are associated with subsequent HIV acquisition.

1.5.5 Initial events in HIV acquisition

While the exact location(s) of the initial site of HIV infection in the FGT is not known, a key
factor that may promote HIV acquisition is the number and phenotype of HIV target cells. In the
female genital tract, these include CD4+ T cells, dendritic cells, and macrophages [72,74,76]. Perhaps the best evidence for the role of HIV target cells in HIV acquisition comes from the observation that male circumcision (removal of the foreskin, which contains putative cellular HIV targets) reduces HIV acquisition by 60% [95-97]. In addition, in a retrospective cohort study in men who were initially HIV-uninfected and were under surveillance for a maximum of 4 years prior to circumcision, the mean surface area of the foreskin tissue measured at circumcision was higher in men who had HIV sero-converted compared to controls who remained HIV-sero-negative [98]. Moreover, men whose foreskin surface area was in the highest quartile had 2.4 fold higher odds of being HIV sero-positive compared to those in the lowest quartile, suggesting that the total number of HIV target cells in the tissue may be an important determinant of HIV risk [98]. However, perhaps the most direct evidence that implicates an immune cell as a target in HIV acquisition is from a rectal SIV challenge model in macaques, where the frequency of activated CCR5+ CD4+ T cells in the rectum prior to SIV challenge correlated with subsequent SIV acquisition and early viremia [99].

There is considerable evidence implicating various cell types as the initial targets of HIV acquisition. Broadly speaking, there are two main lines of thought: 1) that a dendritic cell either binds to HIV and the virus remains attached on the surface or that it is internalized and transported to the draining lymph node [100-102] or 2) that CD4+ T cells are initial targets in the FGT, which amplify infection locally, and then the virus moves to the draining lymph node [103] (Figure 1-8). In either case, upon entry into the lymph node, virus expansion can occur in the densely packed CD4+ T cells and the infection can then rapidly spread to other organs including the GALT via the systemic circulation [74].

Macaque models of vaginal SIV challenge demonstrate that prior to challenge, there is a relative paucity of CD4+ T cells and DCs associated with the epithelium and in the submucosa [104]; however, within 4 days after challenge, there is not only an increase in the number of these cells,
but most of the infected cells in the endocervix, ectocervix, and vagina are CD4+ T cells [73,104,105]. Expansion of the virus population in the genital mucosa tends to occur in clusters rather than in a diffusive manner in the submucosa during the first 10 days after SIV challenge, and recruitment of CD4+ T cells also occurs preferentially into areas of virus expansion, suggesting that target cell recruitment may fuel local virus expansion. T cell recruitment may be in part due to signaling by plasmacytoid dendritic cells (pDCs) that increase in number beneath the endocervical epithelium at day 1 post SIV infection and release the chemokines MIP-1α and MIP-1β, which recruit CCR5+ CD4+ T cells, and MIP-3α, which recruits Th17 cells. The clusters of virus and immune cells have increased expression of IL-8 and MIP-3α and are proposed to be local sites of inflammation that promote local viral expansion. Indeed, vaginal application of the anti-inflammatory drug glycerol monolaurate (GML) prevented mucosal SIV acquisition, accumulation of inflammatory cells and chemokine production [104]. These data demonstrate that CD4+ T cells are critical early targets of HIV and that inflammation and cellular immune activation may fuel local expansion of HIV in the genital tract.


**Figure 1-8. Initial events in HIV infection in the female genital tract**
1.5.6 HIV target cells

As described earlier, expression of CD4 and CCR5 flags key HIV targets cells relevant for sexual transmission. Putative correlates of HIV susceptibility were based on studies done mainly in blood and included the expression of CD69, a marker of both early immune activation [106] and mucosal tissue residence [83,107], homing integrins (α4β7 and α4β1), or CCR6, a marker of IL-17 producing CD4+ T cells (Th17 cells). When I first designed the flow cytometry panel for the HIV pseudovirus assay, the main goal was a) to validate whether the assay could identify known cellular targets of HIV (CCR5+ and activated CD4+ T cells) and b) to characterize in detail the mucosal susceptibility of α4β7+ and α4β1+ CD4+ T cells, since it was proposed that these integrins were HIV attachment factors that may promote viral entry (see below).

1.5.6.1 CD69+ CD4+ T cells

CD69 is a C-type lectin, which can be expressed on all leukocytes [108]. The expression of CD69 is induced early upon immune activation of T cells, NK cells, and B cells, and is used as a correlate of early immune activation in blood. Upon T cell activation due to CD3/TCR signaling, CD69 mRNA is detected within 30-60 minutes, and after termination of the activating signal, diminishes rapidly [6,8]. Cell surface expression of CD69 is detectable as early as 2-3 hours after TCR/CD3 stimulation, reaches peak levels at 24h, and declines with a half-life of 24h once the stimulus is removed [109]. T cells from the peripheral blood express very low levels of CD69, however, in lymph nodes and peripheral mucosal surfaces (gut lamina propria or the female genital mucosa), where there is greater presence of antigen in the context of major histocompatibility complex class I or II (MHC I or II), the frequency of CD69+ T cells is higher [81,110,111]. However, recent studies have described a new role of CD69, which have cast uncertainty over the proportion of CD69+ T cell in the periphery that are immunologically activated. In the wake of a local immune response, elevation in CD69 expression may facilitate mucosal T and B cell retention at the mucosal surface or draining lymph node through its binding to sphingosine-1 phosphate receptor-1 (S1P1) [112], a molecule that allows egress of B and T cells from lymph nodes. Therefore, in addition to being a marker of early immune activation, CD69 is also being defined at mucosal surfaces and lymph nodes as a marker of tissue residence, and it currently remains unclear what proportion of mucosal CD69+ CD4+ T cells are tissue resident or activated or both.
In the context of HIV susceptibility, there is plenty of evidence to suggest that genital inflammation and cellular activation is associated with increased HIV susceptibility (discussed in Section 1.5.4). Preferential SIV and HIV infection of genital activated (CD38+ or HLA-DR+) CD4+ T cells is consistently observed [73,113,114]. Therefore, to validate the HIV entry assay (Chapter 4), I included CD69 as an activation marker in my flow cytometry panel (Chapter 3). Although it has become clearer that CD69 is also a marker of tissue residence along with immune activation, at the time that I designed the flow-cytometry panel in 2012, CD69 was well-defined only as a marker of immune activation, hence it is referred to as such in Chapter 3 (published manuscript).

1.5.6.2 Th17 cells

Th17 cells are critical for host defense and inflammatory responses at mucosal surfaces [115]. The differentiation of Th17 cells from newly primed cells is determined by three key cytokines, transforming growth factor-β (TGF-β), IL-6, and IL-21 although IL-23 further stabilizes this commitment [116]. These cytokines induce the downstream expression of the Th17 lineage-determining transcription factors ROR-γt, and ROR-α to induce the differentiation of Th17 cells [117,118]. IL-17 is the proto-typical cytokine produced by Th17 cells, which plays a complex role in host immunity. IL-17 can be protective against bacterial infections through the production of antimicrobials peptides, chemokines, and recruitment of neutrophils; however, an exacerbation of a Th17 response can inflict tissue damage through the recruitment of neutrophils and effector T cells [115].

Several recent reports suggest that Th17 cells may be key initial targets of HIV in the genital mucosa. A recent study in macaques found that approximately 85% of all infected genital CD4+ T cells express CCR6, a marker of Th17 cells [105], while human genital Th17 cells are also preferentially infected ex vivo [119]. Preferential targeting of Th17 cells may be due to elevated expression of CCR5 [111,119] or lower expression of RNases that have the capacity to inhibit HIV replication [120].

Although Th17 cells appear to be especially susceptible, it is unclear why the targeting of this particular cell subset may benefit the virus. It has been suggested by some reports [111,121] though not another [122] that HIV infection depletes cervical Th17 cells. Th17 cells promote epithelial keratinization, while IL-17 promotes host defense against bacterial and fungal
pathogens through the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides [115,123], and directly promotes epithelial barrier integrity in vitro [124]. By compromising Th17 cellular function or number, HIV may impair epithelial barrier integrity and host defenses, which may allow increased translocation of microbial products and/or HIV from the cervico-vaginal lumen to the submucosa.

1.5.6.3 \( \alpha 4 \beta 7^+ \) and \( \alpha 4 \beta 1^+ \) CD4+ T cells

Another molecule that has received considerable attention as a target of HIV and has been a source of controversy is the integrin \( \alpha 4 \beta 7 \). Integrins are heterodimers comprised of an alpha and beta chain, and the \( \alpha 4 \) integrin subunit pairs with either \( \beta 7 \) or \( \beta 1 \) (discussed below). \( \alpha 4 \beta 7 \) is expressed on B cells, T cells, and NK cells, and binds to mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) on high endothelial venules (HEVs) and mediates lymphocyte migration to the intestine [125]. In 2008, Arthos et al. reported that integrin \( \alpha 4 \beta 7 \) binds to HIV env gp120 [126]. This report was followed by two more reports by the group showing that early transmitted gp120 envelopes bound preferentially to \( \alpha 4 \beta 7 \) and that \( \alpha 4 \beta 7 \) forms a cell surface complex with CD4 and CCR5, which may bind HIV env prior to CD4 engagement by the virion, and thereby facilitate virus entry (Figure 1-9). Moreover, indirect evidence that \( \alpha 4 \beta 7 \) may play a role in increasing HIV susceptibility came from the vaccine trial conducted in Thailand. This randomized, double-blind, placebo-controlled vaccine trial used a prime-boost strategy using gag, pro, and env inserts within an inert canarypox vector as the prime and a gp120 boost. The trial demonstrated 31% protective efficacy after 1 year and the main correlate of protection was binding of IgG antibodies to the variable regions 1 and 2 of HIV env, a region that binds to CD4 and CCR5 and is the putative \( \alpha 4 \beta 7 \) binding site [44,127].
These reports resulted in a flurry of investigation into the role that α4β7 may play in sexual HIV acquisition and was the basis of my rationale for characterizing α4β7+ CD4+ T cells as HIV targets (see chapter 2). However, these early reports were also fraught with caveats, which surfaced due to lack of reproducibility of the initial findings. Most of the gp120 envelopes tested by the Arthos research group were monomeric, while the native env on the surface of an HIV virion is trimeric. Moreover, the gp120 envs that showed binding to α4β7 were produced in cell lines that resulted in surface glycosylation patterns that did not reflect in vivo glycosylation that would be expected during viral budding from CD4+ T cells. The first evidence that α4β7 did not influence the replication of many early-transmitted or chronic strains of HIV in vitro came from the study by Parrish et al [129]. These investigators showed that the addition of saturating concentration of anti-α4β7 antibody, Act-1, did not inhibit HIV replication in CD4+ T cells. Perez et al. employed a similar protocol as Arthos et al in performing integrin-gp120 binding assays and demonstrated that in comparison to lab-adapted SF162 gp120 recombinant env (which bound α4β7 in both studies), a large panel of acute and chronic HIV envs did not bind α4β7 [130]. Despite these mixed and inconclusive findings, significant focus still remains in the field over the role of α4β7 in HIV infection.
Another related integrin that is expressed in CD4+ T cells that may also play a role in genital HIV acquisition is \( \alpha_4\beta_1 \). While \( \alpha_4\beta_7 \) binds to MAdCAM-1 on high endothelial venules and mediates lymphocyte migration to the intestine [125], \( \alpha_4\beta_1 \) binds vascular cell adhesion molecule-1 (VCAM-1) [131] to mediate lymphocyte migration to non-lymphoid tissue and sites of inflammation [132,133]. Leukocytes within cervical tissue express \( \alpha_4\beta_7 \) or \( \alpha_4\beta_1 \) integrins and pre-treatment with either \( \alpha_4 \), \( \beta_7 \), or \( \beta_1 \) blocking monoclonal antibodies (mAbs) caused a substantial reduction in ex vivo HIV replication in cervical explants [134]. These data suggest that \( \alpha_4\beta_7 \) and \( \alpha_4\beta_1 \) integrins may play a role in mucosal HIV infection, although the underlying biological mechanisms are not clear. Therefore, a key focus of my PhD (Chapters 3 and 4) was to characterize whether \( \alpha_4\beta_7^+ \) or \( \alpha_4\beta_1^+ \) CD4+ T cells are preferential targets of HIV entry and whether these integrins facilitated HIV entry.

1.6 Mucosal sampling and the suitability of an HIV entry assay for assessment of HIV susceptibility and HIV target cells

1.6.1 Limitations of current assessment of HIV susceptibility

A key limitation to genital (mucosal) sampling in clinical studies is the low number of immune cells obtained from the tissue (~1000 CD4+ T cells per biopsy or cytobrush) [135]. To identify HIV target cells, an alternative technique that is frequently used is the surgical hysterectomy (removal of the uterus, and sometimes also the cervix), which provides an abundant number of immune cells to allow for the assessment of ex vivo HIV replication. However, surgical hysterectomy is generally not performed on young women who are most at risk of heterosexual HIV acquisition, and the tissue can only be obtained once, which precludes multiple sampling to assess the impact of clinical parameters or clinical interventions on HIV susceptibility in longitudinal studies. A mucosal sampling technique that is conducive to longitudinal clinical studies is the biopsy, which is typically obtained from the ectocervix and/or the vagina. However, its main caveat is that it is more invasive and thus less acceptable to the study participant compared to the endocervical cytobrush (described next). Moreover, some variation in immune cell numbers has been reported in sequential biopsies collected from the contralateral
vaginal wall suggesting that a single biopsy may not be representative of the entire mucosal surface [135].

1.6.2 Appropriateness of endocervical cytobrush sampling in clinical immunology

The endocervical cytobrush sampling addresses the issue of immune cell heterogeneity along the mucosal lining to some extent, as the cytobrush is rotated over a 360° turn along the endocervical wall, thereby collecting cells from all parts of the endocervical mucosa. A multisite study was recently conducted to assess immune cell yield by flow cytometry to compare two endocervical cytobrushes and 1 ectocervical biopsy that was enzymatically digested to extract immune cells [135]. The results of this study showed that the total number of leukocytes were similar between the two sampling techniques; however, the cytobrush and the biopsy were biased towards macrophages and T cells respectively [135]. Although on average, endocervical cytobrushes yielded fewer CD4+ T cells than biopsy, the average yield was ~1000 CD4+ T cells which suggested that if a highly sensitive HIV infectivity assay was employed, ex vivo HIV infection could be assessed using cytobrush sampling. It is important to note that no single sampling technique addresses all caveats of mucosal sampling for human studies, however, endocervical cytobrush sampling is similar to the routine PAP smear and can be performed by a nurse as opposed to gynecologist who is required for biopsy. This addresses a key limitation of patient acceptability and study feasibility in many resource-limited settings and was an important factor for choosing endocervical cytobrush sampling to establish an ex vivo HIV infectivity assay (Chapter 2), and then to apply it to a field setting to assess the impact of a clinical intervention on HIV susceptibility (Chapter 4).

1.6.3 The advantages of using the β-lactamase-vpr HIV entry assay

As mentioned above, the low numbers of CD4+ T cells obtained from endocervical cytobrushes necessitate the use of a highly sensitive assay to measure ex vivo HIV infectivity. Moreover, primary cells obtained from the female genital tract may undergo alterations in ex vivo culture conditions if cultured long term, which also requires exogenous immune activation to ensure cell survival [136]. However, long-term cell culture conditions do not allow an assessment of the impact of in vivo immune activation on cellular HIV susceptibility, which is critical for an
assessment of HIV acquisition risk. It is for these reasons that I established the β-lactamase (BlaM)-Vpr HIV assay for use in endocervical cytobrush derived CD4+ T cells to assess ex vivo HIV infectivity. While the methodology of this assay is described in chapter 3, here, I will describe why this assay achieves high sensitivity, which is critical when the number of mucosal immune cells obtained from genital sampling in typically low.

The BlaM-HIV entry assay was developed by Cavrois et al [137] to assess virion fusion with HIV target cells. Since then, the assay has been used to assess HIV infection in immune cells obtained from blood, though not in mucosal samples. Two fundamental features of the assay enable its application for assessment of virion fusion when immune cell numbers are limited. First the assay utilizes β-lactamase, an enzyme that catalyzes cleavage of a β-lactam bond commonly found in antibiotics such as cephalosporins. The idea of using β-lactamase was developed by Zlokarnik et al, who fused two fluorophores, coumarin and fluorescein, with a β-lactam bond to form a fluorescence resonance energy transfer (FRET) pair [138]. Cleavage of the FRET pair by conversion of β-lactamase inside living cells can be detected with as few as 100 molecules of β-lactamase per cell. Subsequently, Cavrois et al utilized this technique to create a fusion construct between β-lactamase and the HIV protein vpr (Figure 1-10). Approximately 300 vpr molecules are non-covalently linked to the HIV capsid by means of capsid protein p6, and hence, co-transfection of BlaM-Vpr and an HIV backbone into a mammalian cell line results in the production of virions tagged with hundreds of reporter BlaM enzymes [138,139], suggesting that fusion of a few number of virions per cell may be detectable.
Figure 1-10. Overview of the β-lactamase-vpr HIV entry assay. HIV pseudovirions incorporating BlaM are produced by transient transfection of 293T cells with plasmids depicted above. Blood cells are infected with a BlaM-Vpr HIV isolate by spinoculation. Cells are then loaded with cell-permeant CCF2-AM dye, a fluorescent resonance energy transfer (FRET) pair. CCF2 is excited at 409 nm, which triggers FRET and emission of green light at 520 nm. However, viral fusion with target cells allows BlaM into the cytoplasm causing cleavage of CCF2, separation of the FRET pair, and emission of blue light at 447 nm. The ratio of blue versus green light provides a readout of cytosolic HIV entry. Figure modified from Cavrois et al, Nat Biotech, 2002.

1.7 Clinical determinants of HIV acquisition

1.7.1 Viral clade

A significant amount of work has been done to study the role of viral clade on HIV transmission. Early studies suggested that clade C and CRF01_AE (which is common in Thailand), are more transmissible through vaginal sex [140], potentially through enhanced viral replication or higher transmission efficiency across the mucosal barrier [141]. While it remains unclear whether certain clades are more infectious than others in general, based on the existing evidence, subtle virulence differences between circulating clades are not sufficient to explain the significant differences in HIV transmission rates in SSA compared to industrialized nations [55].
1.7.2 Sexually transmitted infections

The STI that arguably has the greatest impact on increasing the number of new HIV infections in SSA is herpes simplex virus - 2 (HSV-2). HSV-2 is 3 times more prevalent in SSA with prevalence well over 50% in sexually-active individuals compared to 17% in North America [142,143]. HSV-2 is a double-stranded DNA virus, closely related to herpes simplex virus-1 (HSV-1) and both of these viruses infect and replicate in epithelial cells [144]. Worldwide ~ 90% of individuals are infected with HSV-1, HSV-2 or both. Although HSV-2 was thought to be responsible for most cases of genital herpes, which is spread through sexual contact, more recent studies suggest that up to 50% of incident cases of genital herpes are caused by HSV-1 [145]. Genital herpes caused by HSV-1 is on the rise in industrialized countries for reasons that are unclear but may be due to earlier sexual debut and increased participation in genital-oral sex during adolescence. Whether this is true in high HIV burden sexual networks is not known.

HSV-2 is thought to be responsible for approximately half of all cases of new HIV infection in SSA. HSV-2 is taken up by sensory nerve endings close to the epithelia and the virus transits through the nerve axon and lays dormant in the dorsal root ganglia and the autonomic nervous system. As HSV-2 reactivates, it travels down the axon and replicates in epithelial cells near the nerve endings and may cause subclinical shedding [146]. HSV-2 infected persons are generally asymptomatic (81% in one large clinical trial) [89], and symptomatic episodes occur due to environmental stressors such as trauma, ultraviolet light exposure or immune suppression. Symptoms of HSV-2 infection include blisters or sores around the genitals or rectum, which break and leave tender sores. Although HSV-2 reactivation is associated with disease symptoms, daily sampling from both symptomatic and asymptomatic individuals showed that approximately 67% of reactivation occurs in the absence of genital lesions [147] and transient shedding occurs roughly every 10 days [148].

The impact of HSV-2 infection on HIV burden in SSA is illustrated by the fact that HSV-2 infection increases the risk of HIV acquisition by 3 fold in both men and women [149] and is thought to contribute to 50% of new HIV infections in this region [143]. One underlying mechanism by which HSV-2 may enhance HIV acquisition risk is the increase in the number of activated CCR5+ CD4+ T cells and dendritic cells in individuals with asymptomatic HSV-2 infection. Recurrent reactivation of HSV-2 is common in individuals using even high dose HSV-
suppressive therapy with acyclovir, likely causing sustained mucosal immune activation marked by elevated CCR5+ CD4+ T cells and DC SIGN+ DCs [59,150] and this may be one reason why HSV-2 therapy does not reduce HIV acquisition.

The prevalence of other STIs including gonorrhea, chlamydia, trichomonas, and syphilis varies not only in different parts of SSA, but there is tremendous heterogeneity in communities within a country or a smaller region. All of these STIs are associated with increased HIV acquisition risk [151], and indeed, treatment of gonorrhea, chlamydia, and syphilis led to a 42% reduction in HIV incidence in a large clinical trial conducted in Mwanza, Tanzania [152]. The success of this trial may be due to the fact that population-based STI treatment in this study was offered during an emerging HIV epidemic when STI treatment services were poor, suggesting that STIs may have been a key driver of new HIV infections. After the success of the Mwanza trial, other trials aimed to repeat these findings in different settings, but did not have the same success as the earlier study. This has been attributed to multiple factors including the notion that STIs may not have been driving the HIV epidemic when the subsequent trials were conducted, or due to design and implementation issues, as all post-Mwanza trials included some treatment in the control arm for ethical reasons, which reduced the statistical power to observe an effect of STI treatment [153]. Stillwagon et al concluded that the shortcomings of published randomized controlled trials make it invalid to conclude that STI treatment cannot reduce HIV incidence [153], and that any future STI treatment trials will face these same difficulties while assessing whether STI treatment can reduce HIV acquisition. In summary, while the impact of STI treatment on HIV acquisition remains unclear, STIs are likely to cause an increase in genital HIV susceptibility.

The increased HIV risk in individuals with an STI is consistent with the association between STIs and genital inflammation. While the particular pro-inflammatory cytokines that are altered may vary in the case of each STI, all promote a pro-inflammatory cytokine milieu [86], which has direct implications on HIV susceptibility (discussed in Section 1.5.3). The prevalence of STIs is high in South Africa, particularly in adolescent women, who mostly present without any clinical symptoms but have equivalent levels of genital inflammation (measured by pro-inflammatory cytokines) compared to their peers with symptomatic STIs [86,154]. The high burden of asymptomatic STIs has a significant impact on HIV risk in SSA [142,143], and improved ways to diagnose asymptomatic STIs are needed.
1.7.3 Bacterial vaginosis

Bacterial vaginosis (BV) is characterized by a reduction in lactobacilli and an increase in several anaerobic bacterial species in the vagina. BV is the most frequent cause of vaginal discharge in women of reproductive age [155], and has been associated with increased susceptibility to several sexually transmitted infections, including HIV [58,156]. In research settings, BV is diagnosed based on Gram’s stain (Nugent score), while it is clinically defined based on symptoms as per the Amsel’s criteria (see below). Microbiologically, diagnosis of BV by Gram’s stain is based on the relative proportion of lactobacilli versus gram-negative (gardnerella and bacteroides spp) and curved gram-variable rods (BVAB-1 and some mobiluncus). A low Nugent score (0-3) reflects an abundance of lactobacilli and low numbers of the gram negative/variable anaerobes, while a high score (7-10) is defined as BV and reflects low numbers of lactobacilli and high numbers of gram negative/variable anaerobes. An intermediate Nugent score (4-6) is defined as altered vaginal flora (AVF).

Clinical evaluation of BV is based on the Amsel’s criteria, which relies on meeting 3 out 4 of the following criteria: a thin and homogenous vaginal discharge, a fishy volatile odor when the discharge is treated with potassium hydroxide, presence of clue cells (squamous epithelial cells lined with bacteria) on Gram’s stain, and vaginal pH > 4.5 [157]. A large proportion of women with BV based on Nugent score do not present with discharge or vaginal odor [154,157,158], suggesting that vaginal dysbiosis is common in the absence of clinical symptoms. Moreover, BV and altered vaginal flora assessed based on Gram stain were each associated with HIV acquisition in a meta-analysis, which demonstrates that diagnosis of BV based on Nugent score has important clinical implications [58]. Several studies have assessed the sensitivity and specificity of the Amsel’s criteria in diagnosing BV assuming the Nugent score as a reference, and while there is significant variability in the findings, the Amsel’s criteria tends to have poor sensitivity and specificity [159-161]. Therefore, the Nugent score is the preferred method of diagnosing BV in research settings; although, newer means of determining the precise composition of the vaginal microbiota using high throughput sequencing tools are now being used in conjunction with the Nugent score (discussed below).
1.7.3.1 Vaginal microbiota and vaginal dysbiosis

The human microbiota is defined as the collection of micro-organisms including those of bacterial, viral, archae, and fungal origin that are present in a defined environment [162,163]. Surfaces and cavities of the human body that are exposed to the environment act as a scaffold for the microbiota, which varies between individuals and over time, and is modulated by host and environmental factors at different anatomical locations [164]. The term microbiome refers to the microbiota, its associated genes and genomes (the metagenome), and the products of the microbiota and host environment [163]. Our current knowledge of the genital microbiome has been restricted by the study of bacteria due to technical limitations, and while future advancements will improve our understanding of other microbial communities including viruses that colonize the FGT, here I will restrict our discussion of the microbiota and microbiome to bacterial species. In addition to the Nugent score, the vaginal microbiota can also be assessed by sequencing of the bacterial 16s rRNA gene of the small ribosomal subunit. Using this technique, processed 16S rRNA amplicon sequences are assigned to species and genera, using a Markov Chain model for precise vaginal species level assignments and an available database that contains all microbes previously observed in the vaginal microbiota. Samples are clustered in community state types (CSTs) using taxonomic composition and abundance and the Jensen-Shannon divergence [165]. Community types associated with BV (based on Nugent score) typically have high bacterial diversity and an abundance of facultative and strict gram negatives anaerobes including gardnererella, atopobium, prevotella, sneathia, BV-associated bacteria (BVAB) 1-3, and mobiluncus spp [165,166], and I will refer to this state of the microbiota as vaginal dysbiosis. However, community types associated with low Nugent scores are commonly characterized by L. crispatus –dominated microbiota, which is strongly associated with positive health outcomes, although L. iners – dominated microbiota is also associated with low Nugent scores in some communities and this is discussed below [167].

1.7.3.2 Clinical importance of BV

BV has been associated with increased susceptibility to genital infections and with adverse obstetrical and reproductive health outcomes. BV is associated with an increased incidence of several bacterial (see below) and viral sexually-transmitted infections (STIs), including HIV (a 1.7-fold increased risk) [58]), HSV-2 (a 3-fold increased risk [168]), and herpes papilloma virus (HPV) risk by approximately 1.2 fold [169,170]. Indeed, BV not only increases the risk of HIV
acquisition but in HIV-infected women, also increases the risk of HIV transmission to their male partners by 3 fold [171]. The increased risk of HIV transmission from an BV/HIV-positive woman to her male partner is apparently independent of effects on the genital HIV viral load; and as a result, BV may be responsible for ~17% of heterosexual HIV transmission in SSA [143,171]. However, despite these epidemiological associations, studies demonstrating that BV therapy and/or prevention can reduce viral STI incidence have not been performed, and would be necessary to demonstrate the causal role of vaginal dysbiosis in viral STI acquisition risk.

One factor that is critical to take into account when assessing the impact of BV treatment on HIV/STI acquisition risk is the generally high rate of BV recurrence, which can be as high as 72% within 3 months [172-174]. Thus, a major challenge for the prevention of adverse clinical outcomes associated with BV is the inability of current treatment modalities to provide a long-term normalization of a lactobacilli-predominant microbiota, with relapse of VM dysbiosis being the rule rather than the exception [172,174]. Perhaps this is best exemplified by the longitudinal assessment of weekly changes in Nugent score conducted over an 18-24 month period in sexually-active women of reproductive age in Rakai, Uganda [175]. In this study, participants provided self-collected vaginal swabs each week, which were assessed for BV by Nugent score, and syndromic treatment of vaginal discharge using metronidazole was provided. A key finding of this study was that over a two-year period, almost all women (95%) had at least 1 episode of BV, and one-third of women spent more than half of this time with BV. This demonstrates that despite syndromic management of BV, this condition is highly prevalent and persistent. The study also divided the participants into tertiles of longitudinal prevalence of BV to assess alterations in the microbiota. Women in the upper tertile (52%-100% longitudinal prevalence of BV) and lower tertile (0-20% prevalence of BV) spent approximately 70% of their time with a diagnosis as BV-positive (Nugent score 7-10) or BV-negative (Nugent score 0-3) respectively, and approximately 30% of their time in other categories. Women in the middle tertile showed the greatest longitudinal fluctuation in the vaginal microbiota as they spent only 20% of their time in an intermediate state and 80% in other states. These data demonstrate the dynamic nature of changes in the vaginal communities over time, and illustrate the challenge in treating BV.

While BV is associated with an 1.8-fold and 1.9-fold increased incidence of the bacterial STIs, *N. gonorrhoeae* (NG) and *C. trachomatis* (CT) [176] respectively, the impact of BV treatment in reducing STI acquisition appears to be dependent on the effectiveness of treatment in preventing
BV recurrence. In one study, HIV/STI-uninfected women who had asymptomatic BV were given standard treatment with topical metronidazole followed by metronidazole treatment twice weekly to prevent BV recurrence [177]. The study reported a reduction in the incidence of chlamydia during 6 months of ongoing treatment/follow-up, and STI incidence only rebounded after cessation of BV treatment after 6 months. In another study, treatment of HIV-uninfected women with BV or vulvovaginal candidiasis (VVC, a yeast infection) with monthly topical metronidazole (for BV) and miconazole (for VVC) for 12 months reduced the combined incidence of NG/CT [178]. These studies suggest that STI prevention is possible if continuous suppression of BV-associated anaerobic bacteria is achieved [172]. This may also explain why in a recent large, multisite, home-based study that treated BV-positive women at baseline and provided intermittent treatment only upon diagnosis of BV at 2 month intervals, no impact of BV treatment was observed on incident bacterial STI [179]. These data reinforce the notion that continual and not intermittent treatment of BV reduces STI incidence, which suggests that vaginal dysbiosis plays a causative role in increasing susceptibility of bacterial STI.

1.7.3.3 Host genital immunology and the vaginal microbiota

Although BV is not characterized by clinical signs of inflammation including redness, pain, or swelling, there is a clear association between vaginal dysbiosis and elevation in pro-inflammatory cytokines in both cross-sectional and observational studies, and broadly speaking this has two possible explanations. First, vaginal dysbiosis may alter genital immunology through several mechanisms, including changes in the barrier properties of mucus and upregulation of pro-inflammatory cytokines by epithelial and dendritic cells, both of which may lead to barrier disruption and increased susceptibility to opportunistic infections. However, an alternative explanation is that elevation in certain pro-inflammatory cytokines is the primary event, with downstream impact on the VM through effects on epithelial cell differentiation and barrier integrity, since these parameters may shape the vaginal microbiota by altering the local availability of key nutrients.

1.7.3.4 Impact of the vaginal microbiota on the mucosal immune system

The lining of the ectocervix and vagina consists of a stratified squamous epithelium, while that of the endocervix and upper genital tract is a single columnar layer. Epithelial barrier protection at all tissue sites is mediated by protein complexes that enable cells to adhere to one another and
through the overlying mucus layer that restricts the movement of microbes and their access to the epithelium [76]. Cross-sectional studies suggest that bacterial community types alter the properties of mucus, which may then affect host susceptibility to opportunistic pathogens. Mucus from women with BV is thin, watery, and has reduced viscosity, possibly due to increased activity of mucin-degrading enzymes [180], which may reduce its ability to trap pathogens. Indeed, mucus obtained from women who are BV-negative [80] or with a L. crispatus-dominated microbiota efficiently traps HIV, whereas that from women with L. iners- or G. vaginalis-dominant microbiota is much less efficient [79]. This activity is optimal at acidic pH (~4) but abrogated at neutral pH [80], which suggests several possible mechanisms by which a L. crispatus-dominated microbiota may protect the host: (i) the formation of hydrogen-bonding interactions between HIV and mucins at low pH [79,80] (ii) direct inactivation of HIV by high levels of D-lactic acid (D-LA) produced by L. crispatus (but not by L. iners or G. vaginalis) [181,182] or (iii) lactic acid induced reduction in the risk of acquiring HIV by inhibition of the growth of BV-associated bacteria and sexually-transmitted microbes such as N. gonorrhea, C. trachomatis [182-184].

Vaginal dysbiosis may also have a direct impact on genital epithelial cells. Epithelial cells express various pathogen recognition receptors (PRRs), including Toll-like-receptors (TLRs) and Nod-like receptors (NLRs), which sense conserved microbial and host danger signals such as lipopolysaccharide (LPS), components of the bacterial cell wall, and microbial DNA and RNA [185,186]. Activation of PRRs stimulates activation of NF-κB, the master transcription factor with broad effects on inflammation, apoptosis and immune responses, resulting in production of mucins, anti-microbial peptides (AMPs), and pro- and anti-inflammatory cytokines and chemokines that induce the recruitment and/or activation of immune cells [185-189]. In ex vivo/in vitro models of a vaginal epithelial monolayer or cell aggregates, addition of bacteria that are associated with dysbiosis (gardnerella, prevotella, mobiluncus, atopobium or sneathia spp) but not a ‘healthy’ microbiota (L. crispatus) stimulated the production of pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α), the chemokine MIP-3α, IL-8 [190,191], AMPs (lactoferrin and SLPI) [188-190], and mucins (MUC1, MUC2 and MUC4) [190], likely through activation of the NF-κB pathway [190,191]. Moreover, high bacterial diversity, dysbiosis or BV were each associated with elevated levels of pro-inflammatory cytokines and chemokines, which were reduced either after a spontaneous return to a lower-diversity microbiota [192] or after successful
metronidazole treatment (Nugent score <4) [193,194]. Despite changes in genital inflammation, vaginal dysbiosis was not associated with changes in the number of endocervical CD4+ T cell or DC subsets [192,195]. However, DC activation was evident by increased gene expression of pro-inflammatory cytokines including IL-1, TNF-α and IL-6, possibly through LPS-sensing and activation [192]. Collectively, these data suggest that changes in the vaginal microbiota are sensed by genital epithelial cells and DCs, which respond by secreting various innate immune factors including cytokines, chemokines, AMPs, and mucus.

Several cross-sectional studies also suggest that genital inflammation might enhance host susceptibility to various pathogens by damaging the genital epithelial barrier. Vaginal dysbiosis [166] and genital inflammation (elevated pro-inflammatory cytokines [85]) were each associated with an overlapping mucosal proteomic signature of increased proteolytic activity and actin cytoskeletal pathways, and of decreased epidermal cell differentiation and cornified envelope pathways, all of which are indicative of epithelial barrier disruption [127]. Genital inflammation also directly reduces epithelial integrity and increases translocation of bacteria across the vaginal epithelium in vitro [78], and in vivo is associated with an increased number of mucosal HIV target cells [85,192] and HIV acquisition risk [94]. In summary, there is a consistent association between the VM and elevated pro-inflammatory cytokines, although the direction of causation is not established, with a downstream impact on susceptibility to opportunistic infections such as HIV and STIs.

1.7.3.5 Impact of genital inflammation on the VM

Mucosal inflammation may not only be triggered by alterations in the vaginal microbiota, but may also mediate them. The apical layer of the stratified epithelium in the vagina and ectocervix is termed the stratum corneum. This layer is comprised of dead flattened cells that have undergone a terminal differentiation program called cornification, which involves the expulsion of large glycogen stores [127]. Glycogen is a critical source of carbon for lactobacilli [196], which metabolize it using α-amylase, a host enzyme expressed in genital epithelial cells [197]. Glycogen metabolism results in the production of maltose and maltodextrin sugars, which are required for the growth of lactobacilli in vitro [196], and the production of lactic acid, which lowers the vaginal pH and creates unfavourable growth conditions for pathogens. α-amylase levels are also associated with matrix metalloprotease-8 (MMP-8) and hyaluronidase [198],
enzymes which target the ECM and may contribute to detachment of glycogen-rich epithelial cells and enhance glycogen availability for lactobacilli.

The local availability of glycogen, which appears to be critical for lactobacilli, may be disrupted by genital inflammation. Vaginal dysbiosis and genital inflammation have an overlapping proteomic signature that includes down-regulation of epidermal cell differentiation, keratinization, and cornification pathways, which are critical for epithelial exfoliation and glycogen availability [85,166]. Therefore, I hypothesize that genital inflammation itself may create conditions that are unfavourable for survival of lactobacilli through the disruption of glycogen availability and/or metabolism, allowing the outgrowth of bacteria that constitute vaginal dysbiosis. This hypothesis is supported by proteomics, metabolomics, and genomics studies, and 16s rRNA-based gene surveys. Dysbiosis-associated bacteria prefer amino acid metabolism as a source of carbon and nitrogen, in contrast to lactobacilli: specifically, genital secretions from women with BV had lower levels of amino acids and increased amino acid catabolites and polyamines, while *L. crispatus* and *L. jensenii* were associated with intact amino acids and dipeptides [199]. Moreover, lactobacilli genomes are underrepresented in amino acid transport and metabolism and over-represented in carbohydrate transport and metabolism [200]. Therefore, I speculate that elevated genital inflammation may trigger the upregulation of proteases and down-regulation of anti-proteases and cornification pathways, which increases the availability of amino acids and reduces glycogen availability, thereby favouring the growth of bacteria associated with dysbiosis.

In keeping with these data, the unique ability of *L. iners* to metabolize both carbohydrates and amino acids may enable it to co-exist in a vaginal environment that also contains diverse bacteria. *L. iners* contains genes for carbohydrate transport and metabolism [200], and although it lacks genes necessary for *de novo* synthesis of most amino acids, ~15% of its genome supports various transport mechanisms so that it may acquire crucial amino acids from the environment [201]. These unique adaptations fit with the observation that the metabolic signature of *L. iners* is intermediate between that of *L. crispatus/jensenii* and BV-associated bacteria [199]. Therefore, *L. iners*-dominated communities may be more adaptable and shift towards other community types in the context of changing growth conditions. Collectively, these data support the model that genital inflammation may trigger vaginal dysbiosis through impairment of epithelial cell differentiation and cornification pathways and increased proteolytic activity, resulting in growth
conditions that are unfavourable for lactobacilli but more conducive for bacteria associated with vaginal dysbiosis.

In summary, there is strong evidence from prospective studies that vaginal dysbiosis is associated with increased pro-inflammatory cytokine production in the FGT, but such studies cannot determine the direction of causation in this relationship. There is a high degree of interrelatedness between host and environmental factors, including the genital microbiota, inflammation, host and microbial metabolites, immune cells, reproductive hormones and host genetics. Future interventional studies that are focused on altering specific microbial or immune parameters (see Chapter 4), combined with state-of-the-art molecular analytical tools, will enable us to determine the causal nature of these relationships and to develop better clinical strategies to improve health outcomes.
Chapter 2

2 Identification of preferential CD4+ T cell targets for HIV infection in the cervix


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Main Objective: To develop a novel ex vivo HIV infection assay to assess HIV susceptibility in cervix-derived CD4+ T cells

Specific objectives

1.Validate the β-lactamase vpr HIV entry assay for use in endocervical cytobrush derived CD4+ T cells

2.Compare HIV susceptibility between blood and the genital mucosa

3.Identify and characterize novel cellular targets of HIV
2.1 Abstract

A better understanding of the cellular targets of HIV infection in the female genital tract may inform HIV prevention efforts. Proposed correlates of cellular susceptibility include the HIV co-receptor CCR5, peripheral homing integrins, and immune activation. We used a CCR5-tropic pseudovirus to quantify HIV entry into unstimulated endocervical CD4+ T cells collected by cytobrush. Virus entry was three-fold higher into cervix-derived CD4+ T cells than blood, but was strongly correlated between these two compartments. Cervix-derived CD4+ T cells expressing CD69, α4β7, or α4β1 were preferential HIV targets; this enhanced susceptibility was strongly correlated with increased CCR5 expression in α4β7+ and CD69+ CD4+ T cells, and to a lesser extent in α4β1+ CD4+ T cells. Direct binding of gp140 to integrins was not observed, integrin inhibitors had no effect on virus entry, and pseudotypes with an env that preferentially binds α4β7 still demonstrated enhanced entry into α4β1+ cells. In summary, a rapid and sensitive HIV entry assay demonstrated enhanced susceptibility of activated endocervical CD4+ T cells, and those expressing α4β7 or α4β1. This may relate to increased CCR5 expression by these cell subsets, but did not appear to be due to direct interaction of α4β7 or α4β1 with HIV envelope.
2.2 Introduction

Heterosexual sex is the most common mode of HIV transmission, and women account for almost two thirds of new infections globally [12]. While sexual transmission is inefficient, with a probability of 1/200 to 1/2000 per episode of penile-vaginal sex [202], the immunopathogenesis of transmission events is incompletely understood. Most sexual exposures do not result in HIV infection, presumably because the virus is repelled by innate mucosal immune defenses. These include an intact genital epithelium, cervical mucus, innate antimicrobial peptides in the genital fluids, and immune cells within the mucosa (neutrophils, αβ and γδ T cells, dendritic cell subsets, and others) [70]. However, preventing HIV infection is a delicate balance, since HIV preferentially replicates in activated CD4+ T cells [73,113] and inflammation in the female genital tract FGT was directly associated with HIV acquisition in young women from South Africa [94]. Increased levels of antimicrobial peptides are associated with an improved ability of genital fluids to neutralize HIV in vitro, but in a prospective study, this ability was paradoxically associated with increased HIV acquisition in vivo [93], perhaps because mucosal inflammation is accompanied by increased mucosal HIV target cells [203] and reduced epithelial integrity [78,204]

While mucosal T cell immune activation enhances HIV susceptibility, there are several other cellular correlates of HIV susceptibility in the FGT. Following gp120 binding to CD4, the HIV co-receptor CCR5 is necessary for viral entry and establishment of infection by sexually-transmitted strains of the virus [205]. The mucosal integrins α4β7 and/or α4β1 may also have a role in sexual transmission of HIV. α4β7 binds to mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) on high endothelial venules (HEVs) and mediates lymphocyte migration to the intestine [125], while α4β1 binds vascular cell adhesion molecule-1 (VCAM-1) [131] to mediate lymphocyte migration to non-lymphoid tissue and sites of inflammation [132,133,206]. α4β7 has been shown in some studies [126,207], although not all [129], to enhance cellular HIV replication in CD4+ T cells derived from peripheral blood mononuclear cells (PBMCs). In cervical explants, addition of α4, β7, and β1 blocking monoclonal antibodies (mAbs) reduced HIV replication of the R5-tropic BaL strain [134], suggesting that α4β7 and/or α4β1 may play a role in the early establishment of HIV infection in the FGT. Collectively, these results suggest that mucosal CD4+ T cell expression of α4β7, α4β1, CCR5, and CD69 may flag
mucosal T cell subsets with increased susceptibility to HIV.

Measuring inflammatory genital biomarkers or mucosal T cell expression of putative susceptibility markers provides a surrogate measure of HIV susceptibility. However, since the optimal mucosal surrogate of HIV susceptibility is not known, measuring actual mucosal T cell HIV infection \textit{ex vivo} would be the most biologically relevant way to assess HIV susceptibility, and might allow for improved identification and characterization of susceptible cells. Currently, most cervical \textit{ex vivo} infection assays are performed on biopsies or cervical tissue obtained during hysterectomies [100,134,208]. However, hysterectomies are rarely performed on young and/or healthy individuals and can only be done once per individual, making them impractical for studying HIV susceptibility in prospective studies. Although sequential ectocervical biopsies or cervical cytobrush specimens can be obtained from healthy individuals, both yield a relatively low number of CD4+ T cells [135] and this poses a challenge for measuring HIV infection \textit{ex vivo}.

Given these concerns, the purpose of this study was to establish a rapid and sensitive assay to quantify HIV entry into freshly isolated and unstimulated cervix-derived CD4+ T cells obtained using cervical cytobrushes, and to identify preferential cellular targets of HIV. Using an R5-tropic β-lactamase (BlaM)-Vpr HIV pseudovirus, we hypothesized that cervix-derived CD4+ T cells were highly susceptible to HIV entry and that immunologically activated, α4β7+, and α4β1+ CD4+ T cells were preferential cellular targets of HIV entry in the FGT.
2.3 Methods

2.3.1 Ethics statement

Informed written consent was obtained from all participants prior to enrolment, and the study was approved by Institutional Review Boards at both Kenyatta National Hospital (Nairobi, Kenya) and St. Michael’s Hospital (Toronto, Ontario), as well as the Universities of Manitoba and Toronto (Canada). The described studies were conducted according to the principles expressed in the Declaration of Helsinki.

2.3.2 Study participants

Female participants were recruited from two outpatient clinics in Nairobi, Kenya. Participants had no vaginal discharge, no clinical signs/symptoms of genital inflammation, were pre-menopausal and were not actively menstruating. HIV rapid testing was performed according to Kenyan national guidelines (using Determine, Inverness Medical, Waltham, MA, USA), and only HIV-uninfected participants were enrolled. Bacterial vaginosis was diagnosed by Gram’s stain based on Nugent’s criteria [209], *Chlamydia trachomatis and Neisseria gonorrhoeae* screened by PCR (Roche, Pleasonton, CA, USA), syphilis by serology (Diagnostics Worldwide, Brooklyn, NY, USA) and *Trichomonas vaginalis* by In-Pouch culture (Biomed Diagnostics, Inc. White City, OR, USA). A semi-structured questionnaire capturing a range of behavioural and reproductive health data was administered to all participants.

For assay development and analysis of surface expression of integrin α4, β7, and β1 on CD4+ T cells, female participants were recruited from a Colposcopy Clinic at St. Michael’s Hospital, Women’s Health Care Centre in Toronto, Canada. Recruited participants self-reported to be HIV-negative, were not actively menstruating at the time of sample collection, and did not present with clinical signs and symptoms of genital inflammation. No formal HIV or STI testing was performed nor was a questionnaire administered.

2.3.3 Sample collection and processing

Ten milliliters of heparinized blood was collected by venipuncture, and two cervical cytobrushes by insertion of the cytobrush into the endocervical os and rotation through 360°. Cytobrushes
were then transferred to a conical vial containing complete media (RPMI 1640, fetal bovine serum (FBS), antibiotic/antimycotic cocktail containing clindamycin, streptomycin, polymyxin B, amphotericin B, and gentamycin) and transported on ice to the laboratory for processing. PBMCs were isolated by Ficoll density separation and reconstituted at 10^7 cells/ml in complete media. Cervical immune cells were removed from the cytobrushes by agitation, washed in complete media, filtered through a 100-µm cell strainer, and reconstituted in 200 µl complete media for use in the HIV entry assay.

### 2.3.4 BlaM-Vpr HIV entry assay

The BlaM-Vpr HIV entry assay is described in detail elsewhere [137,139]. Briefly pseudovirions incorporating BlaM fused to the viral protein R (vpr) were used to infect blood and cervical cells by spinoculation. Samples are then loaded with a cell-permeant dye, CCF2-AM, which is susceptible to cleavage by BlaM. CCF2 is a fluorescence resonance energy transfer (FRET) substrate, which emits green light at 520nm when excited by a 405-nm laser. Upon cleavage by BlaM, blue light is emitted at 447nm. The change in blue:green emission can be monitored by flow cytometric analysis and flags cells with cytosolic entry of virus (Figure 1-10).

Since our primary goal was to develop an infection assay relevant for sexual transmission of HIV, the envelope used for pseudotyping the BlaM-Vpr virions was early-transmitted, CCR5-tropic and Clade A, the most common clade in Kenya [210], where we performed our mucosal studies. As described elsewhere, the \( Q259d2.17 \) envelope had been cloned from an HIV isolate that was obtained from a Kenyan woman, 1 week after HIV sero-conversion. BlaM-Vpr pseudovirions were produced by transfection of 293T cells with 20 µg HIV backbone lacking envelope - \( Q23Aenv\ gfp\ nef \) [211], 10 µg early-transmitted R5-tropic Clade A envelope - \( Q259d2.17env \) [212], 10 µg \( pCMV-BlaM-Vpr \) (Addgene, Cambridge, MA, USA), 5 µg of pAdvantage (Promega, Madison, WI, USA) and 135 µl of the transfection reagent Polyjet (FroggaBio, Toronto, Canada). Forty-eight hours post-transfection, supernatant was collected, filtered through a 0.45 µm filter, and incubated for 15h at 4°C with PEG-it to allow formation of virus-PEG complexes as per manufacturer’s instructions (SystemBio, Mountain View, CA, USA). Virus-PEG-it complexes were precipitated at 1500 xg for 30 min at 4°C and concentrated 100 fold in PBS and stored at -80°C. Viral stocks were titrated using the BlaM-Vpr HIV entry assay on reference PBMCs obtained from an HIV-negative donor and by p24 ELISA (NCI,
Frederick, MD, US). Input BlaM-Vpr pseudovirus used in experiments below was equivalent to approximately 60% of maximum viral entry in the reference PBMCs or 37ng by p24 ELISA. Where specified, pseudotypes expressing a Clade A, CCR5-tropic env - 92Ug037 were produced using the same protocol.

For ex vivo infections, 10⁶ PBMCs were incubated with BlaM-Vpr pseudovirus or media (negative control; mock infection). Processed cervical cell suspension from each participant was divided in two and similarly incubated with either virus or mock. For testing of HIV entry inhibitors, cervical cells were split into 3 equal aliquots and pre-incubated with the appropriate inhibitor or vehicle for 30 min at room temperature followed by addition of virus/PBS. Samples were spinoculated at 1200 xg for 2h at 17°C in 48 well flat-bottom plates to allow viral interaction with cells followed by a 2h incubation at 37°C, 5% CO₂ to allow cell entry [137]. Samples were washed twice in CO₂-independent media (Invitrogen, Carlsbad, CA, USA) and loaded with 1μM CCF2-AM (Invitrogen) for 1.5h. Samples were washed once in CO₂-independent media and incubated for 12h in CO₂-independent media supplemented with 10% FBS, antibiotic cocktail and solution D (Invitrogen), an anion transport inhibitor to prevent leakage of CCF2-AM dye. Samples were then stained with fluorescent-labelled antibodies (as described below) and analyzed using a BD LSR-II Flow Cytometer (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA).

For analysis of HIV entry within each individual, the same flow cytometry gating strategy used for blood was applied to cervix, with the exception of the gates denoting HIV entry, as these were not consistent between the two compartments. Hence, an uninfected mock/PBS control was included for both blood and cervical samples and used to gate on change in blue: green fluorescence (viral entry) between virus- and mock- treated samples.

2.3.5 Flow cytometry

Surface staining of cells with fluorescently labelled monoclonal antibodies was performed for 30 min at 4°C, and then acquired and analyzed using a BD LSR-II Flow Cytometer. Antibodies used for staining were (α4) CD49d-PE, integrin β7-PE-Cy5, CD3-A700, CCR5-APC-Cy7, (Becton Dickinson Bioscience), LIVE DEAD Far Red (Invitrogen), CD4-ECD (Beckman...
Intracellular staining for Ki67 could not be performed on the same cervical samples used for the HIV entry assay because cell permeabilization, which is required for detection of Ki-67 caused leakage of CCF2-AM. Hence, Ki-67-FITC (Becton Dickinson Bioscience) staining was performed on cervical samples from separate participants at 4°C for 30 min after staining for aforementioned cell surface markers including LIVE DEAD Far Red stain. All cellular events were recorded during flow-cytometric analysis of cytobrush samples.

2.3.6 Gp140 production and purification

Mammalian codon-optimized Q259d2.17 gp140 env with a FLAG-tag was cloned into a mammalian expression vector pKF-Foldon. HEK293T cells were stably transfected with the vector using Fugene 6 according to manufacturers recommendation (Promega, Madison, WI, USA) and selected for puromycin resistance. Supernatant was collected from stably transfected cells and purified using anti-FLAG-agarose beads as per manufacturers instructions (Sigma, Oakville, ON, Canada). Purified protein was analyzed by SDS-PAGE. Proper folding of the purified protein was assessed by immune-precipitation (IP) for 1.5 hour at room temperature with several antibodies including PG9, 2G12, F105, 447-52D, b12 against various regions of HIV env and with soluble CD4 dimer (CD4-IgG2). Antibodies used for IP were obtained from the NIH AIDS Reagent Program. This was followed by incubation with Protein G Dynabeads as per manufacturers instructions (Life Technologies, Burlington, ON, Canada) and Western Blot for anti-FLAG M2 antibody (Sigma, Oakville, ON, Canada).

2.3.7 Integrin-binding assay

PBMCs of HIV-negative donors were obtained as previously described. To assess CD4-independent binding of HIV env to surface integrins, CD4+ T cells were depleted by positive selection (Stemcell, Vancouver, British Columbia, Canada). The remaining cells were cultured for 6 days in RPMI, 10% FBS supplemented with 10nM ATRA (Sigma), 1µg/ml OKT3 Anti-human CD3 mAb (Biolegend, San Diego, CA, USA), and IL-2 (10U/ml) (NIH AIDS Reagent Program). Cells were tested for expression of α4, β7 and β1 before and after ATRA-stimulation using aforementioned antibodies to ensure upregulation of β7 expression prior to performing the
integrin-binding assay. As described elsewhere, CD4-depleted PBMCs that were either ATRA-stimulated or unstimulated were incubated with 10μg FLAG-gp140 or 10μg MAdCAM-1-Fc in buffer containing 10mM HEPES, 150mM NaCl, 100μM CaCl₂ (Sigma), 1mM MnCl₂ (Sigma), 0.5% BSA (Sigma), and 0.09% sodium azide (Sigma) [213]. Cells were stained with FITC-anti-FLAG (Sigma) or FITC-anti-Human IgG Fc (Biolegend) as required and then analyze by flow cytometry.

2.3.8 Statistical analysis

Intra-individual differences in percent HIV entry, CCR5 and Ki-67 expression on CD4+ T cell subsets were assessed using the Wilcoxon-matched pairs signed-rank test. Correlations were determined using Spearman rank correlation. All statistical tests were run on Prism 6 (La Jolla, CA, US) on Mac OS. Flow cytometry data were analyzed in FlowJo v. 8.6.6. (Ashland, OR, USA).
2.4 Results

2.4.1 Study participants

Fifty-four participants were enrolled in the Nairobi-based studies. No participants had genital symptoms or clinical findings (see exclusion criteria in Methods), and asymptomatic genital infections were rare: 7/54 had bacterial vaginosis (a Nugent score ≥ 7) [209], 1/54 Neisseria gonorrhoeae, 2/54 Chlamydia trachomatis, 1/54 Trichomonas vaginalis, and 0/54 active syphilis. Of these 54 participants, HIV entry into cervical T cell targets was assessed in 41 participants (median age, 28 years; range, 19-46 years), and 13 participants were enrolled in studies of mucosal Ki-67 expression (median age 30 years; 19-39 years).

2.4.2 Enhanced HIV susceptibility of cervix-derived CD4+ T cells

Cytosolic entry of the BlaM-Vpr HIV pseudovirus was restricted to CD4+ T cells in both blood (Figure 2-1A and C) and cervix (Figure 2-1B and D). In blood, median virus entry was 4.5% (IQR 2.2%-6.4%) in CD4+ T cells, compared to 0.22% (IQR 0.0%-0.6%) in CD4- T cells (P<0.0001; Figure 2-1C). Similarly, in cervical CD4+ and CD4- T cells, median HIV entry was 10.4% (IQR 6.0%-25.6%) and 0.02% (IQR 0.0%-0.1%) respectively (p<0.0001; Figure 2-1D).
Figure 2-1. Enhanced HIV susceptibility of cervix-derived CD4+ T cells compared to blood. HIV entry into blood- and cervix-derived CD4+ and CD4- T cells infected ex vivo with a CCR5-tropic BlaM-Vpr pseudovirus. Gating on from left to right, singlets, live cells, lymphocytes, and CD4+ and CD4- T cells in top rows in blood (A) and the cervix (B). Bottom rows in (A) and (B) show representative cytosolic HIV entry or blue versus green signal in CD4+ and CD4- T cells in mock- or virus-treated samples. Overall % HIV entry into CD4+ and CD4- T cells in 24 blood
(C) and 20 cervical samples (D). Comparison (E) and correlation (F) of HIV entry into CD4+ T cells derived from 18 matched blood and cervical samples. Graph shows median values and statistical comparisons are done by Wilcoxon rank-pairs test. Correlation was performed by Spearman correlation. *p=0.001, **p=0.0003.

**Figure 2-2. Surface expression of CCR5 and CD69 in blood and cervix-derived CD4+ T cells.** Wilcoxon matched-pairs test is applied for all comparisons. B=blood, C=cervix, N=24 and N=20 for blood and cervical samples respectively. *p<0.0001.

We hypothesized that cervical CD4+ T cells would demonstrate enhanced HIV susceptibility compared to blood, since these cells were more activated and express higher levels of CCR5 ([Figure 2-2] [111]). In keeping with this, HIV entry was 2.8 fold higher into CD4+ T cells derived from the cervix (median 11.8%, IQR 6.1%-26.6%) than from the blood (median 4.2%, IQR 2.0%-6.4%, p=0.0003; **Figure 2-1E**). Asymptomatic STIs or asymptomatic BV were diagnosed in 4 participants, but enhanced virus entry into cervix-derived CD4+ T cells compared to blood was seen regardless of genital co-infection or BV status (p=1.0). Since CD4+ T cells were responsible for a smaller proportion of flow cytometry events in cervical samples (mean, 1.7x10^3 CD4+ T cells per participant) than blood (mean, 1.7x10^5 CD4+ T cells per participant), in order to ensure that lower viral entry in blood CD4+ T cells did not simply reflect a lower stoichiometric ratio of input BlaM-Vpr virus per CD4+ T cell, HIV entry was then assessed in six samples matched for actual CD4+ T cell input number from each compartment (**Figure 2-3**). Again, HIV entry into cervix-derived CD4+ T cells (median 5.5%, IQR 1.0%-11.8%) was 7.7 fold higher than CD4+ T cells from blood (median 0.9%, IQR 0.2%-1.2% p=0.03). Overall, there was a consistent increase in HIV entry into cervix-derived CD4+ T cells. However, there was a strong positive correlation between HIV entry into blood- and cervix-derived CD4+ T cells (r=0.748, p=0.0002; **Figure 2-1F**) within a given individual, suggesting shared determinants of susceptibility between these two compartments.
Next we compared expression of Ki67, a marker of metabolic activity and cell proliferation, between blood and cervical compartments, as metabolically active cells are more likely to sustain productive HIV infection. Ki67 expression was 1.9 fold higher in CD4+ T cells derived from cervix (median 4.6%, IQR 2.7%-5.2%) compared to blood (median 2.4%, IQR 1.6%-2.8%; p=0.005; Figure 2-4). These results demonstrate that cervix-derived CD4+ T cells are highly susceptible to HIV, as they not only demonstrate preferential viral entry but also have a higher proportion of actively cycling cells.

Figure 2-4. Enhanced Ki67 expression in cervix-derived CD4+ T cells compared to blood. N=13, * p=0.005
2.4.3 BlaM-Vpr HIV entry is dependent on CCR5 and virus/cell fusion but not CXCR4

The gp160 env in our construct was described as R5-tropic [212], and so to test whether the increased blue:green signal observed in virus-treated samples was a consequence of CCR5-dependent viral fusion with CD4+ T cells, the CCR5-inhibitor maraviroc was titrated on PBMCs (Figure 2-5A and B). A dose-dependent inhibition of HIV entry was observed, with 34% inhibition (IQR 23%-59%) at 10nM maraviroc, 89% inhibition (IQR 78%-96%) at 100nM maraviroc and 94% inhibition (IQR 90%-98%) at 1µM maraviroc (Figure 2-5A). Furthermore, median HIV entry dropped from 11.7% (IQR 4.4%-15.0%) to 0.0% (IQR 0%-1.5%) in cervical CD4+ T cells treated with 1µM maraviroc (p=0.03; Figure 2-5C). As expected, treatment with the CXCR4 inhibitor AMD3100 had no effect on HIV entry into CD4+ T cells, either in the cervix (median 8.9%, IQR 1.1%-13.8% vs. 8.6%, IQR 2.0%-17.5% in untreated; p=0.69; Figure 2-5D) or blood (median 2.9%; IQR 1.9%-4.8% vs. 2.2%; IQR 1.6%-4.5% in untreated; p=0.07; Figure 2-5B).

Figure 2-5. Effect of HIV-entry inhibitors on cytosolic entry of a CCR5-tropic BlaM-Vpr HIV pseudovirus into CD4+ T cells in blood and the cervix. PBMCs were titrated with 0-
1000nM maraviroc (A) and treated with 1µM maraviroc, 1µM fuzeon (T20), or 250nM AMD3100 prior to addition of BlaM-Vpr pseudovirus (B). Cervical cells were treated with mock, virus, or virus plus 1µM maraviroc (C) or 250nM AMD3100 (D). All inhibitors were incubated with cells for 30 min at room temperature prior to addition of virus. Graphs show median values and statistical comparisons are done by Wilcoxon rank-pairs test. NI=No inhibitor. N=6. *p=0.03, **p=0.003, NS=Not Significant.

Next, we tested whether the increased blue: green fluorescence in virus-treated samples was a consequence of fusion-dependent cytosolic entry of HIV, rather than de novo expression of BlaM-Vpr. Treatment with T20 (FUZEON), which prevents fusion of viral and host membranes, resulted in near-complete ablation of viral entry in PBMCs (median 2.2%, IQR 1.6%-4.5% vs. 0.1%, IQR 0.0%-0.2%, p=0.03; Figure 2-5B). However, the reverse transcriptase (RT) inhibitor lamivudine, which blocks the post-viral entry step of reverse transcription of viral RNA into DNA had no effect on HIV entry (data not shown). Collectively, these results demonstrate that the increased blue:green signal in blood- and cervix-derived T cells treated with the R5-tropic BlaM-Vpr pseudovirus was dependent on CD4, CCR5 and fusion of viral and host membranes, and not due to de novo expression of BlaM, suggesting that this assay can measure physiologically relevant HIV entry.

Since our BlaM-Vpr HIV included an HIV envelope that was previously described as R5-tropic, we assessed whether CCR5+ CD4+ T cells were preferential targets of HIV entry (Figure 2-6A). HIV entry into CCR5+ CD4+ T cells was substantially enhanced compared to CCR5- CD4+ T cells in both blood (p<0.0001; Figure 2-6B and Table 2-1) and the cervix (p<0.0001; Figure 2-6C and Table 2-1). Within an individual, however, HIV entry into bulk cervix-derived CD4+ T cells correlated with the frequency of cervix-derived CCR5+ CD4+ T cells (r=0.70, P=0.0005; Figure 2-6D) and the mean fluorescent intensity (MFI) of CCR5 (r=0.46, P=0.04; Figure 2-7A), but not with CD4 MFI (r=-0.07, p=0.77). This suggests that while CD4 and CCR5 are required for cellular viral entry (Figure 2-1D, 2-5A-C), the expression level of CCR5 is a critical determinant of HIV entry into cervix-derived CD4+ T cells. We also observed a strong correlation between HIV entry into bulk cervix-derived CD4+ T cells and the frequency of CCR5+ CD4+ T cells in blood (r=0.64, p=0.007; Figure 2-6E), probably due to the strong correlation of CCR5 expression in CD4+ T cells between the two compartments (r=0.65, p=0.006; Figure 2-7B). These data further support the notion that CCR5 is a strong correlate of susceptibility to HIV, and suggest that expression level of CCR5 in blood may be a reasonable surrogate of the susceptibility of cervical CD4+ T cells to HIV.
Figure 2-6. HIV entry into cervix-derived CD4+ T cells correlates with CCR5 expression. Gating strategy for CCR5+ and CCR5- CD4+ T cells in blood and the cervix (A). Comparison of HIV entry into CCR5- versus CCR5+ CD4+ T cells in blood (B) and the cervix (C). Correlation between HIV entry into cervix-derived CD4+ T cells and % CCR5+ CD4+ T cells in cervix (D) and blood (E). All correlations were performed by Spearman correlation. In (D) and (E), N=19 and 17 respectively. *P<0.0001.

Figure 2-7. Association between expression of CCR5 and HIV entry into cervix-derived CD4+ T cells. Correlation between CCR5 MFI in cervix-derived T cells and HIV entry into cervix-derived T cells (A). Correlation between percent CCR5+ CD4+ T cells between the blood and cervical compartments (B). Correlation was performed by Spearman correlation. N=19.
### 2.4.4 Preferential HIV entry into activated cervical CD4+ T cells

We next assessed HIV entry into CD4+ T cells that expressed several putative correlates of HIV susceptibility, specifically the activation marker CD69, and the integrins α4β7 and α4β1. HIV entry into blood-derived CD69+ CD4+ T cells was 1.6-fold greater than CD69- CD4+ T cells (P<0.0001) ([Figure 2-8A, B, and Table 2-1](#)), and CCR5 expression on these CD69+ CD4+ T cells was increased 2.4 fold (P=0.0002) ([Table 2-2](#)). In the cervix, HIV entry into CD69+ CD4+ T cells was increased 1.9 fold (p=0.001) ([Figure 2-8A, C, Table 2-1](#)), and CCR5 expression was 3.6 fold higher (p<0.0001) ([Table 2-2](#)). These data demonstrate that activated CD4+ T cells are preferential targets of HIV entry in both blood and cervix, potentially due to increased CCR5 expression.

#### Table 2-1. Percent HIV entry into various CD4+ T cell subsets

<table>
<thead>
<tr>
<th></th>
<th>α4β7-</th>
<th>α4β7+</th>
<th>α4β1+</th>
<th>CD69-</th>
<th>CD69+</th>
<th>CCR5-</th>
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<td>Blood</td>
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<td>9.2**</td>
<td>4.1</td>
<td>6.8**</td>
<td>4.6</td>
<td>9.7**</td>
</tr>
<tr>
<td>IQR</td>
<td>0.6-2.6</td>
<td>2.6-6.4</td>
<td>5.5-14.3</td>
<td>2.1-5.6</td>
<td>3.9-10.4</td>
<td>6.9-17.6</td>
<td>2.4-6.0</td>
</tr>
<tr>
<td>Cervix</td>
<td>7.2</td>
<td>12.0*</td>
<td>12.9**</td>
<td>7.5</td>
<td>14.5*</td>
<td>7.0</td>
<td>20.9**</td>
</tr>
<tr>
<td>IQR</td>
<td>4.2-17.3</td>
<td>5.6-26.8</td>
<td>7.7-30.4</td>
<td>4.1-19.2</td>
<td>8.0-26.2</td>
<td>3.7-14.6</td>
<td>13.5-21.2</td>
</tr>
</tbody>
</table>

Values indicate % median, interquartile range (IQR). * p<0.005, ** p <0.0005. Wilcoxon rank-pairs test is applied. N=24 and 20 for blood and cervical samples respectively.
**Figure 2-8.** CD69 and CCR5 expression identifies CD4+ T cells susceptible to HIV. Gating strategy for CD69+ and CD69- CD4+ T cells in blood and cervical compartments (A). Comparison of HIV entry between CD69+ and CD69- CD4+ T cells in 24 blood (B) and 20 cervical (C) samples. Comparison of HIV entry between CCR5- CD69-, CCR5- CD69+, CCR5+ CD69- and CCR5+ CD69+ CD4+ T cells in 24 blood (D) and 19 cervical samples (E). Graphs show median values and statistical comparisons are done by Wilcoxon rank-pairs test. *p<0.05, **p<0.01, ***p<0.0005.

| Table 2-2. Percent CCR5 expression in various CD4+ T cell subsets |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | α4β7- | α4β7+ | α4β1+ | CD69- | CD69+ |
| Blood                            | 2.5   | 5.7** | 5.7*  | 3.1   | 7.7** |
| IQR                              | 1.5-3.7 | 3.0-7.9 | 3.0-7.8 | 1.6-4.9 | 4.4-10.8 |
| Cervix                           | 23.4  | 45.5** | 31.3* | 11.2  | 39.9** |
| IQR                              | 12.2-36.2 | 21.6-67.2 | 20.0-43.6 | 5.3-20.2 | 27.3-55.2 |

Values indicate % median, IQR. *P<0.002, ** P<0.0001. Wilcoxon rank-pairs test is applied. N=24 and 20 for blood and cervical samples respectively.

Next, we assessed the impact of co-expression of CCR5 and CD69 on HIV entry to determine the stronger correlate of susceptibility. In blood, in comparison to CCR5- CD69- CD4+ T cells (median 4.1%, IQR 2.1%-5.2%, P<0.0001), CCR5+ CD69+ CD4+ T cells were most susceptible to HIV entry (median 12.9%, IQR 8.3%-22.4%) followed by CCR5+ CD69- (median 8.1%, IQR 6.3%-15.7%, P=0.0003), and CCR5- CD69+ (median 6.9%, IQR 3.3%-8.9%, P=0.0002) CD4+ T cells respectively (Figure 2-8D). The same associations were seen in the cervix: CCR5+ CD69+ CD4+ T cells were the most susceptible (median 22.8%, IQR 14.0%-28.4%) followed by CCR5+ CD69- (median 16.7%, IQR 7.6%-29.2%, p=0.24), CCR5- CD69+ (median 10.4%, IQR 4.4%-14.4%, p=0.002), and CCR5- CD69- (median 7.0%, IQR 3.4%-13.5%, p=0.0003) CD4+ T cells (Figure 2-8E). HIV entry into CCR5+ CD69- CD4+ T cells was 1.2 and 1.8 fold higher compared to CCR5- CD69+ CD4+ T cells in blood (P=0.001) and the cervix (p=0.04) respectively; this suggests that CCR5 is a stronger correlate of HIV entry and that the enhanced HIV entry into activated CD4+ T cells may be largely due to the higher expression of CCR5.

### 2.4.5 Integrins and CD4+ T cell susceptibility

Next, we assessed the association of integrin expression with cellular HIV entry. First, we demonstrated that the nearly all of blood- and cervix-derived α4+β7- CD4+ T cells expressed the integrin β1 (median 99.8% IQR 96.9%-100.0% and median 94.7% IQR 90.6%-100.0%
respectively; **Figure 2-9A and B**); therefore in subsequent Nairobi-based work, the α4+β7-CD4+ T cell subset was defined as being α4β1+.

While expression of the integrins α4β7 and α4β1 was higher in CD4+ T cells derived from blood than the cervix (median 12.4% vs. 5.2%, p=0.0008; and 25.0% vs. 18.8%, p=0.02, respectively; **Figure 2-9C-E**), the expression of either integrin was associated with enhanced HIV susceptibility in both compartments. In blood, HIV entry was 3.8 fold higher into α4β7+ (p<0.0001) and 7.3 fold higher into α4β1+ (P<0.0001) CD4+ T cells compared to α4-β7- CD4+ T cells (**Figure 2-9F, Table 2-1**), while α4β7+ and α4β1+ CD4+ T cells in the cervix demonstrated 1.7 fold (p=0.003) and 1.8 fold (p=0.0002) higher HIV entry respectively (**Figure 2-9G, Table 2-1**). Expression of CCR5 was higher in both α4β7+ and α4β1+ CD4+ T cell subsets compared to α4-β7- CD4+ T cells in blood and the cervix (all p≤0.002; **Table 2-2**), but differences in CCR5 expression did not completely explain enhanced HIV susceptibility. In blood, while α4β1+ CD4+ T cells had similar CCR5 expression to α4β7+ CD4+ T cells, HIV entry was nearly 2 fold higher in the former subset (**Tables 2-1 and 2-2, Figure 2-9F**). In the cervix, CCR5 expression was ~1.4 fold lower in α4β1+ CD4+ T cells compared to α4β7+ CD4+ T cells (p=0.0006), but HIV entry levels were similar (p=0.54, **Tables 2-1 and 2-2, Figure 2-9G**). The cause of the enhanced HIV entry into α4β1+ CD4+ T cells is not known, but these data suggest that while CCR5 is a critical determinant of HIV entry, CCR5-independent mechanisms may also alter HIV entry into α4β1+ and/or α4β7+ CD4+ T cells. Importantly, while both α4β7+ and α4β1+ CD4+ T cells were preferential cellular targets of HIV in the cervix, α4β1+ CD4+ T cells were 3.6 fold more abundant (**Figure 2-9E**), suggesting that α4β1+ CD4+ T cells may be important early cellular target of HIV in the FGT.
Figure 2-9. α4β7 and α4β1 expression identifies CD4+ T cells susceptible to HIV. Gating strategy for integrin β1 expression in α4+β7- CD4+ T cells from blood (A) and cervical (B) samples obtained from 10 HIV-negative participants in Toronto, Canada. Gating strategy is shown for α4-β7-, α4+β7- (α4β7-) and α4+β7- (α4β1+) CD4+ T cells derived from blood (C) and cervix (D). Percent expression of α4β1 and α4β7 in blood and cervix-derived CD4+ T cells (E) and percent HIV entry into α4-β7-, α4β7+ and α4β1+ CD4+ T cells in blood (F) and cervix (G). In (C) to (G), N=24 for blood and 20 for cervical samples obtained from HIV-negative participants from Nairobi, Kenya. Graphs show median values and statistical comparisons are done by Wilcoxon rank-pairs test. * p <0.05, ** p <0.005, *** p <0.0001.
2.4.6 Enhanced HIV susceptibility of CD4+ T cells expressing α4β7 and α4β1 integrins is not mediated by direct envelope-integrin interaction

To further elucidate the mechanism of enhanced HIV entry into α4β7+ and α4β1+ CD4+ T cells, we tested whether recombinant Q259d2.17 gp140 env could bind to integrins on T cells in a CD4-independent manner. First, we confirmed proper folding of Q259d2.17 gp140 by immunoprecipitation of gp140 with soluble CD4 dimer and various antibodies that target different regions of envelope including the V1/V2 glycan peptide, V3 loop, and the CD4-binding site (data not shown). To assess CD4-independent binding of α4β7 and α4β1 to gp140, CD4+ T cells were removed from PBMCs by positive selection and the remaining cells were stimulated with all-trans retinoic acid (ATRA) to upregulate expression of β7 (as described elsewhere [128]). We first demonstrated that MAdCAM-1 bound to ATRA-stimulated CD8+ CD4- T cells (Figure 2-10A) expressing high levels of α4, β7 and β1 (Figure 2-10B), confirming that our assay conditions were conducive for ligand binding to integrins. In contrast, Q259d2.17 gp140 did not bind to ATRA-stimulated CD8+ CD4- T cells despite high levels of expression of α4, β7 and β1 (Figure 2-10A and B). However, gp140 bound to the small proportion (<2%) of CD4+ CD8- T cells that remained after CD4 depletion (Figure 2-10A), further confirming that the gp140 envelope was properly folded and capable of binding to CD4. Similar results were observed in ATRA-unstimulated blood-derived T cells (data not shown) and in cervical cytobrush-derived cells, where HIV gp140 bound to CD4+ CD8- T cells but not to CD8+ CD4- T cells (Figure 2-11). These data suggest that enhanced HIV susceptibility of CD4+ T cells expressing α4β7 or α4β1 was not mediated by direct binding between the HIV envelope and α4β7 or α4β1.
Figure 2-10. Enhanced HIV susceptibility of CD4+ T cells expressing α4β7 and α4β1 integrins is not mediated by direct envelope-integrin interaction. CD4-depleted PBMCs (A, left panel) cultured for 6 days in ATRA-conditioned media were incubated with PBS (Mock), Q259d2.17 gp140-FLAG or MAdCAM-1-Fc and stained with FITC-anti-FLAG or FITC-anti-human IgG Fc as appropriate (A, right panel). ATRA-stimulated and unstimulated cells stained with antibodies against α4, β7 and β1 (B). Representative figure from three independent experiments is shown in (A) and (B). Blood-derived CD4+ T cells from 9 participants were pre-incubated with integrin blockers 10µg HP2/1 (C) or 33nM Act-1 (D) or isotype control and then infected with Q259d2.17 pseudotypes. Fold difference in virus entry into α4β1+ versus α4β7+ blood-derived CD4+ T cells from 10 participants was compared using BlaM-Vpr pseudotypes that expressed Q259d2.17 or 92Ug037 envelopes (E). NS=Not significant. FMO=Fluorescence Minus One.
Figure 2-11. Lack of binding of HIV env to cervix-derived CD4-CD8+ T cells. Binding of trimeric Q259d2.17 env was tested in cytobrush-derived cells. HIV env bound to CD4+ CD8- T cells in the cervix but not to CD4-CD8+ T cells. Data shows representative figure from 2 HIV-negative participants.

We further tested whether α4β7 and/or α4β1 directly promote HIV infection by pre-incubation of blood-derived CD4+ T cells with the α4β7 inhibitor Act-1 or the α4 inhibitor HP2/1. We targeted the common alpha subunit of α4β7 and α4β1 using HP2/1 because antagonistic antibodies against the α4β1 heterodimer are not available. Neither inhibitor had any impact on virus entry: median virus entry after Act-1 incubation was 3.1%, IQR=1.9%-4.9% vs. 3.8%, IQR=2.9%-4.8% for isotype control (Figure 2-10C, p=0.64), and entry after HP2/1 incubation was 3.7%, IQR=2.4%-4.7% vs. 3.8%, IQR=1.8%-5.1% for isotype control (Figure 2-10D, p=0.9). We confirmed that Act-1 bound to α4β7 on T cells as pre-incubation with unlabeled Act-1 reduced staining with Act-1-PE (data not shown). These results suggest that while expression of α4β7 and α4β1 correlates with HIV susceptibility, these integrins may not play a direct role in HIV entry into CD4+ T cells.

Thus far, the data presented were obtained using BlaM-Vpr pseudotypes containing the Q259d2.17 env, where integrin binding had not been previously assessed. Therefore, we sought to confirm our results using pseudotypes expressing the R5-tropic env – 92Ug037, previously shown to have 3-fold higher binding to α4β7 compared to α4β1[126]. However, despite the use of pseudotypes expressing the 92Ug037 env that preferentially binds α4β7, we still observed approximately 2 fold greater virus entry into α4β1+ CD4+ T cells compared to α4β7+ CD4+ T cells (Figure 2-10E), while CCR5+ and CD69+ CD4+ T cells remained preferential targets of
HIV entry (Figure 2-12). Collectively, these data demonstrate that the integrin binding properties of HIV envs do not influence virus entry into CD4+ T cells, at least in our system.

Figure 2-12. HIV entry of BlaM-Vpr virus pseudotyped with 92Ug037 env correlates with CCR5 and CD69 expression in blood-derived CD4 T cells. N=11.
2.5 Discussion

HIV infection after sexual exposure is thought to be caused by the infection of a small number of highly-susceptible mucosal target cells [214], with subsequent slow local expansion leading to eventual systemic dissemination [73,74]. However, the low risk of HIV acquisition after sexual exposure has hampered the study of mucosal HIV transmission/susceptibility in human cohorts, and the identification of optimal HIV targets in the FGT. Therefore, we applied the BlaM-Vpr HIV entry assay as a means to rapidly quantify viral entry into the cytosol of freshly isolated and unstimulated endocervical CD4+ T cells, and identified several preferential cellular targets of HIV. Cervix-derived CD4+ T cells were more susceptible to HIV entry than those from blood, although within an individual, HIV entry was strongly correlated between the two compartments. Activated (CD69+) CD4+ T cells and those expressing the integrins α4β7 and α4β1 were preferential targets for HIV entry in both the cervix and blood. However, rather than increased susceptibility of the latter subsets being due to any direct interaction of these integrins with the HIV envelope, our results suggest that increased virus entry was likely due to increased CCR5 expression in these subsets and potentially other as yet undefined factors.

In keeping with prior studies [215,216], cervix-derived CD4+ T cells demonstrated enhanced immune activation and increased CCR5 expression compared to blood. Our data supports the hypothesis that this enhances HIV susceptibility at the mucosa, since cervical CD4+ T cells were highly susceptible to cellular HIV entry. In addition, the increased proportion of metabolically active (Ki67+) CD4+ T cells present in the cervix suggests that cervical CD4+ T cells may be more likely to sustain productive HIV infection after virus entry, as ex vivo infected Ki67+ CD4+ T cells in the cervix demonstrated increased de novo SIV-production compared to Ki67-CD4+ T cells [73,113]. In summary, the enhanced HIV susceptibility of cervix-derived CD4+ T cells compared to blood is consistent with differences in expression of several correlates of HIV susceptibility between the two compartments such as CCR5, CD69, and Ki67; this suggests that HIV entry detected using the BlaM-Vpr assay is a good proxy of physiologically relevant alterations in mucosal HIV susceptibility. Furthermore, the strong positive correlation that was observed between HIV entry into cervix-derived CD4+ T cells and CCR5 expression by and/or HIV entry into blood-derived CD4+ T cells suggests that at least in some contexts, differences in
CCR5 expression by and/or HIV entry into blood-derived CD4+ T cells may be a useful endpoint to assess cervical HIV susceptibility.

Increased HIV susceptibility at the mucosa may relate to changes in the phenotype of putative HIV target cells and/or be due to an increase in the number of mucosal target cells [216]. For instance, HSV-2 infection increases the risk of HIV acquisition approximately 3-fold [217], and is associated with an increase in the overall number and expression of CCR5 and CD69 by CD4+ T cells in the cervix [87]. Furthermore, specific genital CD4+ T cell subsets may be preferential targets of productive HIV [73,114], including activated CD4+ T cells [114] and Th17 cells [119]. The frequency of Th17 cells is increased in the mucosa, and these cells not only express higher levels of α4β7 and CCR5 [111], but are depleted from the cervix very early in HIV infection [111]. Therefore, in the current study, the preferential entry of a sexually transmitted R5-tropic BlaM-Vpr HIV pseudovirus into CD69+, CCR5+, and α4β7+ CD4+ T cells demonstrates that enhanced cellular viral entry may be an important mechanism for enhanced HIV susceptibility in activated cervical CD4+ T cells and Th17 cells. This is likely due to increased expression of CCR5 in α4β7+ CD4+ T cells, and potentially to other unknown factors, rather than a direct effect of α4β7 on HIV entry. HIV susceptibility at the genital mucosa may also be associated with the number of target cells (CD4+ T cells), and the protection of macaques from SIV infection after intravenous administration of Act-1 may be in part due to the substantial reduction in cervical CD4+ T cell numbers that were observed early after Act-1 administration [218].

Finally, our data suggests that α4β1+ CD4+ T cells may be preferential cellular targets for HIV in the FGT. α4β1+ CD4+ T cells are common in the endocervix, and in the murine model, this integrin is important in genital CD4+ T cell homing and pathogen clearance during C. trachomatis infection [132]. Consistent with prior studies [111,219], we reported higher expression of α4β7 in blood than cervix, possibly due to down-regulation of the integrin after mucosal homing. Further work will be needed to define the mechanism(s) underlying increased HIV susceptibility in α4β7+ and α4β1+ CD4+ T cells and should also investigate the role of these integrins in the homing of CD4+ T cells to the genital tract in the context of STIs and alterations in the genital microbiota.

Previously, it has been difficult to define cervix-derived CD4+ T cell subsets that are preferentially infected by HIV ex vivo due to the low number of immune cells obtained by
minimally-invasive sampling methods such as endocervical cytobrush sampling or ectocervical biopsies [135]. Assessment of cellular viral entry using the reporter enzyme BlaM fused to Vpr addresses the critical issue of sensitivity because BlaM causes multiple rounds of cleavage of CCF2-AM, thereby amplifying the blue: green signal from ex vivo infected cells. However, our assessment of ex vivo HIV entry does have limitations. We observed some entry of an R5-tropic HIV pseudovirus into apparently CCR5- CD4+ T cells. This occurred despite the absolute requirement of CCR5 for virus entry into blood and cervix-derived CD4+ T cells as demonstrated by the ablation of virus entry by CCR5 (but not CXCR4) blockade. In addition, the complete inhibition of HIV entry by the fusion inhibitor T20 (fuzeon) demonstrates that HIV entry was not due to non-specific effects. The low levels of virus entry into CCR5- CD4+ T cells is in keeping with data from the macaque SIV model, where memory CD4+ T cells that appeared to be CCR5- by flow cytometry harbored similar SIV levels to CCR5+ cells, and were subsequently shown to express 20 fold higher levels of CCR5 mRNA than naïve CD4+ T cells [220,221]. The most likely explanation for maraviroc-inhibited virus entry into CD4+ T cells that appear to be CCR5- by flow is that these cells are in fact expressing low levels of CCR5, below the threshold of detection. This may also account for the observation that percent HIV entry into CD4+ T cells sometimes exceeds the percent CCR5 expression, although other factors on the cell surface, such as G-protein coupled receptors[222], can also modulate HIV entry. Nevertheless, the detection of cellular HIV targets that appear to be CCR5- by flow cytometry may in fact constitute an advantage of the BlaM-Vpr assay, as it can identify cells that might otherwise not be considered as HIV targets.

In order to assess ex vivo HIV infection, we used pseudotypes produced in 293T cells, which display different envelope glycosylation patterns to primary CD4+ T cells. Infection of a small number of unstimulated, cytobrush-derived CD4+ T cells by a T cell-derived virus, while more physiological, could not be detected in our hands by p24 ELISA or intracellular staining (data not shown); the latter would require expansion and activation of cytobrush-derived cells, which would affect our ability to assess the impact of in vivo parameters (immune activation, genital infections, etc.) on HIV susceptibility. Nonetheless, Cavrois and colleagues previously used a replication-competent BlaM-Vpr HIV provirus to demonstrate a direct correlation between HIV entry and de novo viral replication[137]. Furthermore, we found that HIV entry was much higher into cervical than blood-derived CD4+ T cells, directly correlating with cellular immune
activation and CCR5 expression, which strongly suggests that the BlaM-Vpr HIV entry assay measures physiologically relevant HIV entry and is an appropriate tool to assess HIV susceptibility of cervical cells obtained by cytobrush.

2.6 Conclusion

In conclusion, a rapid and sensitive assay to quantify HIV entry into unstimulated endocervical CD4+ T cells demonstrated enhanced susceptibility of cervix-derived CD4+ T cells, with preferential HIV entry into activated endocervical CD4+ T cells and those expressing the integrins α4β7 or α4β1. Enhanced susceptibility of the latter subsets did not appear to be mediated by direct integrin-envelope interaction, and was related in part to increased CCR5 expression. Future studies using the BlaM-Vpr assay will be useful to identify novel cellular targets of HIV and/or assess the impact of clinical interventions such as treatment of STIs or BV on genital HIV susceptibility.
Chapter 3

3  *Ex vivo* virus entry into blood CD4+ T cells from high-risk, HIV-uninfected women does not predict subsequent heterosexual HIV acquisition

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**Submitting to:** *AIDS*

**Overall objective:** To test whether HIV entry into blood CD4+ T cells obtained from HIV-uninfected South African women predicts subsequent heterosexual HIV acquisition.

**Specific objectives**

1. Identify HIV target cells in blood, and assess whether frequency correlates with overall CD4+ T cell susceptibility within an individual.

2. Assess whether virus entry predicts subsequent HIV acquisition before and after controlling for potential confounding factors.
3.1 Abstract

The objective of this study was to test whether ex vivo HIV entry into blood CD4+ T cells predicts the risk of subsequent sexual HIV acquisition. A nested, retrospective, case-control study was performed in blood CD4+ T cells obtained from HIV-uninfected female participants at enrollment in the CAPRISA 004 clinical trial. Cases (n=33) subsequently acquiring HIV were sampled prior to HIV infection and compared with controls (n=55) who remained HIV-uninfected at the conclusion of the trial. All analyses were rigorously blinded. The primary endpoint was ex vivo entry of a CCR5-tropic, clade C HIV founder virus into blood CD4+ T cells. Secondary endpoints included HIV entry into CD4+ central (T_{CM}) and effector (T_{EM}) memory T cells, and into CD4+ T cell subsets expressing CCR5, CD69, CCR6, α4β1 or α4β7. Compared to bulk CD4+ T cells (4.9% virus entry), CD4+ T cells expressing CCR5, CCR6 or α4β1 and T_{EM} were highly susceptible (15.5%, 8.8%, 8.2% and 10.8% entry, respectively, all p<0.0001), while T_{CM} and CD4+ cells expressing CD69+ or α4β7+ were moderately susceptible (6.4%, 6.0% and 5.8% respectively, p ≤ 0.01). Within an individual, virus entry correlated with the proportion of highly susceptible (r>0.44, p<0.0001) but not with moderately susceptible cells. However, virus entry into blood CD4+ T cell subsets did not predict subsequent HIV acquisition in a multivariable model controlling for sexual behaviour and condom use (OR 0.92, 95% CI 0.77-1.11, p=0.4). In conclusion, the virus entry assay identified several highly susceptible CD4+ T cell subsets, but ex vivo HIV susceptibility of blood cells did not predict subsequent sexual HIV acquisition. Such studies may require mucosal sampling at the site of HIV exposure.
3.2 Introduction

There were approximately 1.4 million new HIV infections in sub-Saharan Africa (SSA) in 2014, most of which were acquired in women through receptive vaginal sex [12]. HIV transmission is very inefficient during receptive vaginal sex, with the per-contact risk ranging from 1/200-1/2000 sex acts [202]. This inefficiency likely reflects the effectiveness of various mucosal host immune defenses including an intact mucosal epithelium, cervical mucus, immune cells (neutrophils, macrophages, αβ and γδ T cells, dendritic cells, and others)[70], and innate antimicrobial peptides such as alpha defensins and LL-37 [223]. The risk of HIV transmission is enhanced by various factors including the viral load of the HIV-infected male partner, the presence of sexually transmitted infections (STI) in either partner, alterations in the vaginal flora that include bacterial vaginosis BV [58], and use of the injectable hormonal contraceptive Depo-Provera or DMPA [60,61]. While various host immune defenses are protective, an over-exuberant host immune response can be deleterious, as recent studies have shown that genital inflammation and/or an increased level of several anti-microbial peptides actually enhanced the risk of subsequent heterosexual HIV infection [93,94].

There are several potential mechanisms through which genital inflammation may increase HIV risk. Inflammation directly impairs the integrity of the genital epithelium [78], and also recruits HIV-susceptible CD4+ T cells to the genital mucosa, as well as other cell subsets that may enhance HIV acquisition [85,224]. An ex vivo HIV entry assay used to directly quantify virus entry into unstimulated endocervical CD4+ T cells recently described some of the CD4+ T cell parameters associated with enhanced cellular susceptibility [224]. These included the expression of CCR5 (HIV co-receptor), CD69 (early immune activation and tissue residence), and α4β7 and α4β1 integrins (T cell homing) [224]. Other putative correlates include CCR6 (Th17 cells) [225], and CD45RA and CCR7, which delineate memory CD4+ T cell subsets - T\textsubscript{EM} (CD45RA-CCR7), T\textsubscript{CM} (CD45RA- CCR7+), and T\textsubscript{NAIVE} (CD45RA+ CCR7+) [226].

While the expression of some of these parameters in blood may correlate with their expression in the mucosa [224], it is not clear whether assessing virus entry into blood CD4+ T cells is an adequate surrogate for the risk of sexual (mucosal) HIV acquisition. However, the relative ease of acquiring blood samples means that such an assay would be very useful for field studies of HIV prevention. Therefore, we performed a nested case-control study to compare HIV entry into
peripheral blood mononuclear cells (PBMCs) obtained from HIV-uninfected women in the CAPRISA 004 trial who subsequently acquired / did not acquire HIV infection.
3.3 Methods

3.3.1 Ethics Statement

The protocol for the CAPRISA 004 clinical trial (clinical trials registration NCT 00441298) and informed consent forms were approved by the University of KwaZulu-Natal, Ref: E111/06, the Protection of Human Subject Committee in the Office of International Research Ethics at FHI Ref: 9946, and the South African Medicines Control Council (MCC), Ref: 20060835.

3.3.2 Study Participants

The CAPRISA 004 study was a randomized, placebo-controlled clinical trial that demonstrated 39% efficacy of a tenofovir 1% vaginal gel in preventing heterosexual HIV acquisition [63]. PBMCs were cryopreserved at routine intervals after enrolment, and HIV testing was performed monthly throughout the trial. Our nested, case-control study selected PBMCs from cases acquiring HIV and controls that remained HIV-uninfected for 30 months. Cases and controls were matched for calendar month of enrolment, age (within a 5 year window) and study arm (tenofovir versus placebo).

3.3.3 BlaM-Vpr HIV pseudovirus assay and flow cytometry analysis

BlaM-Vpr HIV pseudovirions were prepared by transfection of HEK 293T cells with 20µg Q23ΔEnv, 10µg CAP45.2.00.G3 env (a CCR5-tropic sexually transmitted Clade C envelope), 10µg BlaM-Vpr and 5µg pAdvantage (Promega) as previously described [224,227]. Virus was harvested after 48h and concentrated 100 fold. Input virus used in ex vivo infections was equivalent to 50% of maximum infection obtained in a reference PBMC stock, thereby maximizing the dynamic range of the assay [224].

The virus infection assay was performed by research personnel blinded to HIV acquisition status using 100,000 input PBMCs/well [224]. To minimize day-to-day variation in sample processing, matched case-control samples were thawed on the same day in RPMI 1640 + supplemented with 10% FBS, rested in a 37°C, 50% CO₂ incubator for 3 hours, and infection assays were performed as described in Section 2.5 and elsewhere [224]. Surface staining of PBMCs was performed with fluorescently-labelled monoclonal antibodies that included CD3 Brilliant Violet (BV) 786, CD4
BV650, CCR5-PE-CF594, CCR7 BV605, CCR6 BV711, CD49d PE, integrin β7 PE-Cy5, and integrin β1 PerCP-eFluor 710 (BD Biosciences), CD69 PE-Cy7 (EBioscience), CD45RA APC-Cy7 (Biolegend) and LIVE DEAD far red (Invitrogen).

3.3.4 Statistical analysis

The *ex vivo* HIV infection assay and flow cytometry analysis was performed by study personnel rigorously blinded to HIV acquisition status; after finalization of virus entry data analysis, the dataset was sealed and the case-control code was broken to allow analysis of the immune associations of HIV acquisition. Flow cytometry data was analyzed using FlowJo X, exported into Microsoft Excel and further analyzed using PRISM6, SPSS (IBM) or SAS Version 9.4 on Mac OS. The impact of HIV entry on subsequent HIV acquisition risk was assessed using univariate and multivariate conditional logistic regression models. The sample size of 33 cases and 55 controls had 80% power to detect a 40% difference in median virus entry.
3.4 Results

3.4.1 Study participants

Among the 33 HIV-sero-converters (cases) and 55 HIV-non-sero-converters (controls) included in the study, demographic data were missing for 1 case and its two matched controls; these participants were excluded from analysis of the primary study endpoint, but were included in assessment of the immune correlates of virus entry. Among the 85 remaining participants, 21 cases and 42 controls were matched in a 1:2 ratio, and 11 cases were matched with 11 controls at a 1:1 ratio due to limited sample availability.

PBMCs from cases had been collected at a median of 110 days (IQR 65 - 182 days) prior to HIV acquisition. HIV sero-converters had a higher number of sex acts in the past month (median 8, IQR 4-12) compared to HIV non sero-converters (median 6, IQR 3-9), OR 1.16, 95% CI (1.01-1.33), p=0.03), but other demographic parameters were similar, including age, HSV-2 infection status, relationship status, vaginal discharge, DMPA use and condom use (Table 3-1).

### Table 3-1. Participant characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV sero-converters</th>
<th>HIV non sero-converters</th>
<th>OR, 95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years, (IQR)</td>
<td>21, IQR (20-24)</td>
<td>22, IQR (19-25)</td>
<td>0.98 (0.59-1.60)</td>
<td>0.92</td>
</tr>
<tr>
<td>Relationship status (stable)*</td>
<td>28 (88%)</td>
<td>51 (93%)</td>
<td>0.31 (0.06-1.72)</td>
<td>0.18</td>
</tr>
<tr>
<td>HSV-2 infected</td>
<td>19 (58%)</td>
<td>29 (53%)</td>
<td>1.32 (0.54-3.27)</td>
<td>0.99</td>
</tr>
<tr>
<td>median number of sex acts/last month (IQR)*</td>
<td>8 (3-9)</td>
<td>6 (4-12)</td>
<td>1.16 (1.01-1.33)</td>
<td>0.03</td>
</tr>
<tr>
<td>DMPA use</td>
<td>22 (67%)</td>
<td>45 (81%)</td>
<td>0.68 (0.26-1.83)</td>
<td>0.45</td>
</tr>
<tr>
<td>Vaginal discharge</td>
<td>12 (36%)</td>
<td>17 (31%)</td>
<td>1.31 (0.47-3.65)</td>
<td>0.6</td>
</tr>
<tr>
<td>Condom use (always)</td>
<td>3 (9%)</td>
<td>17 (31%)</td>
<td>2.40 (0.85-6.73)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Univariate analysis depicting the number (percentage) of participants in each group unless indicated otherwise. Data shows n=33 and 55 for HIV seroconverters and HIV non-seroconverters respectively, except for categories indicated with * (n=32, n=53) due to unavailable data.
3.4.2 Cellular correlates of ex vivo HIV entry

Compared to bulk CD4+ T cells (median virus entry 4.9%, IQR (3.0%-7.7%)), CCR5+ CD4+ T cells were highly susceptible to infection by the R5-tropic pseudovirus (median 15.5%, IQR (12.0%-19.4%), p<0.0001, Figure 3-1A-B, Table 3-2). Other highly HIV-susceptible subsets included T_{EM}, CCR6+ or \(\alpha 4\beta 1+\) CD4+ T cells (medians, 10.8%, IQRs (7.4%-14.4%), 8.8% (5.2%-12.2%), and 8.2% (5.2%-11.1%) respectively, all p<0.0001). Virus entry was also modestly increased into T_{CM}, CD69+ or \(\alpha 4\beta 7+\) CD4+ T cell subsets (medians 5.8%, IQRs (3.3%-9.1%), 6.4% (4.0%-10.8%), and 6.0% (3.4%-9.4%) respectively, p≤0.0004, Figure 3-1 A and B, Table 3-2). Naïve CD4+ T cells were substantially less susceptible to HIV than bulk CD4+ cells (median 0.9%, IQR (0.2%-2.4%), p<0.0001, Table 3-2).
Figure 3-1. Gating strategy for analysis of HIV entry and various blood-derived CD4 T+ cell subsets. Representative plots show gating on singlets, live cells, lymphocytes, and CD4+ T cells (top row, A) and virus entry (bottom row, A). In (B), representative plots show gating strategy for various CD4+ T cell subsets as indicated.

Table 3-2. HIV entry into blood CD4+ T cell subsets

<table>
<thead>
<tr>
<th>CD4+ T cell subset</th>
<th>% HIV entry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk</td>
<td>4.9% (3.0%-7.7%)</td>
</tr>
<tr>
<td>CCR5+</td>
<td>15.5% (12.0%-19.4%)***</td>
</tr>
<tr>
<td>TEM</td>
<td>10.8% (7.4%-14.4%)***</td>
</tr>
<tr>
<td>TCM</td>
<td>5.8% (3.3%-9.1%)**</td>
</tr>
<tr>
<td>T naïve</td>
<td>0.9% (0.2%-2.4%)***</td>
</tr>
<tr>
<td>α4β1+</td>
<td>8.2% (5.2%-11.1%)***</td>
</tr>
<tr>
<td>α4β7+</td>
<td>6.0% (3.4%-9.4%)*</td>
</tr>
<tr>
<td>CCR6+</td>
<td>8.8% (5.2%-12.2%)***</td>
</tr>
<tr>
<td>CD69+</td>
<td>6.4% (4.0%-10.8%)***</td>
</tr>
</tbody>
</table>

Wilcoxon matched pairs signed-rank test was applied to compare percent HIV entry into various indicated cell subsets versus bulk CD4+ T cells. Percent HIV entry is denoted as median (IQR). *, **, and *** indicate p values <0.01, <0.001 and <0.0001 respectively. N=88.

Within a given individual, the proportion of blood CD4+ T cells expressing markers of high HIV susceptibility (T_{EM}, CCR5+, CCR6+ or α4β1+ CD4+ T cells) was strongly correlated with the overall frequency of HIV pseudovirus entry into blood CD4+ T cells (r=0.60, 0.68, 0.47, and 0.67 respectively, p<0.0001 for all, Figure 3-2 A-D). However, virus entry did not correlate with the frequency of more moderately susceptible cell subsets (T_{CM}, α4β7+ or CD69+ CD4+ T cells; all p>0.05, Figure 3-2 B-D), and was inversely correlated with the frequency of T_{NAIVE} cells (r=-0.67, p<0.0001). Therefore, within an individual the overall HIV susceptibility of blood CD4+ T cells was closely correlated with the frequency of highly susceptible cell subsets.
Figure 3-2. Association between various putative correlates of HIV susceptibility and ex vivo HIV entry into blood-derived CD4+ T cells within an individual. N=88

Within an individual, the proportion of CCR5+ CD4+ T cells correlated with the frequency of highly susceptible cells (TEM, α4β1+ or CCR6+ CD4+ T cells, r=0.47, 0.66, and 0.45 respectively, p<0.0001 for all, Figure 3-3) but was either weakly correlated (TCM, r=0.27, p=0.01) or not correlated (α4β7+ or CD69+ CD4+ T cells r= -0.16 and r= -0.2, p>0.04 for both) with the frequency of cell subsets with moderate susceptibility (Table 3-2, Figure 3-3). These data discriminate cell subsets of varying susceptibility to HIV entry and suggest that within an individual, the overall susceptibility of a population of CD4+ T cells is strongly driven by the frequency of highly susceptible cells and by CCR5 expression.
Figure 3-3. Association between expression of CCR5+ CD4+ T cells and various putative correlates of HIV susceptibility. T_EM (blue) and T_CM (red) in (A), α4β1+ (blue) or α4β7+ CD4+ T cells (red) in (B), and CCR6+ (blue) or CD69+ CD4+ T cells (red) in (C).

3.4.3 No association of HIV entry into blood CD4+ T cells with sexual HIV acquisition

Baseline ex vivo HIV entry into blood CD4+ T cells did not differ between cases who subsequently acquired HIV (median 5.0%; IQR 2.7%-8.3%) and controls who remained persistently HIV-uninfected (median 4.8%; IQR 3.0%-7.7%) in univariate analysis (OR 1.02, 95% CI 0.8–1.16, p=0.82, Figure 3-4). This was also the case when the model incorporated previously-defined risk factors such as HSV-2 infection status, history of vaginal discharge, DMPA use, sex acts in the past month, and condom use (OR 0.92, 95% CI (0.77-1.11), p=0.4). Therefore, we conclude that ex vivo HIV entry into blood CD4+ T cells does not predict the subsequent risk of sexual HIV acquisition.
Figure 3-4. HIV entry into blood CD4+ T cells is not associated with HIV risk. Graph shows univariate analysis of percent HIV entry into blood CD4+ T cells of HIV-uninfected participants who subsequently acquired HIV (HIV sero-converters) or did not acquire HIV (HIV non-sero-converters, controls). The two groups were matched for age, study arm (tenofovir or placebo) and time after enrolment.
3.5 Discussion

An improved understanding of the correlates of HIV acquisition would inform HIV prevention efforts. In particular, an assay that could rapidly quantify mucosal (sexual) HIV susceptibility using blood would permit the identification of participants at a high risk of HIV acquisition for subsequent provision of HIV pre-exposure prophylaxis (PrEP), or for enrolment into HIV clinical or vaccine trials. Therefore we assessed the ability of a previously-validated rapid and sensitive HIV entry assay [224] to predict heterosexual HIV acquisition in high-risk South African women enrolled in the CAPRISA 004 clinical trial, using blood samples collected and stored prior to HIV acquisition. We found no evidence that virus entry into blood CD4+ T cells predicted subsequent heterosexual HIV acquisition, either in unadjusted analysis or in multivariable models controlling for sexual behavior, condom use and other risk factors.

The lack of association between blood virus entry and HIV risk in our study can be interpreted in several ways. First and foremost, compartmentalization of biological parameters that alter HIV susceptibility in the female genital tract (FGT) may render virus entry into blood CD4+ cells a poor proxy of heterosexual HIV risk. Some of these compartmentalized factors include the vaginal microbiota [58], genital infections [151], and the use of the injectable hormonal contraceptive Depo Provera [61,228,229], all of which have been associated with HIV acquisition; these and other compartmentalized parameters may reduce epithelial barrier integrity, enhance cell-cell HIV transmission and/or alter post-HIV entry events that are important determinants of persistent HIV infection after sexual exposure [76]. If this is the case, it follows that determination of HIV risk will require mucosal sampling from the compartment where HIV exposure occurs – in this case the female genital tract – although the collection of such samples is more difficult in a field setting. However, an alternative explanation for the lack of association between CD4+ virus entry and subsequent HIV acquisition is that virus entry alone may be an inadequate proxy for HIV susceptibility, since it does not consider the involvement of other infection-enhancing cell types such as dendritic cells, or consider downstream events such as proliferation and migration of infected cells. While our study cannot distinguish between these possibilities, the correct identification by the pseudovirus assay of CD4+ subsets known to be preferential targets in both blood and the cervix [224] suggests that the first explanation is the more likely reason for our negative findings.
Our study does have some limitations. The pseudovirus assay uses a higher viral inoculum than *in vivo* vaginal virus exposure, where infection generally involves the expansion of a single founder virus [68,230]. While this allows for robust detection of virus entry and maximizes the dynamic range of the assay, it is likely that some virus entry occurs in cell subsets that would remain uninfected after a lower dose physiologic virus challenge. Another limitation is that the cryopreservation and subsequent thawing of clinical samples used in this study may have preferentially depleted certain cell subsets. Indeed, cellular recovery was often suboptimal, as the number of live, mononuclear cells recovered were often < 20% of the number of stored cells. Future studies may test the impact of cryopreservation on depletion of cell subsets by comparing the frequency of various cell subsets in the live, T cell fraction. Lastly, this study had a relatively limited sample size, which was powered to detect a 40% difference in virus entry between cases and controls. While our study would not be able to detect more subtle differences in cellular susceptibility, not even a weak trend to enhanced case susceptibility was seen, suggesting that a larger sample size would not have altered our conclusions.

In summary, an *ex vivo* HIV entry assay identified highly-susceptible CD4+ T cell subsets in the blood of high-risk South African women. However, the level of virus entry into blood CD4+ T cells did not predict subsequent risk of heterosexual HIV acquisition, suggesting that sexual acquisition might be driven by compartmentalized mucosal parameters that may be captured by mucosal sampling.
Chapter 4

4 Impact of bacterial vaginosis treatment with metronidazole on genital immunology and HIV susceptibility


Submitting to: Science Translational Medicine

Overall objective: To assess the impact of standard therapy with metronidazole on genital immunology and HIV susceptibility

Specific objectives

1. To assess the impact of BV treatment on HIV entry into cervical CD4+ T cells

2. To assess the impact of BV treatment and clearance on genital immunology
4.1 Abstract

Bacteria vaginosis (BV) increases the risk of HIV acquisition by 50%, perhaps through increased genital inflammation. While population-based BV treatment has been suggested as an HIV prevention strategy, its impact on HIV susceptibility and genital immunology is poorly defined. We hypothesized that standard BV treatment would reduce CD4+ T cell HIV susceptibility and levels of genital pro-inflammatory cytokines and chemokines. In a prospective clinical trial, 45 BV-positive, HIV-uninfected Kenyan women were treated with oral metronidazole; blood and mucosal sampling was performed at baseline and 1 month. Primary endpoints were the frequency and number of HIV-susceptible cervical CD4+ T cells. Secondary endpoints included levels of various genital cytokines and chemokines. Treatment resulted in a moderate reduction in ex vivo CD4+ T cell HIV entry (median virus entry at baseline 9.1% vs. 6.4% post-treatment, p=0.02). BV resolved entirely (Nugent score ≤3) after treatment in 20/45 women. Effective treatment was associated with a reduction in genital pro-inflammatory cytokines IL-1α and IL-1β by >4 fold (p<0.01), but a marked increase in chemokines IP-10, MIG, MCP-1 (21, 6.5, and 8 fold; p<0.0001), MIP-3α and MIP-1α, and cytokines IFN-γ and GM-CSF (6.2, 1.5, 1·7 and 1·9 fold respectively; p<0.05); no changes were observed in women with persistent/recurrent BV. The degree of resolution of BV (based on Nugent score) was associated with the magnitude of changes in genital cytokines and chemokines. In summary, BV treatment reduced genital IL-1 and CD4+ T cell susceptibility to HIV. However, an unexpected increase in several chemokines previously associated with HIV risk suggests that more studies may be needed before the treatment of asymptomatic BV can be recommended for HIV prevention.
4.2 Background

Bacterial vaginosis is the most frequent cause of vaginal discharge in women of reproductive age, and has been associated with various obstetric complications and sexually transmitted infections (STIs) including an increased risk of HIV acquisition by >50% in prospective studies [58]. Microbiologically, diagnosis of BV is based on the Nugent score, an interpretation of a Gram’s stained vaginal swab that is based on numbers of lactobacilli, small gram-negative rods (bacteroides spp), small gram-variable rods (gardenella spp), and curved gram-variable rods (mobiluncus). A low Nugent score (0-3) reflects an abundance of lactobacilli with few gram negative/variable anaerobes, while a high score (7-10) is defined as BV and reflects low numbers of lactobacilli with frequent gram negative/variable anaerobes. A Nugent score between 4 and 6 is defined as intermediate vaginal flora (IVF), and women with either BV or IVF have an increased risk of HIV acquisition (>50%) [58]. The vaginal microbiota can also be defined based on sequencing of the 16s rRNA gene of the small ribosomal subunit and subsequently clustered into different community state types (CSTs) [165]. Women with BV have CSTs characterized by high bacterial diversity and an abundance of facultative and strict Gram negative anaerobes that include gardnerella, atopobium, prevotella, sneathia, BVAB 1-3, and mobiluncus spp [165,166], and we refer to this state of the microbiota as vaginal dysbiosis.

BV is associated with an increased incidence of bacterial STIs including *Neisseria gonorrhoea* (NG), *Chlamydia trachomatis* (CT) (both >1.7 fold increase) [176], as well as viral STIs such as HIV (a 1.5-fold increase) [58], HSV-2 (a 3-fold increase), and HPV (a 1.2-fold increase) [168]. BV not only increases the risk of HIV acquisition, but in an HIV-infected woman, the presence of BV increases the risk of HIV transmission to her male partner by 3 fold [171], independent of any effect of the genital HIV viral load. As a result, BV may be responsible for ~17% of heterosexual HIV transmission in sub-Saharan Africa (SSA) [143,171]. In keeping with this, a recent study from South Africa demonstrated that bacterial community state types with high diversity that had a dominance of gardnerella or various gram-negative anaerobes including prevotella, gardnerella, sneathia etc. were strongly associated with subsequent HIV seroconversion [231]. Similarly, the *L. iners* dominated CST also showed a strong trend towards HIV risk, though this was not statistically significant while *L. crispatus* dominated CST was associated with the lowest HIV risk. These and other data directly associate BV and BV associated gram-negative anaerobes with HIV risk and suggest the *L. iners* dominated VM may
also increase HIV susceptibility; however, studies demonstrating that effective BV therapy and/or prevention can reduce HIV incidence have not been performed. Such studies may be important to prove a causal role of BV in HIV acquisition, as well as to inform new clinical strategies to reduce HIV risk.

HIV is acquired across the genital mucosa during sex, and the genital immune correlates of HIV acquisition include the integrity of genital mucus and the epithelial barrier, the presence and nature of mucosal HIV target cells (CD4+ T cell and dendritic cell subsets), and genital levels of soluble pro-inflammatory cytokines and anti-microbial peptides such as cathelicidins and defensins [232]. Therefore, it is reasonable to assume that BV and/or bacterial dysbiosis may alter HIV risk through effects on some or all of these mucosal immune parameters. BV and vaginal dysbiosis were not associated with a higher number or proportion of genital CD4+ T cell or DC subsets in cross sectional studies [192,195], but were consistently associated with elevated pro-inflammatory cytokines, particularly IL-1α and IL-1β [86,166,192], perhaps through LPS-sensing and activation by genital epithelial and dendritic cells [192]. In addition, increased bacterial diversity [166], microbiota dominated by gardnerella spp [233], and genital inflammation itself (based on elevated pro-inflammatory cytokines) [85] are associated with an overlapping genital proteomic signature indicative of epithelial barrier disruption, suggesting that BV-associated microbiota may also disrupt the epithelial barrier integrity. Indeed, pro-inflammatory cytokines directly reduce epithelial integrity in vitro [78,234], and genital inflammation in vivo is associated with increased mucosal HIV target cells [85,224] and increased HIV acquisition [94]. Based on these findings, screening and population-level treatment of asymptomatic BV with metronidazole was recently recommended to reduce new HIV infections [231], although to date no studies have demonstrated that BV therapy/prevention can prevent HIV infection either in vivo or ex vivo. Therefore, the purpose of the current clinical trial was to define the impact of standard vaginal dysbiosis treatment on genital immunology and ex vivo HIV susceptibility of genital CD4+ T cells.
4.3 Methods

4.3.1 Ethics statement

Informed written consent was obtained from all participants prior to enrolment, and the study was approved by Institutional Review Boards at the Kenyatta National Hospital (Nairobi, Kenya) as well as the Universities of Manitoba and Toronto (Canada). The described studies were conducted according to the principles expressed in the Declaration of Helsinki.

4.3.2 Study Design

The study was powered ($\beta=0.8$) to detect a conservative 15% difference in virus entry into cervical CD4+ T cells after BV treatment since the change in ex vivo HIV pseudovirus entry that would achieve a clinical benefit was not known; this required the enrolment of 50 participants. Co-primary study endpoints for this clinical trial (registration NCT02527941) were the frequency and number of ex vivo HIV-infected cervical CD4+ T cells. Secondary endpoints included vaginal pH and the levels of genital cytokines and chemokines, some of which were previously known to associate with HIV acquisition in prospective studies.

Participants from Nairobi county were recruited into this clinical trial. At screening, informed consent was administered and interested participants answered a short questionnaire to determine study eligibility. HIV-uninfected participants who were BV-positive (Nugent score $\geq 7$) were contacted for potential enrolment. Inclusion criteria for study enrolment was female, age 18-49, and Nugent score $\geq 7$ at screening. Exclusion criterion were current pregnancy, prior hysterectomy, genital ulcer, active menstruation, STI-positive for N. gonorrhea (NG), C. trachomatis (CT), syphilis, or T. vaginalis (TV), contraindication, allergy or intolerance to metronidazole, and participants deemed unfit by study staff to comply with study requirements. Blood and mucosal sampling procedures were performed at the enrolment visit and participants were then offered metronidazole (400mg po tid) for 7 days and followed approximately 28 days after treatment initiation.

In participants reporting a regular menstrual cycle (21-35 days), the baseline visit was scheduled either in the follicular phase of the menstrual cycle (defined as two days after the last day of the next menstrual bleeding) or the luteal phase (7 days prior to the projected first day of the next bleeding), which occurred soonest, and follow-up occurred at the same point in their next
menstrual cycle. Participants not having a regular menstrual cycle due to use of a long-acting hormonal contraceptive were followed up 28 days after enrollment. A short enrollment questionnaire captured a range of socio-economic, behavioral and sexual information.

4.3.3 HIV, BV, and STI diagnostics

Urine was collected for diagnosis of NG and CT by PCR (Xpert CT/NG, Sunnyvale, CA, USA). Vaginal swabs were collected from the posterior fornix of the vagina for Gram’s stain and for TV testing using OSOM Trichomonas rapid test (Sekisui, San Diego, USA). HIV testing and counseling was provided according to Kenyan national guidelines (KHB, Shanghai Kehua Bio-engineering Co Ltd, China), and positive results were confirmed by HIV 1/2 STAT-PAK (Chembio Diagnostics, USA). Discordant results were clarified by a third test, Unigold HIV (Trinity Biotech, Ireland). Syphilis was diagnosed by serology (Diagnostics Worldwide, Brooklyn, NY, USA).

4.3.4 Mucosal and blood sampling

Mucosal samples were collected in the following order: urine, cervico-vaginal secretions (CVS), vaginal swabs, one endocervical swab and two endocervical cytobrushes. Urine and vaginal swabs were used for STI testing (see above). One vaginal swab from the posterior fornix was used to determine the pH using a pH strip with 0.4 log unit gradations (Macherey Nagel). Undiluted CVS were collected using an Instead Softcup (Evofem, San Diego, CA) for 2 min, stored on ice and processed within 6 hours. One endocervical swab was collected and immediately stored in native form at -80°C. Additionally, endocervical cytobrush sampling was performed by gentle insertion of a cytobrush into the cervical os, rotation through 360°, and placement into R10 complete medium (RPMI 1640 with 10% heat-inactivated FBS and antibiotic/antimycotic cocktail containing clindamycin, streptomycin, polymyxin B, amphotericin B, and gentamycin), all from Sigma-Aldrich, Carlsbad, CA. A 2nd endocervical cytobrush was collected in the same manner and both stored at 4°C and processed within 4h. Cells from the two cytobrushes were eluted, combined, passed through a 100-µm filter, washed, and divided into two equal aliquots for use in the virus entry assay.
4.3.5 HIV entry assay

The β-lactamase-vpr HIV entry assay has been described in detail elsewhere [224]. Briefly, BlaM-Vpr pseudovirions were produced by transfection of 293T cells with 20µg HIV backbone lacking envelope - Q23Δenv, 10µg early-transmitted R5-tropic Clade A envelope - Q259d2.17 [212], 10µg pCMV-BlaM-Vpr (Addgene, Cambridge, MA, USA), 5µg of pAdvantage (Promega, Madison, WI, USA) and 135µl of the transfection reagent polyethyl-imine (Polysciences, Warminster, PA, USA). Fourty-eight hours post-transfection, supernatant was collected, filtered, and concentrated 100 fold using PEG6000 [235]. Input pseudovirus used for assessment of ex vivo HIV entry was equivalent to approximately 60% of maximum viral entry in reference PBMCs obtained from an HIV-uninfected donor.

For ex vivo infections, processed cervical cell suspension from each participant was divided in two and incubated with either BlaM-vpr pseudovirus or mock. Samples were spinoculated at 1200xg for 2h at 17°C to allow virus interaction with cells, followed by a 2h incubation at 37°C, 5% CO₂ to allow cellular virus entry [137]. Samples were washed twice in CO₂-independent media (Invitrogen, Carlsbad, CA, USA), loaded with 1µM CCF2-AM (Invitrogen) for 1.5h, washed again and incubated for 12h in CO₂-independent media supplemented with 10% FBS, antibiotic cocktail and solution D (Invitrogen), an anion transport inhibitor to prevent leakage of CCF2-AM dye. Samples were then stained with fluorescent-labelled antibodies (as described below) and analyzed using a BD LSR-II Flow Cytometer (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA).

4.3.6 Flow cytometry

Surface staining of cells with labelled monoclonal antibodies was performed for 30 min at 4°C. Antibodies used for staining were CD49d (integrin α4) -PE, integrin β7-PE-Cy5, CD3-AF700, CCR5-APC-Cy7, (Becton Dickinson Bioscience), LIVE DEAD Far Red (Invitrogen), CD4-ECD (Beckman Coulter, Brea, CA, USA), and CD69-PE-Cy7 (EBioscience, San Diego, CA, USA). Samples were acquired and analyzed using a BD LSR-II Flow Cytometer (Becton Dickinson Bioscience).
4.3.7 Cytokine assays

Cervico-vaginal secretions (CVS) were diluted 10-fold in phosphate-buffered saline (PBS) and stored at −80°C. Levels of 15 cytokines and chemokines including IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, IL-17, IFN-γ, TNF-α, MIP-1α, MIP-1β, MIP-3α, MCP-1, RANTES, and IP-10 were assessed using an electro-chemiluminescence ELISA platform (Meso Scale Discovery, Rockville, MD) by a blinded technician (Kamnoosh Shahabi), and were corrected for dilution to obtain a concentration in undiluted genital secretions [85]. CVS obtained from a given individual pre- and post-metronidazole treatment was analyzed on the same ELISA plate to eliminate plate-to-plate variation in cytokine measurements. Lower limit of detection (LLOD) values were determined based on the standard curve and were the lowest detectable value that had a coefficient of variation (CV) ≤ 30%.

4.3.8 Statistics

The difference in percent HIV entry and percent expression of various cell surface markers in CD4+ T cells before and after BV treatment was assessed using the Wilcoxon-matched pairs signed-rank test. All statistical tests were conducted using SPSS V.23 (Armonk, New York, USA) or Prism 6 (La Jolla, CA, US) on Mac OS X. Flow cytometry data were analyzed in FlowJo v. 10.6 (Ashland, OR, USA). With an expected sample size of 20 who would clear BV after treatment, we had 80% power to detect a 22% difference in virus entry into endocervical CD4+ T cells.
4.4 Results

4.4.1 Screening and enrolment results

The study screened 572 Kenyan women between 10-Aug – 12-Nov-2015; of these 166 tested positive for BV (Nugent score ≥7), and 50 women were enrolled into the study (Figure 4-1). Five participants (10%) did not complete the study: 1 chose not to attend the last study visit, and 4 were excluded due to STI acquisition during follow-up (2 CT, 1 NG and 1 TV), so that 45 participants completed the protocol and subsequent analysis was restricted to these 45 women.

Figure 4-1. Flowchart depicting screening and enrolment statistics. NG = Neisseria gonorrhoea, TV = Trichomonas vaginalis. BV is defined as Nugent score ≥ 7.

4.4.2 Patient demographics

Demographic data for the 45 study participants completing the study are shown in Table 4-1. The median participant age was 26 years, all participants were sexually active and just under half
were married. Roughly half of study participants had a regular menstrual cycle (21-35 days), and the remaining participants were using a long-acting hormonal contraceptive and so were not cycling. The use of hormonal contraceptives was reported by 22/45 (49%) of participants, most commonly DMPA (11/45, 24.4%) and levonorgestrel implant (10/45, 22.2%), or oral contraceptive (1/45, 2.2%) being less common. Over half of participants reported at least one episode of intra-vaginal cleaning in their lifetime, a third reported daily intra-vaginal practices. Approximately a third of participants reported vaginal symptoms including odor, abnormal discharge or irritation.

Table 4-1. Participant characteristics at study enrolment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (%)</th>
</tr>
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<tbody>
<tr>
<td>Age, y, median (range)</td>
<td>26 (19-46)</td>
</tr>
<tr>
<td>Currently married</td>
<td>18 (40%)</td>
</tr>
<tr>
<td>Sexually active (past 12 months)</td>
<td>45 (100%)</td>
</tr>
<tr>
<td>&gt;1 sexual partner in the past year</td>
<td>9 (20%)</td>
</tr>
<tr>
<td>HSV-2 infected</td>
<td>23 (51%)</td>
</tr>
<tr>
<td>Regular menstrual cycle</td>
<td>24 (53%)</td>
</tr>
<tr>
<td>Long-acting hormonal contraceptive use*</td>
<td>21 (47%)</td>
</tr>
<tr>
<td>Vaginal symptoms (physician examined)</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Vaginal symptoms (self-reported or physician examined)</td>
<td>14 (31%)</td>
</tr>
<tr>
<td>Intra-vaginal practices (ever)</td>
<td>25 (56%)</td>
</tr>
<tr>
<td>Intra-vaginal practices (daily)</td>
<td>14 (31%)</td>
</tr>
<tr>
<td>Ever had sex</td>
<td>45 (100%)</td>
</tr>
<tr>
<td>Recent sex**</td>
<td>9 (20%)</td>
</tr>
</tbody>
</table>

Data are number (%) of participants, unless indicated otherwise. Intra-vaginal practices was defined as the use of soap, cloth, bleach, drying agent, herbal product or detergent. * long-active hormonal contraceptive use includes self-reported DMPA use within past 3 months or subdermal implant within the past 5 years. ** recent sex was defined as vaginal sex within the past 3 days. Vaginal symptoms include abnormal discharge, odor, or irritation. Regular menstrual cycle is defined as 21-35 days. N=45 for enrolled participants.
4.4.3 Clinical outcomes after metronidazole treatment

The median time between screening and enrolment was 6d, IQR (4-11d). All 45 enrolled participants who completed the study were BV-positive at screening, however, at the enrolment visit some participants had spontaneously cleared BV prior to the initiation of metronidazole, so that 33/45 (73.3%) women were BV-positive (Nugent scores ≥ 7), 9/45 (20%) had intermediate vaginal flora (Nugent score 4-6), while 3/45 (6.7%) were BV-negative (Nugent score ≤ 3, Figure 4-1). All 45 participants with BV at screening included in the primary study analysis, but some sub-analyses excluded participants who spontaneously cleared BV (Nugent score between 0-3) at enrolment, since women with Nugent score >3 have an increased risk of HIV acquisition. All enrolled participants reported completing one week of oral metronidazole and the median time between the enrolment visit after which metronidazole treatment was initiated and follow-up was 28 days (IQR 27-29). At follow-up, 20/45 (44%) had completely cleared BV (Nugent score ≤3), 7/45 (16%) had intermediate vaginal flora (Nugent score 4-6), and 18/45 (40%) participants had persistent /recurrent BV (Nugent score ≥7).
Figure 4-2. Representative flow cytometry plots show gating strategy for *ex vivo* HIV entry into endocervical CD4+ T cells. Gating on lymphocytes, single cells, live cells, CD3 and CD4 double-positive cells in (A), blue (cleaved CCF2-AM) versus green (uncleaved CCF2-AM) signal in mock or BlaM-vpr HIV pseudovirus-treated endocervical specimens in (B), and expression of CCR5+, CD69+, α4β7+ or α4β7− (or α4β1+) CD4+ T cells in (C).

4.4.4 Impact of metronidazole treatment on *ex vivo* HIV susceptibility and genital immunology

Metronidazole treatment significantly reduced the proportion of cervical CD4+ T cells infected by HIV in the *ex vivo* pseudovirus assay, our co-primary study endpoint, with the median HIV entry falling by 36% (pre-treatment median 9.1% (IQR 4.9%-15.1%) vs. post-treatment median 6.7% (IQR 3.4%-9.9%), n=45, p=0.02, Figure 4-2, 4-3A); there was no impact on the number of
ex vivo infected cervical CD4+ T cells (Figure 4-3 B). BV clearance was not associated with a reduction in virus entry: pre-treatment median 37.9%, IQR (21.7% - 55.0%) vs. post-treatment median 35.0%, IQR (23.4%-42.8%), n=19, p=0.47, Figure 4-4). In analysis stratified for Nugent score after treatment, virus entry declined in 13/18 (72%) participants with low Nugent scores (0-3), 5/7 (71%) of participants with intermediate Nugent scores (4-6), and 12/20 (60%) of participants with high Nugent scores (7-10); and overall, the decline in virus entry occurred across all groups and was not specifically associated with the efficacy of BV treatment ($X^2 = 0.7$, p=0.7).

Figure 4-3. Treatment of BV-positive women with metronidazole causes a moderate reduction in ex vivo HIV entry into endocervical CD4+ T cells. Graphs show percent (A) or number (B) of ex vivo HIV infected CD4+ T cells before (pre) and after (post) metronidazole treatment in all study participants. Scatterplots are used for easy visualization although all analyses were done by paired comparisons using the Wilcoxon matched-pairs signed rank test. N=45.
Figure 4-4. No impact of BV clearance on HIV susceptibility of endocervical CD4+ T cells. Graph shows the frequency of *ex vivo* HIV infected CD4+ T cells before (Pre) and after (Post) metronidazole treatment in participants with BV at baseline who subsequently cleared BV. Analysis was performed using the Wilcoxon matched-pairs signed rank test. N=19.

Consistent with overall changes in the Nugent score, metronidazole treatment resulted in a reduction in vaginal pH (median 5.1 (IQR 4.7-5.5) vs 4.7, (IQR 4.3-5.5), p=0.007, Figure 4-5A), which was driven specifically by women who had cleared BV (median 5.5 (IQR 5.1-5.5) vs. 4.3 (IQR 4.3-4.7), p=0.0001), but was not apparent in women whose Nugent scores remained ≥ 7 (median 5.1 (IQR 5.1-5.5) vs. 5.1 (IQR 4.7-5.5), p=0.49, Figure 4-5B). Women who had intermediate vaginal flora (Nugent score 4-6) at follow-up showed a reduction in vaginal pH, although this was not statistically significant (pre-treatment median 5.1, IQR (4.7-5.5) vs 4.7, IQR (4.3-5.1), p=0.4, n=7).
4.4.5 BV clearance dramatically alters genital cytokine and chemokine levels

Metronidazole treatment substantially reduced genital levels of IL-1α (1.8 fold; p<0.01) while increasing IL-17, IP-10, MIG, MCP-1, and GM-CSF (2.2, 6.2, 3.2, 1.4, and 1.6 fold; all p<0.05, Figures 4-6 and 4-7, left panels). Interestingly, these differences were only apparent in women who completely cleared BV (Nugent score 0-3), with no apparent changes in women whose Nugent scores remained ≥7. Women who cleared BV after treatment had a >4 fold reduction in IL-1α and IL-1β (p<0.01 for both, Figure 4-6B and D), but substantial increases in the chemokines IP-10, MIG, MCP-1 (21, 6.5, and 8 fold; p<0.0001), MIP-3α, and MIP-1α (6.2 and 1.5 fold; p<0.05), and cytokines IFN-γ and GM-CSF (1.7 and 1.9 fold; p<0.05, Figure 4-7 A-H, right panels). In contrast, women who had recurrent/persistent BV at follow-up had no changes in the levels of any cytokines or chemokines that were assessed (Figure 4-6 B and D, Figure 4-7, right panels). In summary, complete resolution of vaginal dysbiosis caused marked alterations in genital cytokines and chemokines.
Figure 4-6. Reduction in genital pro-inflammatory cytokines IL-1α and IL-1β after BV clearance following metronidazole treatment. Graphs show log-transformed values of IL-1α (A and B) and IL-1β (C and D) before (Pre) and after (Post) metronidazole treatment in women who were BV-positive at baseline. In (A) and (C), all 45 BV-positive participants who completed the study are included. In (B) and (D), analysis is stratified according to Nugent score after metronidazole treatment as indicated. Scatterplots are used for ease of visualization of data (left panels) although all analyses were done using the Wilcoxon matched-pairs signed rank test. NS = not significant. N=19 in BV cleared (Nugent score ≤ 3), n=17 in BV not cleared (Nugent score ≥ 7) groups.
Figure 4-7. Marked changes in genital cytokines and chemokines after BV clearance following metronidazole treatment. Graphs show log-transformed cytokine and chemokine levels in BV-positive at baseline (Pre) and after treatment with metronidazole (Post). Scatterplots are used for ease of visualization of data (left panels) although all analyses were done using the Wilcoxon matched-pairs signed rank test. N=45 for analysis of all participants in panels on the left. For analysis stratified according to Nugent score after BV treatment (right panels), n=19 in BV cleared (Nugent score ≤ 3) and n=17 in BV not cleared (Nugent score ≥ 7) groups. * indicates p<0.05, ** p<0.01, ***<0.0001, NS = not significant.
Six women had intermediate vaginal flora after BV treatment, however, due to this limited sample size, longitudinal assessment using paired comparisons would not prove meaningful. Therefore, for these participants, we calculated the fold change in various genital cytokines and chemokines at follow-up compared to baseline, and compared these values with women who completely cleared BV or did not clear BV. Women with intermediate flora after treatment had moderate fold changes in chemokines (IP-10, MIG, MIP-1α, MIP-3α and MCP-1) and the cytokine IL-1β after metronidazole treatment compared to metronidazole-treated women who completely cleared BV (Nugent score 0-3) and those who did not (Table 4-2). Collectively, these data demonstrate that the degree of resolution of vaginal dysbiosis is associated with the magnitude of changes in genital inflammatory cytokines and chemokines; BV clearance after treatment causing dramatic alterations in these factors, change to an intermediate flora generally having moderate effects, and BV persistence/recurrence being associated with the lack of any apparent effect.

Table 4-2. Fold change in genital cytokines and chemokines after BV treatment.

<table>
<thead>
<tr>
<th>Vaginal flora after BV Tx</th>
<th>Fold change in cytokine, IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1α</td>
</tr>
<tr>
<td>BV-negative</td>
<td>0.2</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.6</td>
</tr>
<tr>
<td>BV-positive</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Data are stratified according to Nugent score after treatment. BV-negative, intermediate and BV-positive refer to Nugent scores 0-3, 4-6, and 7-10 respectively. IQR= Interquartile range. Tx = treatment with oral metronidazole. N=19, 6, and 17 for BV-negative, Intermediate and BV-positive groups respectively.

The concentration of IL-10 and TNF-α was below the lower limit of detection (LLOD) for a significant proportion of participants (70% and 49% respectively). Hence, these data were categorized as detectable (> LLOD) or undetectable (< LLOD); however, metronidazole treatment was not associated with percent detectable TNF-α or IL-10 (n=45, p=1.0 for both). Baseline levels of genital cytokines and chemokines were not associated with subsequent BV clearance or persistence/recurrence. Finally, since the number of participants using long-acting hormonal contraception versus those who were regularly having a menstrual cycle were equal, we assessed whether immunological changes differed between the two groups, but did not find any differences.
4.4.6 Impact of metronidazole treatment on putative T cell correlates of HIV susceptibility

Similar to changes in virus entry, we also observed a reduction in the proportion of CD69+ CD4+ T cells after metronidazole treatment (median 45.0%, IQR (23.1% - 55.9%) vs. median 36.8%, IQR (25.0% - 47.4%), p=0.03, **Figure 4-8A**), though not in their number (median 64, IQR (20-141) vs median 56, IQR (15-99), p=0.7, **Figure 4-8B**). In analysis stratified for Nugent score after treatment, the frequency of CD69+ CD4+ T cells declined in 14/18 (77%) participants with high Nugent scores (7-10), 5/7 (71%) of participants with intermediate Nugent scores (4-6), and 13/20 (60%) of participants with low Nugent scores (7-10); and overall, this decline occurred across all groups and was not associated with the efficacy of BV treatment ($\chi^2 = 0.8$, p=0.7). BV treatment did not cause changes in other T cell parameters such as the percent or the number of CD4+ T cells/cytobrush (median 154, IQR (68-307) pre-treatment vs. median 139, IQR (58-267) post-treatment) or specific T cell subsets (CCR5+, α4β7+, or α4β1+ CD4+ T cells, **Figure 4-2C and 4-9**).

**Figure 4-8. Reduction in the proportion of endocervical CD69+ CD4+ T cells after BV treatment.** Graphs show the frequency (A) and number (B) of CD69+ CD4+ T cells before (Pre) and after (Post) metronidazole treatment. Scatterplots are used for ease of visualization of data although all analyses were done using the Wilcoxon matched-pairs signed rank test. NS = not significant. N=45.
Fig. 4-9. No effect of BV treatment on the frequency or number of CCR5+, α4β7+, or α4β1+ CD4+ T cells. Graphs show the frequency (A, C, E) or number (B, D, F) of various *ex vivo* HIV infected CD4+ T cell subsets as indicated prior to (Pre) and after (Post) metronidazole treatment in all study participants with BV at baseline. Scatterplots are used for ease of visualization of data although all analyses were done using the Wilcoxon matched-pairs signed rank test. N=45.
4.5 Discussion

BV increases HIV acquisition risk. This enhanced susceptibility is likely due to the local induction of pro-inflammatory cytokines, which may not only directly mediate epithelial disruption [78,85,166], but also recruit activated CD4+ T cells that constitute the primary HIV mucosal target cell after sexual exposure [85,224]. While it seems intuitive that BV treatment and/or prevention would reduce HIV risk, vaginal dysbiosis generally recurs quickly after standard clinical treatment (generally oral metronidazole), and intermittent provision of metronidazole did not reduce HIV incidence in a large community-based trial [236]. In order to assess the impact of BV treatment on mucosal immune parameters known to enhance HIV susceptibility in the female genital tract, in this study, HIV/STI-uninfected, non-pregnant Kenyan women with BV were provided standard treatment (one week of oral metronidazole), and the impact of therapy on genital immunology and HIV susceptibility was assessed after 1 month. BV treatment caused a moderate reduction in the frequency of cervical CD4+ T cells susceptible to HIV using an ex vivo HIV pseudovirus assay. Moreover, BV therapy caused a significant reduction in genital levels of the prototypical pro-inflammatory cytokine IL-1β, a reduction that was only apparent in women who responded to metronidazole treatment by normalizing their vaginal flora. However, while these results would strongly suggest that BV-therapy should reduce HIV susceptibility, BV clearance also caused a dramatic increase in several cytokines and chemokines that have been directly linked to HIV/SIV acquisition in both human and primate studies [90,94,104], suggesting that further studies of the effect of BV treatment on mucosal immunology and/or actual HIV acquisition are indicated before this can be recommended as an HIV prevention tool.

Emerging data from human cohort studies demonstrate that vaginal community state types dominated by BV-associated bacteria (gardnerella, prevotella, sneathia etc.) [231], or by gram-negative and gram-positive anaerobes (prevotella spp. and peptostreptococci) in the penile foreskin (Liu, personal communication), are key predictors of HIV acquisition. Based on such data, the screening and treatment of asymptomatic BV with metronidazole was recently recommended as an HIV prevention strategy in South Africa [231]. Our study directly addresses the implications of standard BV treatment on genital immunology and HIV susceptibility, and potential benefits of effective treatment were clearly observed. Firstly, the observed reduction in
pro-inflammatory cytokines IL-1α and IL-1β as a result of effective BV treatment was consistent with the association between vaginal dysbiosis and increased pro-inflammatory cytokines in women from SSA [166,192]. Reduced genital IL-1 may have beneficial effects on the epithelium, as this pro-inflammatory cytokine induces epithelial disruption in vitro [234] and is associated with vaginal dysbiosis, bacterial diversity and a mucosal proteomic signature of epithelial barrier disruption [166]. Furthermore, reduced genital IL-1 was associated with the lack of HIV infection among HIV-exposed seronegative (HESN) commercial sex workers in Kenya [90], suggesting that IL-1 reduction through effective BV treatment might reduce HIV susceptibility by enhancing epithelial integrity. Another consistently observed benefit is a ~1 log reduction in vaginal pH after effective BV clearance. Acidic vaginal pH may reduce HIV susceptibility through the virucidal effect of lactic acid on HIV [181], and it may also alter the surface properties of cervico-vaginal mucus and enhance virus trapping [79] to reduce HIV access at the epithelial barrier.

Dysbiosis-induced elevation in pro-inflammatory cytokines may recruit mucosal CD4+ T cells that are immunologically activated and highly susceptible to HIV. Although we observed a moderate and significant reduction in endocervical CD4+ T cell susceptibility to HIV after metronidazole treatment, the mechanism for this reduction is less clear than for the observed changes in cytokines. Specifically, there was an overall reduction in cellular virus entry and in the frequency of mucosal CD69+ CD4+ T cells that was not associated with the efficacy of BV treatment. These data are consistent with observational studies that have found minimal differences in the number or proportion of endocervical T cell subsets in women with vaginal dysbiosis [192,195], and the mechanism(s) underpinning the changes seen may include an overall reduction in bacterial load, or of specific species after BV treatment, and these will be the subject of future investigation based on 16s rRNA deep-sequencing.

The potential for clinical benefit of BV treatment must be tempered by the fact that successful BV clearance led to a striking increase of several cytokines and chemokines, including IL-17, IFN-γ and the γ-inducible chemokines IP-10, MIP-3α, MIG, and MCP-1. The potential impact of these increases on HIV susceptibility is not clear. An increase in IFN-γ and the γ-inducible chemokines may trigger a broad state of pathogen alert and antiviral host defense towards HIV-infected cells through the recruitment, activation, and/or maturation of immune cells including T
cells, NK cells, dendritic cells and neutrophils [82,83]. Elevated IL-17 may potentiate the effects of the IFN-γ response through the production of chemokines (IP-10, MIG, MIP-3α and MIP-1α) and antimicrobial peptides by epithelial cells, as well as through improved epithelial barrier repair [115,237]. However, genital IP-10 and MIP-1α levels in South African women correlated with increased HIV risk [94], as did genital IL-8 and MIG levels in Ugandan men [238], and lower genital levels of IP-10 and MIG were associated with reduced HIV susceptibility in Kenyan women [90]. The underlying mechanism(s) are not well understood, but these chemokines are potent T cell chemoattractants, and their genital levels have been associated with an increased density of highly activated HIV target cells in the genital mucosa [74,239]. While the number of endocervical CD4+ T cells did not increase in conjunction with these chemokines after BV treatment in our study, women were only assessed one month after treatment; future studies might perform a more comprehensive assessment of genital cells obtained from several sites along the FGT at multiple time points after metronidazole treatment. Overall, successful BV therapy induced very substantial changes in multiple genital immune parameters, and on the face of it, these changes might be expected to have opposing effects on HIV susceptibility. Therefore, additional clinical studies may be warranted before the treatment of asymptomatic BV can be recommended as an HIV prevention measure at a population or community level.

Despite these clear effects on BV therapy on genital immunology, our study does have certain limitations. We assessed the impact of BV treatment on HIV pseudovirus entry, but could not simultaneously characterize the number or functionality of other important immune cell subsets, including Th17 and Th1 cells, γδ1 T cells, DCs, macrophages, or neutrophils. Th17 and Th1 cells are important sources of IL-17 and IFN-γ respectively, and γδ1 T cells may be especially important for BV pathogenesis, as they are significant producers of IL-17, are abundant in the endocervix [240], and their numbers are substantially reduced in women with BV and in HIV-infected individuals [240,241]. Therefore, a more comprehensive assessment of the aforementioned immune cells should be performed after BV treatment in future studies, including the impact of elevated chemo-attractant cytokines/chemokines on the mucosal influx of various immune cell populations.

In summary, this study has important implications for HIV prevention. BV treatment appears to have certain benefits through reduced cellular susceptibility to HIV and genital IL-1 and vaginal
pH. However, the upregulation of a broad range of cytokines and chemokines, some previously associated with HIV risk suggests that a more nuanced understanding of the effects of BV treatment is necessary before rollout of asymptomatic BV treatment is recommended as an HIV prevention strategy.
Chapter 5

5 Discussion and future directions

5.1 Contextualization of my thesis in the framework of HIV prevention

The scale up and increased provision of ARTs worldwide has altered the nature of HIV/AIDS disease from a fatal and devastating illness to a chronic and manageable disease. However, people living with HIV, particularly in SSA still face barriers including limited or inconsistent access of ARTs, adherence to lifelong treatment, and the stigma of living with HIV and urgent strategies to prevent new HIV infections are needed. To this end, studies are ongoing to understand how biological and/or behavioral factors including vaginal douching, STIs, bacterial vaginosis, or the use of hormonal contraceptives such as DMPA influence HIV acquisition and will be instrumental in determining which behavioural modifications or clinical interventions can reduce new HIV infections.

Assessment of genital immunology and HIV susceptibility in human studies has historically been performed by measuring soluble cytokines in genital secretions, cellular correlates of susceptibility such as expression of CCR5 or CD69, or by measuring ex vivo HIV replication as an endpoint. The key gap in the field was a lack of an assay that could rapidly assess ex vivo HIV susceptibility in a sensitive manner that did not require in vitro cellular activation, and used a minimally invasive technique for participants in clinical studies. I chose to establish the β-lactamase-vpr HIV entry assay as it satisfied these conditions, and offered an intermediate endpoint to assess HIV susceptibility in settings with low HIV prevalence. To validate this assay, I demonstrated that cervical cells were more susceptible to HIV than blood and that the HIV pseudovirus preferentially infected cells (activated CCR5+ CD4+ T cells) that were previously characterized HIV targets. This observation is important for assay validation as the frequency of activated CCR5+ CD4+ T cells was directly associated with early viremia and SIV acquisition in macaques [99]. Then, I applied the assay to characterize novel HIV targets. I demonstrated that α4β7+ or α4β1+ CD4+ T cells were also preferential HIV targets of HIV entry; however, they were not HIV entry receptors as was previously suggested, and this directly informed the mechanisms through which the two integrins may influence in HIV susceptibility. After the
completion of assay validation, I have also been involved in projects in the lab that were led by my colleagues; these aimed to identify other novel HIV target cells subsets in mucosal tissue and to address the impact of clinical interventions on HIV susceptibility.

The next question to address was whether virus entry could predict real life HIV acquisition. Performing this study using genital samples would be ideal; however, these samples were not available. Moreover, the more practical application of the virus entry assay was to test this question using blood samples as they can easily be obtained in the field to identify individuals at high risk of HIV for potential enrolment in clinical trials. However, I discovered that virus entry in blood could not predict subsequent genital HIV acquisition. This probably relates to compartmentalized differences in immune parameters between blood and genital tract and the most direct way to prove this would be to do a prospective study in real time using cervical samples. It also became clear that using mucosal tissue was necessary to address the impact of compartmentalized (genital) factors on genital HIV risk.

One such compartmentalized factor was the vaginal microbiota. Based on observational studies that showed that the abundance of particular BV-associated bacteria increased HIV risk, treatment of asymptomatic BV was recently recommended to reduce HIV risk. However, the impact of standard treatment on genital immunology and HIV susceptibility was not defined for HIV-uninfected non-pregnant women. To address this gap in knowledge, I treated women with BV at baseline using standard therapy (oral metronidazole) and observed a reduction in the prototypical pro-inflammatory cytokine IL-1 and virus entry into cervical CD4+ T cells after 1 month. While this suggests that BV treatment may be effective in reducing HIV susceptibility, the dramatic elevation in a broad range of chemokines previously associated with HIV risk suggests the need for further studies before recommendations can be made regarding BV treatment as an HIV prevention measure. Possible avenues for further research are outlined in Section 5.4.

I will now discuss and synthesize the findings of my studies in more detail and describe how they advance our knowledge of early cellular targets of HIV and of the impact of the microbiota on genital immunology.
5.2 Cellular HIV targets

Cellular susceptibility to HIV at the genital mucosa depends on both the number and phenotype of CD4+ T cells (Figure 5-1). Closely associated with epithelial cells are intra-epithelial T cells and Langerhan cells [242,243], which is a specialized type of dendritic cell that can extend its dendrites and sample luminal contents (Figure 5-2). Underneath the epithelium is the submucosa, which contains various immune cells including neutrophils, macrophages, dendritic cells, αβ and γδ T cells, and B cells. Potential HIV target cells within the submucosa include CD4+ T cells, dendritic cells, and macrophages (Figure 5-2), and specific CD4+ T cell subsets are illustrated in this figure as their susceptibility to HIV has been recently characterized in my work and by others.

Mckinnon and Kaul, *Curr Opin HIV AIDS* [216]

**Figure 5-1. Both the number and the phenotype of immune cells can affect HIV susceptibility.**

In the genital tract, apart from the HIV receptor CD4, CCR5 appears to be the most important determinant of HIV entry. CCR5+ CD4+ T cells not only had the highest level of HIV entry on a per cell basis, but within an individual, the level of CCR5 expression and MFI (though not CD4 MFI) correlated with overall virus entry into blood and endocervical CD4+ T cells. These data
suggest that the expression of CCR5 is a limiting factor for virus entry at the genital mucosa. In contrast to CCR5 expression, the expression CD69, $\alpha 4\beta 7$, or $\alpha 4\beta 1$ did not correlate with overall virus entry into CD4+ T cells within an individual, although each of these cell types was a preferential target of the HIV pseudovirus. Therefore, among the mucosal (genital) cell subsets that I examined, I define CCR5+ CD4+ T cells as highly susceptible to HIV, while the expression of CD69, $\alpha 4\beta 7$, or $\alpha 4\beta 1$ in genital CD4+ T cells flags moderately susceptible cells.

**Figure 5-2. HIV target cells in the female genital tract.** Close to the single layer of cell in the columnar epithelium are intra-epithelial T cells and langerhan cells [88,242,243]. Underneath the epithelium is the submucosa, which contains various immune cells and among these, the putative HIV target cells that I assessed are illustrated. For the sake of simplicity, CD4+ T cells expressing only a single correlate of HIV susceptibility are depicted, however, in reality, the expression of these markers is not mutually exclusive and cells commonly express multiple putative markers of HIV susceptibility.

The situation is slightly different in blood where although the proportion of CCR5+ CD4+ T cells was again the strongest correlate of HIV entry, the frequency of other cell subsets also correlated with overall virus entry within an individual. These included TEM, $\alpha 4\beta 1+$, or CCR6+ CD4+ T cells, which had high levels of virus entry on a per cell basis; and their expression correlated with overall susceptibility of the individual; however, cells with intermediate levels of virus entry (TCM, $\alpha 4\beta 7+$, or CD69+ CD4+ T cells) did not correlate with the overall CD4+ T
cell susceptibility to HIV within an individual. This demonstrated that virus entry on a per cell basis was a good predictor of overall CD4+ T cells susceptibility to HIV, at least in blood. Still, the main driver of susceptibility was the expression of CCR5, as the frequency of highly susceptible TEM, $\alpha_4\beta_1+$, or CCR6+ CD4+ T cells but not TCM, $\alpha_4\beta_7+$ or CCR6+ CD4+ T cells correlated strongly with CCR5 expression within an individual. Overall, both the blood and genital (mucosal) data demonstrate that CCR5 expression is the strongest correlate of HIV entry.

An important application of the viral entry assay involved an assessment of whether $\alpha_4\beta_1$ or $\alpha_4\beta_7$ mediated virus entry, as some studies [126,134,244], but not others [129,130], had suggested that the binding of HIV env to $\alpha_4\beta_7$ (though not $\alpha_4\beta_1$) and/or downstream signaling promotes HIV infection in CD4+ T cells. Although $\alpha_4\beta_7+$ or $\alpha_4\beta_1+$ CD4+ T cells were equally susceptible to HIV in the cervix, the two-fold elevation in HIV entry into $\alpha_4\beta_1+$ CD4+ T cells, even after using an HIV pseudotype that preferentially bound $\alpha_4\beta_7$ compared to $\alpha_4\beta_1$, questioned the role of $\alpha_4\beta_7$ as an entry receptor. Furthermore, effective blockade of $\alpha_4\beta_7$ did not reduce HIV entry, and use of the recombinant trimeric HIV env that I had used for production of $\beta$-lactamase-vpr HIV pseudovirus failed to bind to T cells that expressed a functional $\alpha_4\beta_7$ heterodimer on the cell surface. These data strongly suggest that $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins are not HIV entry receptors. Indeed, others have also reported that HIV env binding to $\alpha_4\beta_7$ binding is not a general property of most HIV envs [130]; hence, the increase in HIV entry that I observed into CD4+ T cells expressing either $\alpha_4\beta_7$ or $\alpha_4\beta_1$ integrin is likely at least in part due to elevated CCR5 expression, and potentially due to other cell surface proteins such as chemokine receptors [222] or calcium- and integrin-binding-A (CIB-A) and/or calcium- and integrin-binding-B (CIB-B), which have recently been described as potential HIV attachment factors [245]. In summary, my studies described for the first time that $\alpha_4\beta_7+$ or $\alpha_4\beta_1+$ CD4+ T cells were preferential HIV targets, however, these integrins were unlikely to act as HIV entry receptors.

As is the case for most experimental techniques, the BlaM-Vpr HIV entry assay has certain limitations. The assay does not allow for an assessment of certain in vivo mucosal factors that affect HIV acquisition such as tissue architecture, the epithelial barrier, and cervico-vaginal mucus. Furthermore, our observation that virus entry into blood-derived CD4+ T cells did not predict subsequent heterosexual HIV acquisition suggests that blood immune parameters may
not be reflective of local mucosal factors; indeed, in contrast to my work in Kenya, in an independent study conducted by my colleague (Sergey Yegorov), no correlation was evident between virus entry in blood and endocervical CD4+ T cells. The cause for this discrepancy is not known but may be due to technical differences or biological or behavioural differences in the study cohorts that alter susceptibility of mucosal CD4+ T cells. Another reason why blood may have been a poor predictor of mucosal HIV susceptibility in my study is that the cryopreservation performed by the CAPRISA research team may not have been ideal as dramatically reduced cell viability was apparent upon subsequent thawing of clinical samples. Alternative cryopreservation techniques have been described elsewhere [246], and future studies performed ideally in genital cells may include either optimized cryopreservation methods or live cervical cells in real time to assess HIV entry in genital cells as a predictor of subsequent HIV acquisition.

5.3 The vaginal microbiota and HIV susceptibility

The implications of the impact of treatment of vaginal dysbiosis on genital immunology and HIV susceptibility are discussed in depth in section 4.5; however, I will discuss the most significant contributions along with outstanding questions and approaches to address them. Observational studies have previously demonstrated that vaginal dysbiosis is associated with HIV acquisition, presumably through high levels of pro-inflammatory cytokines which may disrupt the epithelial barrier. My study demonstrated that effective BV therapy caused seemingly opposing changes in genital IL-1α and IL-1β and various chemokines known to attract immune cells to mucosal sites. An association of high genital IL-1 and low IP-10 with BV had been previously observed in cross-sectional studies [86,195,247,248], however, our data shows a causal role of the vaginal microbiota in altering in the aforementioned parameters as well as others. BV was not associated with classically defined inflammation, which is characterized by elevated cytokines and chemokines, but a marked down-regulation of a broad range of chemokines, and an elevation in the pro-inflammatory cytokine IL-1 though not TNF-α or IFN-γ. This observation would suggest that BV is likely to be only moderately pro-inflammatory. These complex changes in cytokines/chemokines in humans challenge the relevance of some ex vivo/in vitro reductionist models where specific BV-associated bacteria or bacterial products induce pro-inflammatory
cytokine and chemokine production, including TNF-α, IL-6, IL-8, and MIP-3α [184,190,192]. These in vitro data would predict that BV increases genital inflammation in vivo, however, BV seems to be only mildly pro-inflammatory in vivo, and effective treatment is associated with a distinct and seemingly broader pro-inflammatory signature with an elevation in levels of IFN-γ, IL-17 and various highly chemotactic factors. Genital inflammation is considered a common underlying factor that amplifies HIV transmission risk [64,94,249], so the observed higher levels of several cytokines and chemokines in women with BV after effective treatment may present challenges for HIV prevention efforts. We believe that further detailed studies are necessary to characterize the changes in genital immunology after BV treatment, and to address its potential impact on HIV prevention.

5.4 Future directions

Using genital samples obtained from this study, we will perform two analyses in the near future. Our collaborators, Drs. Jacques Ravel and Pawel Gajer, will perform 16s rRNA sequencing of the vaginal microbiota that will enable us to better understand how changes in specific microbial communities and/or bacterial species are associated with genital immunology and HIV susceptibility. Specific bacterial species or genera associated with changes in virus entry or cytokines may be selected for quantitative PCR analysis to target bacteria of interest for future studies that are outlined below. We will also perform proteomic studies in collaboration with Dr. Adam Burgener to address whether BV treatment alters a previously validated mucosal proteomic signature of epithelial disruption [85,233]. These studies will explore the impact of vaginal dysbiosis and particular bacterial species on genital immunology and epithelial integrity.

To advance the findings of my study, I propose a clinical trial to characterize in more detail, the short and medium term impact of bacterial vaginosis treatment on genital immunology and HIV susceptibility. This study will have the following objectives:

1. To monitor changes in immune cell subsets including HIV target cells in the endocervix in the first few weeks after treatment and for several months thereafter, and in the vagina at baseline and 1 month after treatment
2. To monitor changes in genital cytokines/chemokines in the first few weeks after treatment and for several months thereafter; an instead cup, which collects secretions from both the endocervix and the vagina (as in Chapter 4) will be used.

3. To assess differences in soluble cytokines and chemokines in the endocervix versus the vagina using swabs collected from the respective sites.

I hypothesize the following:

1. BV treatment will cause an increase in the number of highly susceptible Th17 cells and CCR5+ CD4+ T cells, and macrophages and dendritic cells in the vagina and the endocervix after 1 month. These numbers will remain elevated up to 3 months.

2. Standard BV treatment will cause marked elevation in various genital chemokines at 2 weeks after treatment and these levels will persist over 3 months.

3. The effect of BV treatment on changes in genital cytokines and chemokines will be predominantly seen in the vagina rather than the endocervix.

4. There will be an increase in the number and frequency of γδ1 T cells and IL-17 producing γδ1 T cells in the vagina and the endocervix in women who clear BV.

**Study Design:** Seventy-five HIV/STI-uninfected women with BV based on Nugent score will be treated with oral metronidazole (400mg po tid) for 7 days. More frequent mucosal sampling compared to my study discussed in Chapter 4 will be performed, starting at baseline and at day 7, 14, 21, and 28 to assess the early effects of BV treatment, and every month thereafter for 3 months to explore the medium-term effects. Genital sampling at each visit will involve the collection of vaginal swabs for analysis of the microbiota and STIs, 1 endocervical and vaginal swab will be stored for cytokine analysis, two endocervical cytobrushes for immune cell phenotyping, and a softcup to assess cytokines in genital secretions. Moreover, this protocol will also include the collection of two vaginal biopsies from the contralateral vaginal wall at baseline and at 1 month after BV treatment. In addition to endocervical cytobrush sampling, vaginal biopsies will be used to assess cellular changes at a different site in the FGT; baseline and 1 month time points are chosen for biopsy based on changes in genital immunology described in Chapter 4. At baseline and 1 month after treatment, one vaginal biopsy will be used for
immunohistochemistry analysis, which provides a more accurate quantification of immune cells compared to tissue digestion and flow cytometry analysis [250]. Specific cell types that we will enumerate will be highly susceptible HIV target cells, CD3+ CD4+ CCR5+ and CD3+ CD4+ CCR6+ (Th17 cells). The second biopsy will be used for more in depth immune cell phenotyping. The biopsy will be processed by collagenase IV digestion [135], and immune cell phenotyping will be performed by multi-parameter flow cytometry to assess the frequency and number of αβ (CCR5+, CD69+ or CCR6+) CD4+ T cells, γδ T cells, macrophages, DCs, and neutrophils; the same panel will be used for analysis of endocervical immune cells obtained using two cytobrushes.

5.5 Conclusion

The initial focus of my PhD was the development a novel ex vivo HIV entry assay to assess genital (mucosal samples) HIV susceptibility in samples that can be easily obtained in clinical studies. I established and validated the BlaM-Vpr assay in endocervical cytobrush derived unstimulated CD4+ T cells and found that CCR5-dependent entry occurred preferentially in activated CD4+ T cells. Expression of mucosal integrins was also associated with increased HIV entry, however this was likely due to increased CCR5 expression, and α4β7 and α4β1 did not appear to be HIV entry receptors. Secondly, in the absence of genital (mucosal) samples for assay validation, I tested whether HIV entry into blood derived CD4+ T cells could predict subsequent HIV seroconversion. However, HIV entry did not predict subsequent HIV risk, suggesting that perhaps compartmentalized immune parameters may influence local (mucosal) HIV risk that is not captured by a blood-based virus infection assay. I then tested the impact of one such mucosal factor, the vaginal microbiota, on genital immunology and HIV susceptibility using the virus entry assay that I had developed as the primary study endpoint. Treatment of vaginal dysbiosis using standard therapy using metronidazole reduced cellular susceptibility to HIV and genital IL-1 suggesting some potential benefits of currently recommended treatment. However, treatment also increased a range of chemokines, and some of which had been previously linked to HIV acquisition in both women and men; suggesting that population based treatment of BV to reduce HIV acquisition should be approached with caution, and only after further research to better understand the impact of the vaginal microbiota on HIV susceptibility.
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