The impact of herpes therapy on genital and systemic immunology

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

HIV infects more than 34 million people globally. Herpes simplex virus type 2 (HSV-2) has been associated with a 3-fold increase in the rate of HIV acquisition, which may be due to physical breaks in the mucosal barrier during symptomatic episodes and/or an increased number of HIV target cells being exposed in the genital tract. Although clinical trials have demonstrated that herpes suppression in HIV uninfected individuals had no impact on HIV incidence, acyclovir was associated with a decrease in plasma HIV viral load and delayed disease progression in HSV-2 co-infected individuals. In addition, many HIV infected individuals display persistent systemic immune activation, which correlates with disease progression, despite successful antiretroviral therapy (ART). It is hypothesized that HSV could be one of the drivers of this activation. To further assess these relationships, my thesis focused on examining the impact of herpes therapy on genital tract immunology and systemic immune activation in HIV uninfected women and a comparison of systemic immune activation in individuals co-infected with HIV and HSV-2 on ART randomized to valacyclovir or placebo.

A randomized, placebo-controlled, double-blinded, cross-over trial was conducted in HSV-2 infected, HIV uninfected, women using valacyclovir. No differences were observed in
the number of various HIV target cells in the endocervix between valacyclovir and placebo phases. Further, valacyclovir had no impact on systemic immune activation in the same cohort. In a second clinical trial, the role of herpes therapy on systemic immune activation and inflammation in HIV and HSV-2 co-infected individuals on ART was evaluated. It was concluded that valacyclovir therapy had no impact on these parameters in this population.

In summary, we were unable to demonstrate that herpes therapy was able to reduce the number of HIV target cells in the endocervix of HSV-2 infected, HIV uninfected women or systemic immune activation in either HIV uninfected or HIV infected individuals. These findings demonstrate that currently available herpes therapy in standard doses does not appear to reverse the immunological changes associated with HSV-2 infection.
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List of Abbreviations

3-O HS – 3-O sulfated heparin sulfate
ACD – Acid Citrate Dextran
AIDS – Acquired Immunodeficiency Syndrome
Ang – Angiopoietin
APC – Allophycocyanin
ART – Antiretroviral Therapy
AVF – Altered Vaginal Flora
BV – Bacterial Vaginosis
CAPRISA – Center for the AIDS Programme of Research in South Africa
CCR5 – CC Chemokine Receptor 5
CD – Cluster of Differentiation
CI – Confidence Intervals
CLR – C-type Lectin Receptors
CVD – Cardiovascular Disease
CXCR4 – CXC Chemokine Receptor 4
C.trachomatis – Chlamydia trachomatis
DC – Dendritic cell
DC-SIGN – Dendritic cell Specific Intracellular Adhesion Molecule-3 grabbing Non-integrin
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribose Nucleic Acid
dNTP – Deoxyribonucleotide Triphosphate
ELISA – Enzyme Linked Immunosorbent Assay
ENV – HIV envelope glycoprotein
FACS – Flow Cytometry
FBS – Fetal Bovine Serum
FGT – Female Genital Tract
FITC – Fluorescein isothiocynate
GAG – Group Specific Antigen
GEE – General Estimating Equation
GP120 – Envelope Glycoprotein 120
GP41 – Envelope Glycoprotein 41
GUD – Genital Ulcer Disease
HIV – Human Immunodeficiency Virus
HR – Hazard Ratio
hsCRP – highly sensitive C Reactive Protein
HSPG – Heparin Sulfate Proteoglycans
HSV – Herpes Simplex Virus
HVEM – Herpesvirus Entry Mediator
H/P complex – helicase and primase complex IL – Interleukin
IP-10 – Interferon Inducible Protein-10
IQR – Interquartile Range
MCP-1 – Monocyte Attractant Protein-1
MDC – Macrophage Derived Chemokine
MHC – Major Histocompatibility Complex
MIG – Monokine Induced by Gamma Interferon
MIP-3α – Macrophage Inflammatory Protein 1-beta
NEF – HIV Negative Factor
NHP – Non-human Primate
NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI – Nucleoside Reverse Transcriptase Inhibitor
NSAID – Non-Steroidal Anti-Inflammatory Drug
NUD – Nonulcerative Disease
*N. gonorrhea* – *Neisseria gonorrhea*
OR – Odds Ratio
PBMC – Peripheral Blood Mononuclear Cell
PCR – Polymerase Chain Reaction
pDC – plasmacytoid Dendritic Cell
PE – Phycoerythrin
PerCP – Peridinin Chrolophyll Protein
PI – Protease Inhibitor
POL – HIV Polymerase gene
PrEP – Pre-Exposure Prophylaxis
RANTES – Regulated Upon Expression Normally T cell Expressed and Secretes
REV – Regulator of Expression of Viral proteins
RM – Rhesus Macaque
RNA – Ribonucleic Acid
RR – Risk Ratio
RPMI 1640 – Roswell Park Memorial Institute media
SAMHD1 – SAM domain and HD domain-containing protein-1
sICAM-1 – Soluble Intercellular Adhesion Molecule-1
SIV – Simian Immunodeficiency Virus
SM – Sooty Mangabey
SMART – The Strategies for Management of Antiretroviral Therapy
STI – Sexually Transmitted Infection
sVCAM-1 – Soluble Vascular Adhesion Molecule-1
TAT – Transactivator of Transcription
TDF – Tenofovir Disoproxyl Fumarate
TFV – Tenofovir
TNF – Tumour Necrosis Factor
Treg – T Regulatory Cells
UL – Unique Long Sequence
US – Unique Short Sequence
UNAIDS – Joint United Nations Program on HIV/AIDS
VALIANT – Valaclovir for Inflammation Attenuation Trial
VIF – Viral Infectivity Factor
VL – Viral Load
VPR – Viral Protein R
VPU – Viral Protein U
Chapter 1

1 Introduction
1.1 The Global HIV Epidemic

In 1981, the first cases of acquired immunodeficiency syndrome (AIDS) were reported when infections, which were uncommon in healthy individuals, were increasingly observed in homosexual men in New York City and Los Angeles [1]. Shortly after, independent labs in France, lead by Drs. Barre-Sinoussi and Montagnier, and the US, lead by Dr. Gallo, identified the etiological agent responsible for causing AIDS – the human immunodeficiency virus (HIV) [2, 3]. Drs. Barre-Sinoussi and Montagnier eventually shared the 2008 Nobel Prize in physiology and medicine for their discovery.

Since the start of the epidemic, the United Nations Program on HIV/AIDS (UNAIDS) estimates HIV has claimed over 45 million lives [4]. At the end of 2011, 34 million people were living with HIV globally [4]. The prevalence of HIV infection differs greatly from one region to another, with sub-Saharan Africa accounting for 69% of people living with HIV [4]. Despite its high prevalence, it should be noted that the HIV incidence has been declining in sub-Saharan Africa for the past decade [4]. However, the incidence of HIV infection has been increasing slowly in other regions, such as Eastern Europe, Central Asia, Middle East and North Africa [4]. Overall, there was a 20% decrease in new HIV infections in 2011, compared to 2010 [4]. Despite the decrease, the goal of eradication remains out of reach.

Along with a steady decrease in incidence, AIDS-related deaths have been reduced over time [4]. Compared to 2005, there has been a 24% decline in AIDS-related mortality in 2011 (1.7 million) [4]. This reduction is largely due to antiretroviral therapy (ART) and recent collaborative efforts to increase the availability of antiretroviral drugs to the regions that are in the most need [5]. HIV therapy has dramatically reduced mortality rates and transformed this
once fatal disease into a manageable chronic condition. However, these drugs have side effects and even with treatment, HIV infected individuals display increased morbidity and mortality when compared to those who are uninfected. Cardiovascular disease (CVD) is one of these complications that will be discussed in more detail in Chapter 4 of the thesis.

1.2 The HIV Virus

1.2.1 The Structure of HIV

HIV is a member of the Retroviridae family within the Lentiviruses genus. Lentiviruses are characteristically known for their long incubation period and duration of illness. Being a retrovirus, HIV has a single stranded RNA genome that is transcribed into DNA within the host cell. The genome is 9.2kb comprised of nine genes – gag, env, pol, nef, vif, vpu, rev, tat and vpr – that encode 15 proteins. Env, gag and pol are the major genetic domains of the virus. Env proteins (gp120 and gp41) are responsible for forming the viral envelope, gag proteins are responsible for structural proteins within the viral core and pol proteins are responsible for viral replication. The HIV virion itself has approximately 72 spikes of HIV envelope glycoprotein, resulting in a spherical virus with a diameter of approximately 100nm. Figure 1.1 below depicts the viral structure and lists all the HIV genes and their functions.
Figure 1.1 The viral structure (top), viral genome (middle) and a list of viral genes and their functions (bottom) of HIV.
1.2.2 HIV Infection and Target Cells

In order for HIV to establish infection, it first needs to bind to a target cell. HIV glycoprotein, gp120, readily binds to CD4 molecules on CD4+ T cells and macrophages. Binding of gp120 to CD4 leads to a conformational change in the viral capsid and leads to the binding of gp41 with one of the two co-receptors: either CCR5, predominantly expressed on macrophages, or CXCR4, predominantly expressed on T cells. Hence, viruses that utilize CCR5 receptors are known as R5-tropic, while those that utilize CXCR4 receptors are known as X4-tropic; however, many viruses can utilize both of these receptors and are dual or mixed tropic. Binding of gp41 to one of its co-receptors leads to the fusion of viral and cellular membranes allowing viral RNA to enter the cell.

It should be noted that in addition to macrophages and T cells, other cell types known as dendritic cells (DCs) may play a role in establishing HIV infection. DCs facilitate HIV infection in two different ways: either by cis-infection or by trans-infection. Cis-infection occurs when a DC is productively infected by HIV and subsequently infects T cells by presenting newly formed – de novo – viruses. Various types of DCs – Langerhans, myeloid DCs and plasmacytoid DCs – have been shown to be infected by HIV. However, HIV replication in these subsets are less productive [6] and the frequency of HIV infected DCs are substantially lower when compared to T cells in vivo. Details regarding the preferential infection of T cells are described in-depth in section 1.2.3. Trans-infection is another mechanism in which DCs play a role in establishing HIV infection. Once DCs bind to HIV, it can facilitate HIV infection by presenting the virus to T cells both in the mucosa or regional lymph nodes. During trans-infection, DCs themselves do not get productively infected by the virus, but rather, act as a vehicle in providing HIV to other cell types. C-type lectin receptors (CLR), such as DCSIGN, langerin and mannose receptors, on
DCs have been found to be key players in *trans*-infection. It has been shown *in vitro* that infection of CD4 T cells are more efficient if HIV is presented by DCs [7, 8]. Multiple T cells can bind to a single DC and this may lead to greater viral production and infection than presenting HIV alone in the media [9, 10].

1.2.3 Immune Activation and HIV Susceptibility

As mentioned previously, HIV can infect a variety of cells. After viral exposure, the virus must infect a target cell and initiate sufficient viral replication to establish infection. Although DCs and resting CD4+ T cells are possible targets, activated CD4+ T cells are the preferential target cells for localized viral replication at the site of infection [11, 12]. There are a couple of key factors that may explain why activated CD4+ T cells are likely to be initial target cells and favor viral replication upon HIV exposure.

Activated CD4+ T cells express more HIV co-entry receptors. Both the expression of CCR5 and CXCR4 are inducible and cell activation leads to transcriptional upregulation and increased expression of these co-receptors on the surface of the cell. With an increased number of these co-receptors, it makes it easier for the virus to bind to and initiate viral entry into these cells compared to resting CD4+ T cells.

It has been shown that reverse transcription of the HIV genome is less likely in resting, or immunologically quiescent, CD4+ T cells [13]. Recently, a new host defense mechanism against HIV has been discovered and this could possibly explain why resting CD4+ T cells and DCs are less prone to HIV infection than activated CD4+ T cells [14-17]. The molecule is a deoxynucleoside triphosphate (dNTP) triphosphohydrolase, known as SAMHD1. SAMHD1
converts nucleotide triphosphates into a nucleoside and triphosphate [14]. This depletes the pool of nucleotides in the cellular and nuclear compartments to very low levels and does not permit HIV replication in resting CD4+ T cells and DCs. When SAMHD1 expression was suppressed in resting CD4+ T cells, these cells were able to propagate HIV replication, indicating the importance of SAMHD1 as a host defense mechanism. However, it should be noted that unlike resting CD4+ T cells where expression of SAMHD1 was associated with decreased levels of dNTPs, the levels of dNTPs remained high despite the expression of SAMHD1 in activated CD4+ T cells [17]. Thus, the authors believe that it is possible for SAMHD1 to have different enzymatic activity in resting CD4+ T cells compared to activated CD4+ T cells that dictates HIV permissivity. It is likely that the factors mentioned above work in concert to allow activated CD4+ T cells to be more readily infected by HIV than resting CD4+ T cells.

The above findings lead to a term “HIV target cells” to describe specific types of cells that are preferentially infected by HIV. Some of these cells include cells that express HIV coreceptors, such as CD4+ T cells that express CCR5 and DCs that express CLR5, and activated cells, such as CD4+ T cells that express CD69 – a marker of early activation.

1.3 Natural History of HIV Infection

1.3.1 Clinical Aspects of HIV Infection

The acute phase of HIV infection is characterized by high levels of viral replication, leading to a viral load peak that may reach as high as $10^7$ copies/mL. About 50% of infected individuals display various symptoms during this time, such as fever, lymphadenopathy,
headaches and rash [18]. However, since these symptoms are very nonspecific, it is difficult to identify HIV infection at the early stages. Also, about half of acutely infected individuals do not display these symptoms, making it even harder to detect acute HIV infection [19, 20].
Figure 1.2 The magnitude of HIV viremia, HIV-specific CD8+ T cells and antibody response to HIV (top) and CD4+ T cell count (bottom) throughout infection.

The host responds to this peak in viral load through the generation of both a cellular and humoral immune response. Roughly two weeks after infection, the peak in HIV-specific CD8+ T cells coincides with a decrease in plasma HIV viral load. Shortly after this time, seroconversion occurs and anti-HIV antibodies can be detected in the plasma. Following cellular immune responses mediated by CD8+ T cells, viremia decreases to a steady state level, known as the “viral set point” and the host enters the chronic phase of infection.

The chronic phase is largely asymptomatic and duration is variable, anywhere from 3 months to >20 years, depending on viral phenotype, [21] host genetics and unknown factors [22, 23]. The viral set point correlates with the rate of disease progression [24, 25]. Despite achieving a stable viral load and blood CD4+ T cell levels initially, there is a steady decline in CD4+ T cells at a rate of 25-60 cells/uL per year and a steady increase in plasma HIV viral load.

Eventually, an individual will be diagnosed of AIDS, defined by the following:

1. Positive test for HIV

2. Having one or more clinical disease or illnesses that characterize AIDS

(Note: In the US, clinical definition of AIDS is met when a person has a CD4+ T cell count that is less than 200 cells/uL.)

As an individual progresses with immune deficiencies, there is a rise in viremia, clinical immunodeficiency and increased chance of opportunistic infections. Individuals may also develop nonspecific clinical symptoms, such as fatigue, weight loss and fever [26]. If left
untreated, AIDS will eventually lead to death, most commonly due to opportunistic infections or malignancies.

1.3.2 Immune Activation and HIV

The host develops a vigorous HIV-specific immune response in an attempt to clear the virus. Initially, this immune response may be beneficial in promoting proliferation and maintaining HIV-specific cellular immune responses to control viral replication. However, HIV infected individuals cannot fully clear the virus and HIV-specific immune responses persist from the acute phase to the chronic phase of infection. Factors contributing to these immune responses may be the ongoing presence of HIV viremia or elevated levels of bacterial products in blood plasma, such as LPS that are translocated form the gastrointestinal tract. This leads to increased expression of activation markers – such as CD38 and HLADR – on both CD4+ and CD8+ T cells in blood. CD38 is a surface glycoprotein and HLADR is MHC class II antigen and expression of both of these molecules is increased in activated T cells. The correlation between immune activation and disease progression has been so closely linked that it is a better prognostic marker for disease progression than HIV viral load or CD4 count in HIV infected individuals [25, 27, 28].

Non-human primate studies demonstrated the importance of immune activation in SIV disease progression. Simian immunodeficiency virus (SIV) – the equivalent to HIV in non-human primates – can infect its natural host, sooty mangabeys (SMs), and non-natural host, rhesus macaques (RMs). In both primates, SIV infection leads to high viremia accompanied by systemic immune activation and generation of SIV-specific CD8+ T cell responses [29-31].
However, chronic SIV infection in SMs does not progress to AIDS, while RMs ultimately succumb to an AIDS-like illness [29]. In the natural hosts of SIV, SMs maintain high viremia upon infection indicating that high level viremia itself may not be responsible for pathogenesis. However, SMs display low levels of systemic immune activation and maintain a normal CD4 count [32]. Unlike the natural hosts, SIV infection of RMs is characterized by a lower level SIV viremia but an associated chronic immune activation that leads to AIDS. The studies from NHP models illustrate the important relationship between systemic immune activation and disease progression and that chronic immune activation may actually drive pathogenesis rather than high viremia.

1.3.3 Antiretroviral Treatment

Antiretroviral therapy (ART) has not only reduced HIV related morbidity and mortality, but has also managed to transform what was once a fatal disease into a chronic condition. The first antiretroviral agent, zidovudine, was introduced in 1987 [33]. Currently, there are more than 20 different drugs from the following classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors, fusion inhibitors and integrase inhibitors [34]. The nomenclature of these drugs indicates the various stages of the HIV life cycle that each drug targets. In ART, three active agents are used in combination to reduce the HIV plasma viral load below the level of detection and reduce the risk of resistance through replication and mutation – which is common during HIV replication, due to the high error rate ($10^{-4} \sim 10^{-5}$) of HIV reverse transcriptase. Conventional ART uses a “cocktail” of three antiretroviral drugs taken together, either two NRTIs complemented with a NNRTI or a PI or integrase inhibitor [35]. ART dramatically prolongs
survival in HIV infected individuals [36, 37]. However, no drugs can target the integrated virus, meaning that it does not lead to a cure.

Once an infected individual starts ART, the plasma viral load decreases rapidly. Before reaching undetectable levels in blood (<50 copies/mL), two phases of viral decay are observed, also known as a bi-phasic decay. The first phase occurs within the first two weeks of therapy, characterized by a rapid drop of the HIV RNA level in blood. This is largely attributed to prevention of de novo production of virus from CD4+ T cells [38]. A much slower and gradual phase of viral decay follows, associated with clearance of viremia sourced from chronically infected CD4+ T cells and macrophages [39, 40]. Most individuals achieve undetectable blood viral levels within 6 months of therapy [39, 41]. However, strict adherence to ART regimens is crucial, as therapy interruptions have been associated with emergence of drug-resistant viruses [42].

Without a doubt, ART has transformed the lives of those infected with HIV in preventing AIDS-related illnesses. However, HIV infected individuals on ART have higher rates of non-AIDS morbidities, such as cardiovascular, liver and renal disease [43, 44]. The Strategies for Management of Antiretroviral Therapy (SMART) trial observed the effects of the episodic use of ART [5]. The criteria for ART usage was as follows: defer therapy until CD4+ T cell count decreased below 250/mm³ and start on therapy until CD4+ T cell count increases above 350/mm³. The study demonstrated that CD4 count-guided intermittent ART was associated with higher incidences of both opportunistic disease and major cardiovascular, liver or renal diseases compared to continuous ART [45, 46]. The plausible explanation for this observation is that intermittent ART allows viral replication and results in an increased inflammation and systemic immune activation [47]. While HIV treatment leads to a decrease in immune activation, levels
are still elevated compared to those who are HIV-uninfected [45, 47-49]. Persistent immune activation is observed in blood despite ART and can be measured in many different parameters: markers of systemic immune activation, inflammatory cytokines, acute phase reactants, endothelial activation markers and markers of microbial translocation. More specifically, a study comparing markers of inflammation, coagulation and renal functions in participants from the SMART trial with HIV uninfected individuals showed that these markers were significantly increased in HIV infected individuals on ART compared to those not on therapy [50].

1.4 HIV Susceptibility in Women

Women are disproportionally infected by HIV globally, accounting more than 50% of these infections [51]. Within HIV endemic countries, where heterosexual sex is the predominant mode of virus transmission, this predominance is even more striking, with women accounting for 58% of HIV infection [51]. Furthermore, when one looks at HIV prevalence according to age and gender, a striking observation is that women tend to acquire HIV at a much younger age than men [52], with young women aged 15-24 years being eight times more likely than men to be HIV-infected [53]. Various factors, both behavioural and biological, appear to predispose women to acquire HIV.

1.4.1 Behavioral Factors Altering Transmission of HIV in Women

HIV is a sexually transmitted infection that is transmitted through unprotected sex. As such, there is no question that sexual behaviour and patterning are very important determinants
of the high HIV incidence in women. Women have a similar or later age of sexual debut compared to men [52], but young women more often enter into a sexual relationship with older partners [52, 54, 55]. In addition, a less experienced partner may be less able to successfully negotiate condom use with her partner. In keeping with this, HIV rates can be high within young women reporting only a single lifetime partner and very few episodes of sexual intercourse [52]. Indeed, this may also be one reason for the startlingly high per-act HIV incidence of up to 3% that was observed within women from the CAPRISA 004 study [56], although data regarding male partners in that study are not (or are not yet) available. Lastly, other behavioural factors may also play a role. For example, douching has been associated with altered vaginal flora and an increased risk for HIV acquisition [57, 58].

1.4.2 Biological Factors Altering Transmission of HIV in Women

1.4.2.1 Mucosal Immunology and HIV Acquisition in Women

The female genital tract (FGT) is the most common site of HIV acquisition (Table 1.1) and between 30-40% of all new HIV infections are acquired in the FGT [59]. With such high incidence of HIV attributable to the FGT, many studies have been conducted to better understand the modes of HIV infection, using non-human primate, in vitro and ex vivo models.
Table 1.1 Contribution of HIV invasion sites to global HIV infections.

<table>
<thead>
<tr>
<th>HIV invasion site</th>
<th>Anatomical sub-location</th>
<th>Type of epithelium</th>
<th>Transmission medium</th>
<th>Transmission probability per exposure event</th>
<th>Estimated contribution to HIV cases worldwide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female genital tract</td>
<td>Vagina</td>
<td>Squamous, non-keratinized</td>
<td>Semen</td>
<td>1 in 200 – 1 in 2,000</td>
<td>12.6 million</td>
</tr>
<tr>
<td></td>
<td>Ectocervix</td>
<td>Squamous, non-keratinized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endocervix</td>
<td>Columnar, single layer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Various</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male genital tract</td>
<td>Inner foreskin</td>
<td>Squamous, poorly keratinized</td>
<td>Cervicovaginal and rectal secretions and desquamations</td>
<td>1 in 700 – 1 in 3,000</td>
<td>10.2 million$^1$</td>
</tr>
<tr>
<td></td>
<td>Penile urethra</td>
<td>Columnar, stratified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Various</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal tract</td>
<td>Rectum</td>
<td>Columnar, single layer</td>
<td>Semen</td>
<td>1 in 20 – 1 in 300</td>
<td>3.9 million$^1$</td>
</tr>
<tr>
<td>Upper GI tract</td>
<td>Various</td>
<td>Semen</td>
<td>Maternal blood, genital secretions (intrapartum)</td>
<td>1 in 5 – 1 in 10</td>
<td>960,000$^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast milk</td>
<td>1 in 5 – 1 in 10</td>
<td>960,000$^3$</td>
</tr>
<tr>
<td>Placenta</td>
<td>Chorionic vili</td>
<td>Two layer epithelium (cyto- and syncytiotrophoblast)</td>
<td>Maternal blood (intrauterine)</td>
<td>1 in 10 – 1 in 20</td>
<td>480,000$^3$</td>
</tr>
<tr>
<td>Blood stream</td>
<td></td>
<td>Blood products, sharps</td>
<td></td>
<td>95 in 100 – 1 in 150</td>
<td>2.6 million$^3$</td>
</tr>
</tbody>
</table>

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The female genital tract can be divided into the upper genital tract (endocervix, endometrium and fallopian tubes), and the lower genital tract (vagina and ectocervix) [60]. Figure 1.3 illustrates the general anatomy of the FGT with a focus on specific epithelial cell types that make up the endocervix and ectocervix, which will be discussed in detail.
Despite having different anatomic and physiological properties, the vagina, ectocervix and the endocervix have all been shown to be susceptible to HIV transmission [59, 61-64]. The vaginal and ectocervical epithelium are comprised of multilayered squamous epithelial cells and possibly offer a more robust barrier to pathogens than that of the endocervix, which is comprised of a mono-layer of columnar epithelial cells [61, 62]. Although the exact relative risk of HIV acquisition is difficult to compare between sites, the cervical transformation zone between the squamous epithelium and single-layer columnar epithelium (Figure 1.3) is believed to be the most susceptible site, partly due to the presence of CD4+ T cells [59]. To support this, recent non-human primate (NHP) studies show that SIV infections occur around this transformation zone and the endocervix [65]. Once NHP are exposed to SIV vaginally, the virus can cross the mucosal barrier within hours [66] and lead to infection in couple days. Li et al. found that 4 days post-inoculation, all 9 NHPs infected with SIV had a predominant focus of infected cells in the endocervix and three animals displayed additional cluster of SIV infected cells in the transformation zone, illustrating the rapid infection of the virus [65]. In addition, HIV acquisition is higher in women with cervical ectopy, defined as the extension of the columnar epithelial cells to the ectocervix [67, 68]. The role of mucus plug in relation to HIV acquisition remains unclear. The layer of mucus may immobilize HIV and prevent its contact with epithelial cells [69]; on the other hand, this may lead to entrapment of HIV and prolong its survival in the host thereby enhancing HIV transmission.

However, the endocervix is not the only site of HIV entry within the FGT. HIV transmission was observed in hysterectomized animals [70] and in women with congenital
absence of a cervix [71]. Compared to the endocervix, the vaginal tract has a much greater surface area (>15 times), potentially providing more access sites for HIV entry. Also, the epithelial integrity of the vagina and ectocervix can be compromised due to trauma, such as micro-abrasions during sexual intercourse that may facilitate HIV entry [61-63]. There are apparent physiological differences between the lower and upper FGT, but it has been shown that HIV can take advantage of almost any site to establish infection.

The mucosal immune environment is another important determinant of HIV transmission, in addition to the physical characteristics of the FGT. Reduced genital immune activation among women has been associated with lower HIV susceptibility. Specifically, increased levels of pro-inflammatory cytokines and regulated on activation, normal T cell expressed and secreted (RANTES) in the female genital tract have been associated with HIV acquisition [72, 73]. In conjunction with this finding, our lab has previously shown a strong correlation between genital RANTES levels and the number of CD4+ T cells and CD4+ T cells that expressed CCR5 in the endocervix of female sex workers [74]. The above findings indicate the important relationship between genital inflammation – both cytokines and presence of HIV target cells – and HIV acquisition. However, it is not the mere presence of HIV target cells in the mucosal environment that may dictate HIV infection: both the quantity and quality of the HIV target cells are important [75]. The sheer increase in the number of HIV susceptible cells may increase the likelihood of acquiring HIV, but the activated status of these cells are also be important. As discussed in section 1.2.3, activated target cells are more prone to HIV infection and the figure below (1.4) depicts how changes in the quantity and quality of these cells may promote HIV infection. The left section of the figure illustrates the normal condition of mucosal environment; the middle section illustrates how HIV infection can be enhanced by recruitment of all three types of cells despite the similar ratio in the type of cells; the right section illustrates how HIV
infection can be favored by increasing the proportion of highly susceptible cells despite similar number of cells to the normal mucosa.

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**Figure 1.4 Three possible scenarios of mucosal environment for HIV infection.**
Panel 1 illustrates the normal mucosa. Panel 2 and 3 illustrate the mucosal environment that may favour HIV infection, the formal by increasing the number of all three types of cells and the latter by increasing the frequency of highly susceptible cells.
1.4.2.2  Sexually Transmitted Co-infections

Sexually transmitted infections (STIs) are associated with increased risk of HIV acquisition. STIs can be divided into two broad categories: those that cause genital ulcer disease (GUD) such as HSV-2, chancroid and syphilis and those that cause nonulcerative disease (NUD) such as gonorrhea, chlamydia, candidiasis and trichomonas [76]. In a meta-analysis in which 31 studies were included, it was determined that GUD was associated with increased risk of acquiring HIV with odds ratio (OR) of 3.29 (1.9-5.8 95% confidence intervals) while NUD had an associated OR of 1.7 (1.0-2.8 95% CI) [76].

Genital inflammation plays a critical role in HIV acquisition, and these STIs are associated with increased inflammation in the genital tract. Among the STIs, HSV-2 is one of the most well documented and studied diseases in terms of HIV acquisition. GUDs by HSV-2 cause ulceration, directly breaking epithelial integrity in the mucosa. The disruption in the physical breach may provide an easier portal of entry for HIV into the host. Another mechanism in which GUDs can facilitate HIV transmission is to increase inflammation in the genital tract. The specific details regarding possible mechanism and impact of HSV-2 in HIV transmission will be discussed in-depth in section 1.8. NUDs, such as gonorrhea, chlamydia, trichomonas and bacterial vaginosis do not directly break epithelial integrity but are capable of inducing inflammation and may lead to the recruitment of HIV target cells to the site of HIV exposure, such as the genital tract, and ultimately, enhancing HIV transmission [77]. With a clear link between STIs and increased HIV transmission, many studies have sought to determine the potential impact of STI treatment on reducing HIV acquisition. However, the results of most of the studies demonstrated that treatment of STIs did not decrease HIV incidence [78] and the reason for failure are unclear.
1.5 HSV-2 Virion

1.5.1 The Structure of HSV

Herpes simplex virus (HSV) 1 and 2 are from the family of Herpesviridae within the genus Simplexvirus. HSV-1 and HSV-2 share 82% of their amino acid sequence and subsequently demonstrate very similar structures [79]. HSV is an enveloped, double-stranded DNA virus belonging to the subgroup Alphaherpesviridae. As shown in Figure 1.3, the viral structure consists of four major elements: core, capsid, tegument and envelope. The core contains the viral double-stranded DNA. The capsid is structured in icosahedral symmetry and contains viral proteins that facilitate DNA encapsidation. The space between the capsid and the viral envelope is known as the tegument and it is largely unstructured containing proteins that are required for host shut-off mechanisms. Lastly, the envelope is made up of a lipid bilayer with viral glycoproteins that play a crucial role in binding to target cells.

1.5.2 HSV Infection

1.5.2.1 HSV Cell Entry

To initiate HSV infection, the virus must come in close proximity to the target cell, such as an epithelial cell. The HSV envelope contains five viral glycoproteins that are involved in the entry process: gB, gC, gD, gH and gL [80, 81]. Among the five proteins, gB and gC are the first proteins to interact with heparin sulfate proteoglycans (HSPGs) on the target cell, facilitating binding of the virus. After binding of the virus to the cell, successful fusion of the virus is required to produce a successful infection. There are two methods of entry: through the plasma membrane or through the vesicular membrane of the cell. In addition to the viral glycoproteins – gB, gD, gH and gL – cellular receptors such as nectin-1, herpesvirus entry mediator (HVEM) and 3-O sulfated heparin sulfate (3-O HS) are necessary for fusion [80, 82]. Once gD binds to one of the cellular receptors a conformational change occurs, leading to the formation of multiprotein complexes with other glycoproteins [83, 84]. This multiprotein complex induces fusion of the viral membrane with the plasma membrane allowing delivery of viral tegument proteins and viral capsid into the cytoplasm [84]. In a similar mechanism, HSV can enter the cell through a mechanism using endocytosis [81, 85]. After endocytosis, the viral particle is placed into an endosome with acidic pH environment, but it can still fuse with the vesicular membrane and lead to a productive infection [86-88]. The fusion between the endosomal membrane and the viral membrane occurs through a similar mechanism to entry through the plasma membrane.
1.5.2.2 Cell to Cell Spread of HSV

After successful infection of a target cell, HSV establishes a productive infection through cell to cell spread. Just like HSV entry through the plasma membrane, initial interaction between gD with its cellular receptors are required. However, unlike initial entry, formation of gE and gI heterodimers are required for cell to cell infection [89]. The gE and gI heterodimers move to epithelial cell junctions from the trans-Golgi network with other viral glycoproteins and virion particles. Once near the cell junctions, the viral particles can bind to either nectin-1, HVEM or 3-O HS to spread from one cell to another.

1.5.2.3 HSV Replication

After gaining access to the cytoplasm of a cell, nucleocapsids and teguments are transported to the nuclear pores and then the DNA is released and enters the nucleus. Once the viral genome enters into the nucleus, it is able to start replication. The HSV genome has 3 origins of replication [90], one oriL (located in a long unique region) and two oriS (located in a short unique region). To initiate DNA replication, origin-binding protein UL9 and single-strand DNA binding protein ICP8 must bind to the origin of replication and unwind the DNA [91]. This unwinding exposes the viral DNA in a single stranded form and recruits the viral helicase and primase (H/P) complex. The HSV H/P complex is comprised of three proteins: UL5, UL8 and UL52 [90]. Once H/P complex is bound to the site of DNA replication, the UL8 subunit interacts and recruits viral polymerase [92]. The HSV polymerase is made up of UL30 (pol), the catalytic subunit, and UL42, the processivity subunit [90]. Recruitment of the viral polymerase allows replication of the HSV genome. It should be noted that a common anti-HSV drug, acyclovir,
targets the viral polymerase by acting as a guanosine analogue and terminates DNA replication. This will be discussed in further detail in section 1.7.

1.6 Natural History of HSV-2 Infection

1.6.1 Primary HSV-2 Infection

HSV-2 is a lifelong infection that is the leading cause of genital ulcers worldwide. Infection occurs when the virus comes in contact with non-intact skin or a mucosal surface. It can cause ulcerative lesions that can heal with or without crusting, depending on the location of the lesions. Since it is sexually transmitted, consistent condom use has been associated with a 30% decrease in HSV-2 acquisition [93, 94]. Many studies indicate that the incidence of HSV-2 per 100 person-years is roughly 7.4 (5.1-16.2) [94, 95]. Once infected, symptoms appear usually 4-7 days after exposure to the virus and include itching, burning, painful sensations and lymphadenopathy [96]. However, 80% of individuals who are seropositive have no history of genital lesions [97]. The prevalence of HSV-2 varies from region to region; well over 50% of sexually active adults are infected in sub-Saharan Africa, while only 20% of sexually active adults are infected in North America [98]. Since HSV-2 is a life-long infection, its prevalence increases with age [99].
1.6.2 HSV-2 Reactivation

After initial contact with the mucosal surface of the host, HSV-2 needs to enter the sensory nerve ending and establish a latent infection. Once taken up by the sensory nerve ending, the virus travels to the nerve axon. It is within the neurons of the dorsal root ganglia and autonomic nervous system that HSV-2 stays in its dormant state – non-replicating and unintegrated. HSV-2 successfully evades the immune response by augmenting the CD8+ T cell mediated apoptosis in infected cells [100, 101]. As HSV-2 reactivates in the ganglia, it travels down the axon and towards the genital epithelium, leading to subsequent replication within epidermal cells and subclinical shedding [96]. HSV-2 DNA detected in genital swabs has been suggested to be due to viral replication within epithelial cells rather than viral release from the neurons [102]. Two findings support this idea: (1) Nerve endings are present only as far as the mid-epidermis and are separated by at least 10 epithelial cells from the site of swab collection [102]; and (2) HSV-2 DNA replicates in better in epithelial cells compared to the neurons [103, 104]. It is thought that symptomatic episodes occur when environmental stimuli, such as trauma, ultraviolet light or immune suppression, elicit reactivation. A mathematical model for HSV-2 reactivation suggests that rather than having bursts of HSV-2 reactivation, it is likely that the infected neurons constantly release small amount of virus [105]. In line with this model, it has been shown that there is persistence of HSV-2 specific lymphocytes in the neuronal termini of sensory nerve roots [106].

One of the major discoveries made regarding HSV-2 within the last two decades is that HSV-2 shedding occurs even in the absence of visible ulcers. This finding was possible due to the advent of PCR and the ability to detect HSV-2 DNA on skin or genital swabs. Although HSV-2 PCR detection is no less specific than the traditional method of viral culture, it is more
sensitive [107, 108]. Before using PCR technology, the general notion was that HSV-2 shedding resulted in ulcerative lesions and that shedding was rare in the absence of such lesions. However, daily sampling from both symptomatic and asymptomatic individuals found that 67% of reactivation occurred in the absence of genital lesions [109]. Another study that sampled HSV-2 infected individuals, both symptomatic and asymptomatic, every 6 hours for at least a month discovered that transient shedding occurs roughly every 10 days [110].

1.7 HSV-2 Treatment and Suppression

1.7.1 HSV-2 Suppressive Therapies

HSV infection can be treated with antiviral drugs such as acyclovir, valacyclovir, penciclovir and famciclovir. All these drugs are classified as nucleoside analogs as they mimic nucleosides in order to terminate or slow down viral DNA replication. Acyclovir was one of the first antiviral drugs to be discovered more than three decades ago. Although all four drugs are efficient in treating HSV-2 symptoms, the mechanism of action differs subtly and the antivirals can be separated into two groups: Acyclovir and valacyclovir vs. penciclovir and famciclovir.

Acyclovir is an acyclic guanosine analogue that terminates DNA synthesis during viral DNA replication [111]. It needs to be phosphorylated by viral thymidine kinase into its monophosphate form. Viral thymidine kinases have higher affinity to acyclovir than cellular kinases (>3000 fold), making it a very specific drug. Acyclovir monophosphate can then be further phosphorylated by cellular kinases to its triphosphate form. Acyclovir triphosphate then competes with deoxyguanosine triphosphate for uptake by viral DNA polymerase. Acyclovir
lacks the 3’-hydroxy group for chain elongation and thus, once incorporated, it is able to terminate DNA replication. Even though acyclovir is very effective, it is limited by its low bioavailability (10-30%) [112]. This requires frequent dosing of acyclovir in HSV-2 infected individuals and an increased pill burden compared to other drugs, such as valacyclovir. Valacyclovir is a prodrug of acyclovir. Valacyclovir is synthesized by adding an aminoacid, L-valine, to acyclovir through valine esterification. This leads higher bioavailability (54%) [113] and similar therapeutic effect can be reached even with less frequent dosing thus, lowering pill burdens. Many studies have compared the effects of acyclovir and valacyclovir and concluded that valacyclovir is at least as effective as acyclovir [114, 115].

Penciclovir also resembles guanosine and it is acyclic [116]. Just like acyclovir, penciclovir needs to be phosphorylated – initially by viral thymidine kinase and then followed by cellular kinases – before it can successfully compete with endogenous deoxyguanosine triphosphate. However, there are differences in the mechanism of action between penciclovir and acyclovir. Unlike acyclovir which terminates viral DNA replication, penciclovir can only delay DNA synthesis once it is incorporated into the viral DNA strand [117, 118]. Acyclovir has higher affinity (>100 times) for viral DNA polymerase than penciclovir but penciclovir makes up for its less affinity with a faster rate of phosphorylation and much greater half life (> 10 fold) [118]. Famciclovir is a prodrug of penciclovir that has undergone esterification to increase its bioavailability [116]. Overall, both acyclovir and penciclovir and their prodrugs are very potent treatments for HSV-2 infections.

In addition to drugs related to acyclovir and penciclovir, several other antivirals exhibit anti-herpes activity: ganciclovir, cidofovir and foscarnet [119]. Ganciclovir is mainly used to treat cytomegalovirus, another type of herpes virus. The mechanism of action is similar to
acyclovir in that the drug needs to be phosphorylated by viral thymidine kinase and then act as deoxyguanosine triphosphate analogue [119]. However, cidofovir and foscarnet do not require thymidine kinase and can be used as an alternative drugs to treat acyclovir-resistant HSV infection [119]. Compared to acyclovir and penciclovir derivatives, these drugs are not generally used to treat for HSV due to their increased toxicity.

Herpes antiviral therapy is used in four main situations; first, to treat primary HSV-2 infection; second, to treat recurrent episodes; third, to suppress symptoms in those who have frequent HSV-2 recurrences; and fourth, to prevent transmission. The dosing for primary episodes is higher than for recurrent episodes. When a patient presents with genital herpes ulceration for the first time, the suggested dosing includes: acyclovir 200mg five times daily or 400mg three times daily, valacyclovir 1000mg twice daily and famciclovir 250mg three times daily, each for anywhere from 7-10 days [120-122]. For recurrent episodes, regimens include acyclovir 200mg five times daily or 800mg twice daily, valacyclovir 500mg daily and famciclovir 125mg twice daily, each for about 5 days [120-122]. Compared to untreated individuals, these treatments lead to accelerated healing of the lesions and a shortened duration of viral shedding [120-122]. Lastly, in individuals that with frequent clinical reactivation of HSV, the regimens are as follows: acyclovir 400mg twice daily, valacyclovir 500 or 1000mg once daily and famciclovir 250mg twice daily with the length of treatment varying between individuals. [120-122].

Even though herpes suppressive therapy is efficient – decreasing lesion rates by 75% and shedding rates by 80% – and decreases transmission, it cannot fully suppress herpes reactivation nor cure the disease [123, 124]. A recent study demonstrated that despite maximal doses of acyclovir and valacyclovir, herpes shedding cannot be fully suppressed [125]. In this study
researchers compared the impact of various doses of acyclovir and valacyclovir: acyclovir 400mg twice daily, 800mg three times daily, valacyclovir 500mg twice daily and 1000mg three times daily. The participants in the study were required to provide four swabs – each swabbed around genital and perianal area – daily to assay for viral shedding. They showed that even in the group that received valacyclovir 1000mg three times daily, well above the recommended dose, HSV-2 shedding occurred 16.5 times a year – meaning that shedding occurred at least once a month [125]. This study highlights the limits of current herpes therapy and that complete herpes suppression cannot be achieved despite using high doses of valacyclovir.

1.8 Negative Synergy of HIV and HSV-2

Considering that both HIV and HSV-2 are sexually transmitted, it is not surprising that there is an overlap in their epidemiology. As mentioned in section 1.4.2.2, STIs have been associated with increase risk in HIV acquisition. HSV-2 is one of the most characterized STIs in terms of HIV transmission and a meta-analysis indicates that it increases the rate of HIV acquisition by ~3 fold [126]. HSV-2 may also affect HIV disease progression in individuals who are co-infected with both viruses. HIV infection also impacts the clinical course of HSV-2 infection. With the complicated relationship between HSV-2 and HIV, it was designated the term “negative immuno-synergy” and this section will describe three aspects of this synergy.
1.8.1 Impact of HSV-2 on HIV Acquisition and Transmission

In sub-Saharan Africa, >60% of the general population is infected with HSV-2 and it has been estimated that HSV-2 play a role in increasing HIV incidence. Studies have estimated that up to half of HIV incidence within the region can be attributed to HSV-2 infection [98, 127]. This is due to HSV-2 both increasing HIV susceptibility in those infected by HSV-2, and also increasing HIV infectiousness in HIV and HSV-2 co-infected individuals to their HIV-uninfected partner.

Several biological mechanisms may explain the increased rate of HIV acquisition in individuals with HSV-2. HSV-2 reactivation causes ulcers, a disruption of the physical mucosal barrier that would normally provide some protection against HIV. These ulcers may provide an easier portal of entry for HIV to infect the host. In addition, HSV-2 has been associated with increased number of HIV susceptible cells, CCR5+ CD4+ T cells and DCs that express HIV binding receptors, both in the endocervix of HSV-2 infected, asymptomatic women and in HSV-2 ulcerative lesions [128, 129].

Two large randomized clinical trials have been conducted to test whether the increase in HIV acquisition by HSV-2 infected individuals can be reversed upon HSV-2 treatment [130, 131]. Both clinical trials used 400mg of acyclovir twice-daily to suppress HSV-2 and compared the rate of HIV acquisition in the treatment group vs. the matching placebo group. However, neither studies demonstrated an impact on the rate of HIV acquisition with the hazard ratio (HR) for HIV infection trended to be (non-significantly) higher in those treated with acyclovir: HR of 1.16 and 1.08. In one of the trials, the lack of effect was thought possibly associated with poor adherence to drugs [131]. Although the adherence was 90% according to pill count, random urine analysis showed that only 33% to 67% of samples were detected for acyclovir [131]. Zhu
et al. demonstrated that increased number of HIV target cells in the ulcerative sites of HSV-2 persisted even after 8 weeks of therapy, hinting that HSV-2 has long lasting effects on immune parameters [129]; and with individuals displaying HSV-2 shedding at least once a month despite high dose of acyclovir [125], it is likely that herpes therapy is insufficient to reverse the increased rate of HIV transmission seen in these individuals.

Higher HIV transmission incidence is associated with GUD in HIV infected individuals. Symptomatic GUD increased the risk of HIV transmission by 4 fold in monogamous HIV-1 serodiscordant couples in Uganda [132]. Since HSV-2 is the leading cause of GUD, HSV-2 likely played a role in increasing HIV incidence [98, 133, 134]. Symptomatic and asymptomatic reactivation of HSV-2 has been associated with increased levels of HIV VL in plasma and genital secretions [134-138] and both plasma HIV VL and genital HIV VL have been associated with HIV transmission. Thus, it seems that HSV-2 may fuel the HIV epidemic by increasing susceptibility to HIV infection in HIV uninfected individuals and increasing the likelihood of HIV transmission in HSV-2 and HIV co-infected individuals.

1.8.2 Impact of HIV and HSV-2 Co-infection

The prevalence of HSV-2 is high in HIV infected individuals (50-90%) [139-143]. More specifically, in Canada, it has been shown that HSV-2 prevalence is 54.7% vs 18% in HIV infected and uninfected individuals, respectively [144-146]. In HIV infected people, HSV-2 reactivation in the genital tract is greater in both frequency and quantity when compared to HSV-2 mono-infected individuals [139-143]. Asymptomatic genital HSV-2 shedding was increased 4 fold in HIV infected vs. uninfected women [147]. Similar findings were found in MSM, where
HIV infected individuals displayed a 3 fold increase in asymptomatic anogenital HSV-2 shedding [148]. In conjunction with these findings, HSV-2 reactivations occur more frequently with increased level of plasma HIV VL [98]. Recent studies have demonstrated impaired HSV-specific CD4+ and CD8+ T cell responses in HIV infected individuals [149], illustrating a mechanism by which HIV may exacerbate HSV-2 reactivation and clinical disease. Furthermore, HSV-2 is one of the opportunistic infections that can cause serious illness in those with AIDS.

In turn, HSV-2 also has a negative effect on HIV infection. HSV-2 reactivation has been associated with an increase in plasma HIV VL [134-138]. A possible mechanism for this increase in plasma HIV VL has been shown using in vitro models. In these studies, it was shown that certain HSV-2 gene products - ICP-0, ICP-4 and ICP27 - can transactivate proviral HIV by allowing cellular activators, such as nuclear factor kappa B (NFκB), to bind to HIV’s long terminal repeats [150-155]. Since HIV and HSV-2 can co-infect the same CD4+ T cell, this transactivation may have physiological relevance [156]. In addition, HSV-2 can alter the cell tropism of HIV by forming a viral particle that is a mixture of both viral constructs, such as a virus with HSV-2 surface glycoproteins that contains the HIV genome [157, 158]. This has been observed in clinical samples, shown by infection of keratinocytes, which are not the usual targets of HIV [157, 158]. Lastly, HSV-2 seropositivity has been associated with increased systemic immune activation of CD4+ T cells [159].

Thus, HSV-2 and HIV work in concert, demonstrating a complex negative synergy between the two diseases. Not only do they share similar modes of transmission, they are both also life-long infections.
1.8.2.1 HIV Treatment and its Effect on HSV-2

HIV infection increases HSV reactivation and studies have observed the impact of ART on HSV-2. One study compared 28 ART-treated and 49 untreated HIV infected individuals and found that there was no significant difference in the frequency and quantity of HSV-2 shedding between the two groups [160]. However, ART was associated with fewer days with clinical HSV lesions [160]. Another study showed that ART did not reduce the occurrence of GUD in HIV-infected women compared to their pre-ART visits [161]. A more in-depth longitudinal study compared three groups of women over 24 weeks: HIV uninfected, HIV infected taking ART and HIV infected not on ART [143]. This study concluded that the presence of ulcers (1.9%, 3.1% and 7.2%) and the number of visits with cervicovaginal HSV-2 DNA (4.3%, 9.7% and 15.5%) was different among the three groups, with the lowest being HIV uninfected, followed by ART treated individuals and then ART naïve group. However, when they conducted a sub-analysis, women who were not on ART with a CD4 cell count of 500 cells/uL or greater had a similar risk of HSV-2 shedding as women on ART (RR 0.95) and those with 200-500 cells/uL were more likely to shed HSV-2 (RR 1.71) [143]. Overall, most studies have concluded that ART has minimal effects on HSV-2 reactivation, but reduces HSV-2 clinical disease.

1.8.2.2 HSV-2 Treatment and its Effect on HIV

Both acyclovir and valacyclovir, have been associated with an improved clinical outcome in HIV infected individuals. Five placebo-controlled, randomized trials have demonstrated that HSV-2 therapy caused a decrease in plasma HIV VL of 0.25 to 0.5 log\text{10} copies/mL [140, 142, 162-164]. In conjunction, the Partners in Prevention trial, in which 3408 serodiscordant couples...
were recruited to observe the impact of acyclovir 400mg twice daily on reducing HIV transmission from HIV and HSV-2 co-infected partner to HIV uninfected individuals, found that HSV-2 treatment was associated with a decrease in plasma HIV VL [165]. In addition, acyclovir treatment caused a 16% decrease in disease progression – defined by first occurrence of CD4 cell count < 200/mm$^3$ – in HIV and HSV-2 co-infected individuals, highlighting the impact on disease outcome [166]. Another study demonstrated that acyclovir 400mg twice daily delayed disease progression – defined by CD4 cell count <250/mm$^3$ – but greater in HIV and HSV-2 co-infected individuals who had HIV viral load > 50,000 copies/mL [167]. Although the benefits of acyclovir and valacyclovir are clear, their mechanism remains unclear. It is likely that herpes therapy works both indirectly and directly against HIV. HSV-2 reactivation will lead to localized immune activation and will result in an influx of CD4+ T cells [129]. This provides an opportunity for HIV to replicate in these activated CD4+ T cells and may lead to the increase in plasma HIV VL [147]. By reducing the frequency of HSV-2 reactivation, herpes treatment could indirectly benefit HIV pathogenesis. Recent *in vitro* studies have also demonstrated that acyclovir can act directly against HIV [168, 169]. Acyclovir can be incorporated into HIV DNA and reduce viral replication [168, 169]. Although the exact mechanism may be hard to replicate *in vivo*, it is likely that the combination of both indirect and direct effects of HSV-2 therapy on HIV are responsible for its anti-viral activities against HIV.
Chapter 2

2 Clinical trial protocol: the impact of herpes therapy on the genital and systemic immunology of HSV-2 infected, HIV uninfected women
Note: This chapter covers the introduction and methods of the clinical trial that observed the impact of herpes therapy on the genital tract and systemic immunology of HIV uninfected, HSV-2 infected women. The results for the genital tract immunology are shown in chapter 3 and systemic immunology in chapter 4.

2.1 Introduction

The primary route of HIV transmission is sexual [170]. Although the great majority of sexual exposures to HIV do not result in productive infection, transmission is enhanced in the presence of various genital sexually transmitted infections (STIs) [171, 172]. Chronic infection by herpes simplex virus type 2 (HSV-2) is generally asymptomatic, and is associated with a roughly 3-fold increase in HIV acquisition risk after exposure [126]. Therefore the high prevalence of HSV-2 infection in sub-Saharan Africa, where over 50% of sexually active adults are infected in many countries [98], means that HSV-2 may be an important driver of HIV transmission in the region [127].

The exact sequence of events that culminates in productive HIV infection after a sexual exposure to HIV at the genital mucosa remains unclear. Genital inflammation, as indicated by elevated levels of pro-inflammatory cytokines and RANTES in genital secretions, is associated with an increased risk of HIV acquisition [72, 73]. Such inflammation is associated with the recruitment of susceptible target cells to the genital tract, as demonstrated by the strong correlation between genital RANTES and IL1β levels and the number of CD4+ T cells in the cervical mucosa [74]. However, it is not known whether specific CD4+ T cell subsets are needed for the establishment of productive infection, such as activated T cells, Th17 cells or those expressing CCR5 and/or α4β7 [75]. Local dendritic cell (DC) subsets may also play an
important role in susceptibility, particularly those expressing C-lectin receptors such as DC-SIGN and mannose receptor that can bind HIV gp120 and enhance subsequent T cell infection and dissemination.

In keeping with these cell subsets being key to mucosal HIV acquisition, the number of activated CD4+ T cells and DC-SIGN expressing dendritic cells was increased in the cervix of HSV-2-infected asymptomatic female sex workers from Nairobi, Kenya [128], a population where asymptomatic HSV-2 infection was prospectively associated with a six-fold increase in HIV incidence [173]. In addition, sequential biopsies at the site of a clinical herpes ulcer demonstrated prolonged increases (beyond two months) in CD4+ T cell and dendritic cell numbers at the prior ulcer site [129]. However, despite the strong epidemiological and immunological evidence that HSV-2 infection enhances HIV susceptibility, two clinical trials demonstrated that HSV-2 suppression by acyclovir had no impact on HIV incidence in HSV-2-infected individuals, with a non-significant trend to increased risk in the acyclovir arm of both trials [130, 131]. This suggests that, while HSV-2-associated increases in HIV susceptibility may be mediated by increases in HIV target cells within the genital mucosa, persistence of these changes despite acyclovir therapy may underlie the failure of herpes suppression to reduce HIV incidence.

Recent trials suggest that valacyclovir may be more potent than acyclovir in terms of HSV-2 suppression [125, 174]. Therefore, to investigate a possible role for valacyclovir in HIV prevention among asymptomatic, HSV-2-infected individuals, we examined the impact of valacyclovir therapy on the immune correlates of HIV susceptibility in the cervix, using a randomized, double-blinded, placebo-controlled, cross-over study format. We found that two
months of oral valacyclovir was not associated with any alteration in putative HIV target cells, either T cells or dendritic cell subsets.

Furthermore, the genital immune alterations observed in HSV-2 infected women are associated with systemic immune activation and increased expression of the mucosal homing integrin α4β7 on blood T cells. The latter is important since T cells expressing this integrin are not only more activated (and hence thought to be more HIV susceptible) [175], but α4β7 may also interact directly with HIV-1 gp120 to enhance HIV entry and cell-cell spread [176]. While the genital immune changes persist despite antiviral treatment of acute HSV-2 clinical episodes or longer-term suppressive therapy, the impact of herpes treatment on systemic immune alterations associated with HSV-2 infection has not yet been described to our knowledge.

In herpes-HIV co-infected individuals, herpes therapy has consistently decreased the HIV plasma viral load – by approximately ~0.5 log_{10} copies/mL – and also slows HIV disease progression to a modest degree [166, 167]. The mechanism(s) of these benefits in herpes-HIV co-infected people is not clear. Specifically, it is not known whether the reduction in HIV viral load (VL) and disease progression is a consequence of a direct effect of herpes antivirals (acyclovir or valacyclovir) on the HIV virus itself, or whether benefits are mediated through a reduction in virus reactivation and the associated mucosal and systemic immune activation. However, the rapid drop in HIV VL upon initiation of valacyclovir therapy suggests a direct antiviral effect, and such an effect can be demonstrated in vitro [168, 169]. Furthermore, cross sectional studies in herpes-HIV co-infected individuals have not shown an effect of valacyclovir therapy on systemic immune activation [177].

To further explore this issue, as well as the potential host benefits of herpes suppression in HIV-uninfected individuals, we conducted a secondary analysis of the trial to examine the
systemic immune impact of herpes suppression. In this secondary analysis we find that daily valacyclovir therapy had no impact on systemic immune activation or the expression of the mucosal homing integrin α4β7 on blood T cells.
2.2 Methods

2.2.1 Participant enrolment and inclusion criteria.

Participants were recruited from a larger study defining the epidemiology of several genital co-infections in clinic-based cohort of African / Caribbean women in Toronto, Canada [178]. HIV-uninfected participants who were found to be HSV-2 infected based on adjusted threshold value of 3.5 using HerpeSelect® gG-1 and gG-2 ELISA (Focus Technologies, Cypress, CA) and who had consented to be contacted regarding future research studies, were approached. Exclusion criteria included any symptomatic genital infection during the past three months; asymptomatic Trichomonas vaginalis, Neisseria gonorrhoea or Chlamydia trachomatis infection at screening; and the use of any herpes suppressive medication (see ClinicalTrials.gov: #NCT00946556). Informed written consent was obtained from all participants, and the study protocol was approved by the HIV Research Ethics Board at the University of Toronto.

2.2.2 Study protocol and sampling.

An external pharmacist disbursed 30 tablets of active drug or identical placebo into 4 containers, marked only with a study identification code and study period “A” (two containers) or “B” (two containers) based on study codes that were randomly assigned 1:1 by the study biostatistician to either oral valacyclovir 1 g once daily or identical placebo during period “A”. After a one-month washout period, each code crossed over to the alternate regimen during period “B”. Drug and placebo were purchased from Apotex Inc. (Toronto, Canada). Only the study biostatistician had access to the randomization codes; both research personnel and participants were blind to group allocation.
Participants were followed up monthly during the study protocol for a total of six visits, with blood and cervical cytobrushes collected within 10-18 days of the last day of menstrual period. At each visit, three 8ml vials of venous blood were collected into acid citrate dextran (ACD; BD Biosciences), undiluted cervicovaginal secretions were self-collected into an Instead Softcup (Evofem) (Figure 2.2a), and a first-void urine sample was provided. Participants placed the Instead Softcup in to the vagina for at least a minute before removing it. The study physician then performed an internal exam to collect two cotton-tipped and one Dacron vaginal swabs from the posterior fornix, and one Dacron swab and two cytobrush samples from the endocervix. Each cytobrush was gently inserted into the cervical os, rotated 360° (Figure 2.2b), placed into R10 medium at 4°C and transported to the lab within 3 hours. A short questionnaire (see Appendix) regarding sexual behavior, contraceptive and other medication use and genital symptoms was completed at each visit, and adherence was estimated by pill count.
2.2.3 Diagnostic tests.

At each visit, two vaginal swabs were collected to test for Bacterial Vaginosis, and *Trichomonas vaginalis*. A vaginal swab was sent to Mount Sinai to test for Bacterial Vaginosis according the Nugent score and the other vaginal swab was used to test for *Trichomonas vaginalis* using OSOM Trichonomas Rapid Test (Sekisui). A urine sample was collected to test for *Neisseria gonorrhoea* and *Chlamydia trachomatis* for presence of antigens (Mount Sinai). Cervical mucus collected from the Instead cup was used to detect HSV-2 shedding from the participants. DNA from the samples were extracted using Miniprep Qiagen DNA extraction kits (Qiagen) and following their instructions. HSV-2 shedding was detected using real-time PCR assay described elsewhere[179] with minor adjustments. For master mix, SuperMix (Invitrogen)
was used. Also, instead of using the LC instrument, Rotorgene 6000 (Qiagen) at Mount Sinai Hospital, Microbiology Laboratory in Toronto ON was used.

2.2.4 Cervical immune cell populations.

Cells from the two cervical cytobrushes were combined, filtered through a 100µm filter, washed and divided into two equal aliquots for staining with a panel of T cell or dendritic cell (DC) markers. The T cell panel consisted of α4-FITC (Miltenyi), CD4-ECD (Beckman Coulter), CCR5-PE, β7-APC, CD38-AlexaFluor700, HLA-DR-APC-Cy7, CD69-eFluor450 (BD Biosciences), Live/Dead Aqua (Invitrogen), CD25-PerCP-Cy5.5, CD39-PE-Cy7, CD3-eFluor650 (eBioscience). The DC panel consisted of BDCA2-FITC (Miltenyi), CD207-PE (Beckman Coulter), DC-SIGN-PerCP-Cy5.5, CD206-APC (BD Biosciences), CD83-Streptavidin, CD123-PE-Cy7, CD11c-AlexaFluor700, CD14-AlexaFluor780, CD1a-v450, CD3-eFluor650 (eBioscience), Live/Dead Aqua (Invitrogen). Cells were enumerated using a BD LSR-2 flow cytometer (BD Biosciences) and analyzed with FlowJo 9.0 software (Treestar).

2.2.5 Chemokine and cytokine assays/analysis.

Undiluted cervicovaginal secretions (see above) were diluted 10 fold in phosphate buffered saline (PBS) and stored at -80. Levels (pg/mL) of IL-1α, IL-8, MCP-1, MDC, MIG, MIP-3α, RANTES, IL-1β and IP-10 were then measured in thawed samples using an electrochemiluminescent ELISA platform (Meso Scale Discovery). Levels were then corrected for dilution, to give the actual concentrations present in undiluted cervical secretions.
2.2.6 Blood T cell phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll-hypaque density centrifugation and then counted and washed twice. Freshly-isolated PBMCs collected at baseline and the end of each study phase (i.e.: at months 0, 2 and 5) were stained by blinded research personnel using the following panel of antibodies: α4-FITC (Miltenyi), CD4-ECD (Beckman Coulter), CCR5-PE, β7-APC, CD38-AlexaFluor700, HLA-DR-APC-Cy7, CD69-eFluor450 (BD Biosciences), Live/Dead Aqua (Invitrogen), CD25-PerCP-Cy5.5, CD39-PE-Cy7, CD3-eFluor650 (eBioscience). The panel did not include CD8 and, therefore, CD3+CD4- lymphocytes were analyzed as a surrogate for CD8 T cells. Cryopreserved samples from the same time point were used to enumerate regulatory T cells (Tregs). The Treg panel consisted of CD3-PerCP, CD4-APC (BD Biosciences), FOXP3-AlexaFluor488, CD25-PE, CD127-AlexaFluor780 (eBioscience) and Live/Dead Aqua (Invitrogen). Cells were enumerated using an LSR-2 flow cytometer (BD Biosciences) and analyzed with FlowJo 9.0 software (Treestar).

2.2.7 Statistical analysis.

The sample size was calculated based on the standard deviation in the log_{10} absolute CD4+ T cell numbers of 0.62 and with 30 participants, the study will be able to calculate a detectable difference of 0.289 log_{10} CD4+ T cells per cytobrush at 8 weeks, with a significance level of 0.05 and 80% power. Once the predetermined sample size had been reached and the results of all diagnostic tests, immune assays and questionnaires had been entered into a study database, the study codes were broken and analysis performed by the data team. The predefined primary endpoint of the study was the change in the absolute number of CD4+ T cells per
cytobrush during the valacyclovir vs. the placebo phase. The secondary endpoints were changes in (1) the absolute number of immature dendritic cells and (2) the proinflammatory cytokine/chemokine levels in cervicovaginal secretions between placebo and valacyclovir phase. All statistical analyses of immune cell subsets were performed using SAS® Software Version 9.3 (SAS Institute Inc., Cary, NC, USA). The comparison of the difference in percentage and absolute number of T cell and DC population in the genital tract during each phase was done using a paired-T parametric test. The repeated measurements of these cells were also analyzed using generalized linear regression models with random effect to account for the carryover effect and the period effect inherent in the cross-over study. The chemokine and cytokine analysis used a paired Wilcoxon test to compare baseline levels with those at the completion of placebo or valacyclovir phases (SPSS version 19; IBM). All analyses were performed by a biostatistician independent of the main research team.

Secondary analysis of the trial assessed the impact of valacyclovir on systemic immunology: the changes in frequencies of T cells subsets in blood between the placebo and valacyclovir phases. The Wilcoxon Signed-Ranks test was used to compare the change in the frequencies of T cells subsets during the valacyclovir versus placebo phases. All statistical analyses were performed using SPSS version 20 (IBM).
3 Valacyclovir suppressive therapy does not alter herpes-associated alterations in genital immunology: a randomized, placebo-controlled crossover trial
3.1 Abstract

**Background.** Asymptomatic infection by herpes simplex virus 2 (HSV-2) increases HIV acquisition risk more than 3-fold, partly due to long-lived alterations in genital immunology. Since clinical trials of herpes suppression have failed to reduce HIV incidence, we performed a clinical trial to assess the impact of valacyclovir suppressive therapy on genital immunology.

**Methods.** HSV-2 infected, HIV-uninfected African/Caribbean women from Toronto, Canada were administered 1g of oral valacyclovir or an identical placebo daily for 2 months in a double-blind, placebo-controlled fashion [#NCT00946556], followed by a one month washout period and crossover to the alternate regimen. Genital co-infection diagnostics were performed monthly, cytobrush-derived cervical CD4 T cell numbers and subsets assessed by flow cytometry, and genital cytokines were assessed by multiplex chemiluminescent ELISA. The primary endpoint was the change in cervical CD4+ T cell and dendritic cell numbers/cytobrush after two months.

**Results.** A total of 30 participants completed the study protocol and were included in the final analysis. The median age was 44 years (range, 24-66y), and 3/30 (10%) had a previous history of clinical herpes. No clinical herpes outbreaks were observed during the trial, and asymptomatic HSV shedding was detected at 4/148 (3%) of visits. Valacyclovir was not associated with any change in the absolute number of cervical CD4 T cells or dendritic cells/cytobrush (P>0.45). There were no changes in cervical pro-inflammatory cytokine levels, and a trend to increased expression of CCR5 and the activation marker CD69.

**Conclusion.** Valacyclovir therapy did not alter genital immune parameters previously linked to HSV-2 associated increases in HIV susceptibility. More potent regimens may be needed if HSV-2 suppression is to reduce HIV acquisition.
3.2 Results

3.2.1 Participant demographics

Participants were recruited from 419 African / Caribbean women who had participated in a prior clinic-based study of the epidemiology of HIV and genital co-infections (see above). Of these 419 participants, 136 met key eligibility criteria of HSV-2 seropositivity and were HIV-1 seronegative; an additional 17 were excluded due to the diagnosis of a classical sexually transmitted infection or prior herpes medication use, leaving 119 potential participants (Figure 1). All 119 women were contacted and invited to participate in the study protocol; 35 consented and were enrolled. Five participants (9%) did not complete the protocol: one participant became pregnant, one participant withdrew due to headaches during the valacyclovir phase, and 3 participants were lost to follow-up after their first visit.

Only data from the 30 participants (91%) who completed both phases the protocol were included in the final analysis, since the analysis involved a comparison of genital immune changes on and off valacyclovir, and could not be performed for participants with incomplete follow up. Table 1 shows the enrollment characteristics of the 30 participants included in the final analysis. The median age was 44 years (range 24-66 y). All participants were dually-infected by HSV-1 and HSV-2. Ten participants (33%) were post-menopausal and 2 (7%) were taking oral contraceptives. At baseline, the prevalence of bacterial vaginosis (BV) and abnormal vaginal flora (AVF) were 14% and 40% respectively. All participants were uninfected by Trichomonas vaginalis, Neisseria gonorrhoea and Chlamydia trachomatis. Adherence to the study medication was 89%, as estimated by pill count at monthly follow up visits, and did not vary by study phase.
3.2.2 Impact of valacyclovir on the absolute number of cervical CD4 T cells and dendritic cells.

The mean number of CD4 T cells per cytobrush at the start of placebo and valacyclovir phases was 2.57 log_{10} and 2.47 log_{10} respectively (Table 2; representative plots illustrating the flow cytometry gating strategy used are shown in Figure 2). The change in the absolute number of CD4 T cells from baseline to 8 weeks was −0.06 log_{10} cells/cytobrush during the placebo phase and +0.08 log_{10} cells/cytobrush during the valacyclovir phase, with no difference between these two phases (P=0.45; Figure 3). Four distinct dendritic cell subsets were assessed in the cervix: Langerhans cells, defined by the co-expression of CD207 (Langerin) and CD1a; plasmacytoid dendritic cells, defined by the co-expression of CD123 and BDCA2; monocytes, defined by the expression of CD14; and myeloid derived dendritic cells, defined by the expression of CD11c. Insufficient plasmacytoid dendritic cells were present on a cytobrush for meaningful analysis (data not shown). The mean number of Langerhans cells per cytobrush was 1.84 log_{10} at the start of the placebo phase and 1.86 log_{10} and the start of the valacyclovir phase, with no difference between phases in the change over 8 weeks (+0.07 log_{10} and +0.08 log_{10}/cytobrush, respectively; P=0.92; Figure 3). The mean number of monocytes per cytobrush was 3.03 log_{10} and 3.00 log_{10} at the start of placebo and valacyclovir, respectively, and there was no difference in the change over 8 weeks (-0.13 log_{10} for placebo and -0.03 log_{10} for valacyclovir; P=0.71; Figure 3). Lastly, the mean numbers of myeloid derived dendritic cells were 3.20 log_{10} and 3.22 log_{10} per cytobrush at the start of therapy, placebo and valacyclovir, respectively; again, numbers remained similar in the placebo and valacyclovir phases (-0.29 log_{10} and -0.09 log_{10}, respectively; P=0.45; Figure 3).
3.2.3 Impact of valacyclovir on T cell and dendritic cell subsets relevant to HIV susceptibility.

HIV preferentially infects activated T cells expressing the co-receptor CCR5, and HIV infection may be facilitated by dendritic cells expressing the lectin receptors DC-SIGN and CD206 (mannose receptor). Therefore we examined the impact of valacyclovir therapy on these specific T cell and DC subsets. Representative flow gating algorithms for these subsets are depicted in Figure 2. T cell subset analysis demonstrated a trend to an increase in the proportion of cervical CD4+ T cells expressing the HIV co-receptor CCR5 during valacyclovir therapy (-5.11% on placebo vs. +9.31% on valacyclovir, P=0.10; Figure 4) and a significant increase in expression of the early activation marker CD69 (-7.16% vs. +7.81% respectively, P=0.01; Table 2). However, this change did not remain significant in a subsequent multivariate analysis that controlled for study period and cross over effects (Table 2). No differences between the placebo and valacyclovir treatment phases were seen in the proportion of cervical T regulatory CD4+ T cells co-expressing CD25 and CD39 (-2.26% on placebo vs. -1.21% on valacyclovir, P=0.69), CD4+ T cells co-expressing the chronic immune activation markers CD38/HLA-DR (-4.81% on placebo vs. -2.28% on valacyclovir, P=0.69), or cervical expression of the mucosal homing integrin α4β7 on CD4+ T cells (-0.92% on placebo vs. -1.40% on valacyclovir, P=0.90).

Co-expression of each of DC-SIGN and CD206 (mannose receptor) was then assessed on CD11c+ myeloid-derived dendritic cells (mDCs) and CD14+ monocytes. The change in the absolute number of CD11+ mDCs expressing DC-SIGN did not differ between the placebo and valacyclovir treatment phases (-0.05 log₁₀ on placebo vs. -0.03 log₁₀ on valacyclovir, P=0.91; Figure 4) and nor did the absolute number of CD11+ mDCs that expressed CD206 (-0.17 log₁₀ placebo, 0.19 log₁₀ valacyclovir, P=0.30; Figure 4). There was also no change in the absolute
numbers of CD14+ monocytes that expressed either DC-SIGN (0.06 log_{10} placebo, 0.01 log_{10} valacyclovir, P=0.92; Figure 4) or CD206 (0.26 log_{10} placebo, 0.00 log_{10} valacyclovir, P=0.49; Figure 4) during the placebo or valacyclovir treatment phases.

### 3.2.4 Cytokine and chemokine levels in the female genital tract

Cytokine and chemokine levels were measured in cervicovaginal secretions that had been self-collected using the Instead Cup™, and are presented as pg of cytokines per g of undiluted secretions. Levels were measured in samples collected at study baseline and at the end of each treatment phase (ie: after 8 weeks of placebo and valacyclovir). The mean levels of cytokines present at the baseline were as follows (Figure 5): IL-1α (8584.2 pg/mL), IL-8 (12139.6 pg/mL), MCP-1 (917.4 pg/mL), MDC (3928.0 pg/mL), MIG (1377.9 pg/mL), MIP-3α (1181.9.3 pg/mL), RANTES (86.5 pg/mL), IL-1β (955.6 pg/mL) and IP-10 (1637.9 pg/mL) (Figure 5). There were no significant changes in the levels of any cytokine/chemokine at the end of two phases (placebo and valacyclovir): IL-1α (10282.1 pg/mL and 6870.4 pg/mL), IL-8 (12238.8 pg/mL and 15102.4 pg/mL), MCP-1 (854.3 pg/mL and 1300.1 pg/mL), MDC (4315.8 pg/mL and 3749.9 pg/mL), MIG (1423.8 pg/mL and 1783.7 pg/mL), MIP-3α (1057.1 pg/mL and 1362.0 pg/mL), RANTES (88.8 pg/mL and 91.5 pg/mL), IL-1β (1487.5 pg/mL and 1894.5 pg/mL) and IP-10 (1786.4 pg/mL and 3382.7 pg/mL)
3.2.5 Genital HSV-2 shedding

PCR was used to screen for HSV-2 shedding in cervico-vaginal secretions at 174 asymptomatic participant visits out of 180 total visits. HSV-2 shedding was detected at 4/180 study visits, 1/60 visits on valacyclovir therapy and 3/120 visits on no treatment or placebo.
3.3 Discussion

HSV-2 infection is consistently associated 3-6 fold increase in HIV susceptibility [126, 173] and genital immune alterations. Studies demonstrate that HSV-2 was associated with recruitment of HIV target cells both at the site of a clinical ulcer [129] and more diffusely in the foreskin and endocervix in the absence of clinical disease [128, 180, 181]. These immune alterations include an increase in overall CD4+ T cell numbers, increased immune activation and expression of the HIV co-receptor CCR5, and increased expression by dendritic cells of HIV-binding lectin receptors such as DC-SIGN and mannose receptor (CD206). Therefore the failure of acyclovir in reducing HIV acquisition in two large randomized clinical trials was a surprise and both trials results were clear with a non-significant trend to increased HIV acquisition [130, 131]. In order to clarify the potential biological rationale for further trials of herpes suppression for HIV prevention, we performed a randomized, placebo-controlled, double-blinded crossover trial to examine the impact of valacyclovir in standard suppressive doses on these genital immune parameters in women with asymptomatic HSV-2 infection. Our primary endpoints were the change from baseline in the absolute number of CD4+ T cells and of dendritic cells in the cervix, and we found that two months of valacyclovir therapy had no significant effect on these parameters.

Our secondary endpoints examined sub-properties of these cervical cells that included their immune activation and expression of molecules previously described to enhance HIV infection, including T cell molecules that directly enhance infection (CCR5 and α4β7) [182] and the expression by dendritic cells of lectin receptors that may enhance their ability to infect T cell in trans [183-185]. Again, eight weeks of valacyclovir therapy was not associated with a
reduction in any of these parameters, and tended to increase the expression of the early activation marker CD69 and the HIV co-receptor CCR5. Although these associations were not significant in a multivariate analysis that controlled for study period and cross over effects, it is interesting to consider the direction of this association in light of the slight trend to increased HIV acquisition among participants receiving acyclovir in both trials of HSV-2 suppression (hazard ratios of 1.16 and 1.08) [130, 131]. Such an effect, if real, could be mediated by enhanced host anti-HSV immune responses in the context of effective therapy. However, testing of this hypothesis will require further research. The fact that cervico-vaginal levels of inflammatory cytokines were not elevated, including those linked with HIV acquisition in prospective studies (IL6, IL1 and others) [72, 73], argues against the induction of a significant inflammatory response by valacyclovir.

The great majority of participants in our study had asymptomatic HSV-2 infection, and it is tempting to hypothesize that this may contribute to the lack of immune effect seen in our study. While asymptomatic HSV-2 infection has been strongly associated with both HIV acquisition and altered cervical immunology in previous studies [126, 128], it remains possible that a greater immune effect might be seen in women with symptomatic disease. Two months of valacyclovir therapy is a relatively short course of suppressive therapy. However, if one assumes that HSV-associated mucosal immune alterations are induced by asymptomatic virus reactivation, the fact that an average of 16.5 HSV-2 shedding episodes were seen per year despite high-dose (1000 mg three times daily) valacyclovir therapy in a recent clinical trial [125] – a rate of more than one episode per month – suggests that simply prolonging therapy is unlikely to induce an effect. Rather, we hypothesize that the antiviral effect of valacyclovir, either in the standard dose that we used (1g daily) or in higher doses, just does not lead to potent enough virus suppression to reverse HSV-induced immune changes. Indeed, we performed a
paired analysis on the cervical immune cell composition during HSV-2 reactivation visits with the non-shedding visits and found no difference.

Our participants displayed compliance to the study drug of 89%, according to a pill count by a research coordinator. In one of the large clinical trials, they demonstrated that the study participants demonstrated high adherence through self-report; however, when they tested random urine samples for the presence of the study drug, adherence was significantly lower [131]. Although we have not performed a urine analysis, only 1 HSV-2 reactivation was detected during the valacyclovir phase while 3 were detected during placebo or no-therapy, possibly reflecting on the pill count adherence levels we have reported.

In summary, this randomized, placebo-controlled double-blinded crossover trial demonstrates that standard herpes suppressive therapy with valacyclovir is unable to ameliorate the genital immune alterations associated with HSV-2 infection. This may explain the inability of this clinical strategy to reduce HIV acquisition in recent clinical trials, and coupled with recent findings that even high dose valacyclovir therapy provides suboptimal herpes suppression, suggests that currently available antivirals are unlikely to reduce HIV susceptibility.
Figure 3.1 Trial profile

419 participants screened from previous clinic based study of HIV epidemiology in Toronto

300 excluded:
- 127 HIV+
- 156 HSV-2-
- 17 had other STIs

119 eligible for the study criteria

84 declined

35 consented, enrolled

3 lost to follow up
- 1 pregnancy
- 1 side effects

30 completed
Table 3.1 Summary of enrolled study participants

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<th>Completed Participants (N=30)</th>
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<tr>
<td>Age (range)</td>
<td>44 (24-66)</td>
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<tr>
<td>Menopause (%)</td>
<td>10 (33.3%)</td>
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<tr>
<td><strong>Behavioral Characteristics</strong></td>
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<tr>
<td>Sexually active</td>
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<tr>
<td>Last intercourse within a week</td>
<td>3 (10%)</td>
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<tr>
<td>Hormonal contraceptives</td>
<td>2 (6.7%)</td>
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</tr>
<tr>
<td>Intravaginal practices (douching)</td>
<td>5 (16.7%)</td>
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<tr>
<td><strong>Clinical Characteristics</strong></td>
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<tr>
<td>Bacterial vaginosis</td>
<td>4 (13.3%)</td>
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<tr>
<td>Altered vaginal flora</td>
<td>12 (33.3%)</td>
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Table 3.2 Changes in the number or proportion of HIV target cells in the cervical mucosa by treatment arms

<table>
<thead>
<tr>
<th>Variable</th>
<th>Change in the number of cells per cytobrush during valacyclovir (SD)</th>
<th>Change in the number of cells per cytobrush during placebo (SD)</th>
<th>Unadjusted P value</th>
<th>Adjusted P Value</th>
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</thead>
<tbody>
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<td>CD3+CD4+</td>
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<td>-0.06(0.85)</td>
<td>0.45</td>
<td>0.57</td>
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<tr>
<td>Langerhans</td>
<td>0.08(1.08)</td>
<td>0.07(0.78)</td>
<td>0.92</td>
<td>0.93</td>
</tr>
<tr>
<td>CD14+</td>
<td>-0.03(1.02)</td>
<td>-0.13(1.19)</td>
<td>0.71</td>
<td>0.81</td>
</tr>
<tr>
<td>CD11c+</td>
<td>-0.09(1.00)</td>
<td>-0.29(1.39)</td>
<td>0.45</td>
<td>0.39</td>
</tr>
<tr>
<td>CD69+</td>
<td>0.10(1.03)</td>
<td>-0.10(0.99)</td>
<td>0.25</td>
<td>0.56</td>
</tr>
<tr>
<td>CCR5+</td>
<td>0.20(0.89)</td>
<td>-0.11(0.80)</td>
<td>0.09</td>
<td>0.15</td>
</tr>
<tr>
<td>CD11c+DCSIGN+</td>
<td>-0.03(0.99)</td>
<td>-0.05(1.30)</td>
<td>0.91</td>
<td>0.51</td>
</tr>
<tr>
<td>CD11c+CD206+</td>
<td>0.19(1.33)</td>
<td>-0.17(1.44)</td>
<td>0.30</td>
<td>0.48</td>
</tr>
<tr>
<td>CD14+DCSIGN+</td>
<td>0.01(0.95)</td>
<td>0.06(1.21)</td>
<td>0.92</td>
<td>0.58</td>
</tr>
<tr>
<td>CD14+CD206+</td>
<td>0.26(1.35)</td>
<td>0.00(1.41)</td>
<td>0.49</td>
<td>0.58</td>
</tr>
<tr>
<td>%CD69+*</td>
<td>7.81(28.42)</td>
<td>-7.16(27.20)</td>
<td>0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>%CCR5+*</td>
<td>9.31(36.75)</td>
<td>-5.11(28.67)</td>
<td>0.10</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Data are mean(SD).
All absolute numbers are measured in log_{10} cells/cytobrush.
*Reported in proportion (%).
Figure 3.2 Gating strategy for cervical mononuclear cells

Each dot represents a single cell. SSC-A indicates the membrane density of cells and FSC-A indicates the size of cells. The circles and various forms of boxes indicate specific “gates” and these gates are drawn to specifically select certain population of cells. For instance, the oval gate in a) represents lymphocytes. From these lymphocytes, siglet cells are selected (b) followed by a selection of live cells (c). d) represents CD4+ T cells and gates in e) and f) represents the proportion of CD4+ T cells that express CD69 and CCR5 respectively.
c) 

---

SSC-A

Live Dead

10^5

10^4

10^3

10^2

10^1

10^0

0

0

10^5

10^4

10^3

10^2

10^1

10^0

0

0

---

d) 

CD4

CD3

28.3

---

e) 

CD4

CD69

44.2

---

f) 

CD4

CCR5

32
Figure 3.3 Changes in the number of a) CD4+ T cells, b) Langerhans cells, c) myeloid derived dendritic cells and d) monocytes between valacyclovir vs. placebo.

The straight line represents the change in the number of cells per cytobrush over the period of 2 months during valacyclovir phase, while the dotted line represents the placebo phase.
Figure 3.4 Changes in the number and expression of CCR5+ (a, c) and CD69+ (b, d) T cell subsets valacyclovir vs. placebo

The straight line represents the change in the number of cells per cytobrush (a, b) or the proportion of cells that express CCR of CD69 (c, d) over the period of 2 months during valacyclovir phase, while the dotted line represents the placebo phase.
Figure 3.5 Comparison of cytokine levels in the cervico-vaginal fluid between baseline, end of placebo and end of valacyclovir.
Chapter 4

4 Valacyclovir therapy does not alter systemic immune activation in HIV-negative individuals infected by herpes simplex virus type 2
4.1 Abstract

**Background.** Herpes simplex virus 2 (HSV-2) treatment in HIV co-infected individuals is associated with decreased plasma viral load and delayed HIV disease progression. However, it is not clear whether this is related to a direct anti-HIV effect or is mediated through a reduction in herpes-associated immune activation. In HIV uninfected individuals, HSV-2 has been associated with increased systemic immune activation and $\alpha 4\beta 7$ expression on CD4+ T cells, which correlated with the number of activated CD4+ T cells in the genital tract. To better understand the role of herpes therapy in systemic immune activation, we examined the impact of valacyclovir therapy on systemic T cells in HSV-2 infected individuals in the context of a prospective clinical trial.

**Methods.** HSV-2 infected African and Caribbean women from Toronto were recruited and administered either valacyclovir (1g po) or placebo daily for 2 months in a double-blinded, placebo-controlled crossover trial [registration #NCT00946556], followed by a one month washout period and 2 months on the alternate regimen. Blood was collected at baseline and monthly, with blinded assessment of systemic immune activation, defined as expression of CD69, CD38/HLADR on T cells in blood. The Wilcoxon Signed-Ranks test was used to compare the change in the frequency of these T cell subsets during the valacyclovir versus placebo phases.

**Results.** 30 HSV-2 infected women completed the study protocol and were included in the final analysis. Valacyclovir therapy had no impact on systemic T cell activation, the frequency of T regulatory cells or expression of the mucosal homing integrin $\alpha 4\beta 7$. 
**Conclusion.** Short-term valacyclovir therapy in women with HSV-2 infection had no impact on systemic immune activation or Treg populations.
4.2 Results

4.2.1 Participant Demographics

Detailed participant demographics have been described elsewhere [186]. In brief, participants consisted of 30 African / Caribbean women who completed both phases of the clinical trial protocol. The median age of participants was 44 years (range 24-66 years) and all participants were dually-infected by HSV-1 and HSV-2. None of the participants were taking herpes therapy prior to enrollment and 3 participants either had previous episodes of herpes or were unsure. Adherence to the study medication was 89%, as estimated by pill count at monthly follow up visits, and did not vary by study phase.

4.2.2 Association between valacyclovir and systemic immune activation

The primary endpoint of the analysis was to compare changes in the frequencies of activated T cells (CD38/HLADR and CD69), CD4 T cells expressing mucosal homing receptor α4β7 and Tregs (CD4+CD25+FOXP3+) between placebo and valacyclovir phases. There was no significant difference observed in the change of CD38+HLADR+ expression on CD4 or CD8 T cells when patients were on therapy (-0.67% vs 0.05%; P = 0.658, 0.04% vs 0.41% P = 0.614, respectively) (Fig. 2a, b). Likewise, no differences were observed in the change of proportion of CD4 T cells expressing the activation marker CD69 (-0.67% vs -0.31%; P = 0.861), the gut homing integrin α4β7 (0.53% vs 0.11%; P = 0.572) (Fig. 2c), the frequency of blood Tregs (0.01% vs 0.06%; P = 0.350) (Fig. 2d, e). Overall, treatment had no impact on T cell subsets measured in the blood.
4.3 Discussion

Clinical trials have consistently demonstrated that herpes therapy in HSV-2 and HIV co-infected individuals has been associated with a decrease in plasma HIV viral load and a modest delay in disease progression [166, 167]. However, it is unknown whether this was due to the direct anti-HIV properties of acyclovir or through the suppression of herpes reactivation and the associated inflammation. A recent study has demonstrated an association between HSV-2 infection and systemic immune activation [175]. Specifically, HSV-2 infected individuals displayed increased frequencies of activated T cells and CD4 T cells expressing α4β7 in blood compared to HSV-2 uninfected individuals. In addition, the expression of α4β7 on CD4 T cells in blood was associated with T cell activation in the blood and genital immune alterations. In a different cohort, our lab has also shown that HSV-2 infection was associated with an increase in systemic Tregs [159]. Therefore, to investigate the impact of valacyclovir on the systemic immune properties associated with HSV-2 infection, we recruited 30 HSV-2 infected individuals who underwent a randomized, placebo-controlled, double-blinded crossover trial. We concluded that valacyclovir treatment had no impact on systemic immune parameters associated with HSV-2 infection.

To our knowledge, this was the first study to examine the impact of valacyclovir on systemic immune activation in an HIV uninfected, HSV-2 infected population. Although we have specifically examined the T cell subsets that were significantly different between HSV-2 infected and uninfected individuals, valacyclovir was not able to reverse these immune changes. No effect of valacyclovir on systemic immune activation demonstrated in our study indicates that decreased plasma HIV viral load and delayed disease progression by herpes therapy in HIV co-
infected individuals is likely due to direct anti-HIV properties of acyclovir, as demonstrated in vitro [168, 169]. To support this, a cross-sectional study examining the effect of valacyclovir in HIV and HSV-2 co-infected women also concluded that valacyclovir had no impact on CD4 or CD8 T cell activation [177].

We have previously shown that T cells expressing α4β7 were more activated in blood and associated with increased number of activated T cells in the endocervix [175]. Our finding that valacyclovir had no impact on α4β7 expression on T cells in blood mirrors the primary analysis of our clinical trial, which concluded that valacyclovir had no impact on the T cell subsets in the endocervix. Although we cannot determine the causal relationship between HSV-2 associated systemic and genital immune changes, taken together, these findings suggest that valacyclovir therapy may simply not be potent enough to reverse HSV-2 associated immune changes in the blood and genital tract. Indeed, a recent study highlighted the limitations of herpes therapy, demonstrating that, on average, HSV-2 seropositive individuals shed the virus 16.5 times a year despite the use of high dose valacyclovir (1g three times a day) [125].

Overall, valacyclovir therapy had no impact on reducing systemic immune activation in HSV-2 infected women. Further studies are required to fully elucidate the mechanisms behind the association of herpes treatment and beneficial outcomes in HSV-2 and HIV infected individuals. However, our study suggests it is likely not due to a reduction in herpes-associated systemic immune activation and that other factors likely underpin the valacyclovir-induced reduction in HIV viral load and delayed disease progression.
Figure 4.1 Flow gating strategy used to identify CD4+ and CD8 T cell subsets.

Each dot represents a single cell. SSC-A indicates the membrane density of cells and FSC-A indicates the size of cells. The circles and various forms of boxes indicate specific “gates” and these gates are drawn to specifically select certain population of cells.
Figure 4.2 No difference in the change of A) CD38+HLADR+ on CD4 T cells, B) CD38+HLADR+ on CD8 T cells, C) CD69+ on CD4+ T cells, D) α4β7 on CD4+ T cells and E) FoxP3+CD25+ Tregs during valacyclovir vs placebo phases.
Chapter 5

5 A randomized controlled pilot trial of valacyclovir for attenuating inflammation and immune activation in HIV, HSV-2 co-infected adults on suppressive antiretroviral therapy:

The VALIANT study
5.1 Abstract

**Background:** HIV is associated with increased systemic inflammation and immune activation that persist despite suppressive antiretroviral therapy (ART). Herpes simplex virus type 2 (HSV-2) is a common co-infection that may contribute to this inflammation.

**Methods:** Sixty HIV, HSV-2 co-infected adults on suppressive ART were randomized 1:1:1 to 12 weeks of placebo, low-dose (500mg BID) or high-dose (1g BID) valacyclovir in this 18 week trial. Co-primary outcome measures were the percentage of activated (CD38+HLADR+) CD8 T-cells in blood, and highly sensitive C-reactive protein, interleukin-6 and soluble intercellular adhesion molecule-1 in plasma. Secondary outcomes included additional immune, inflammatory cytokine, and endothelial activation markers. The impact of valacyclovir (both groups combined) on each outcome was estimated using treatment*time interaction terms in generalized estimating equation regression models.

**Results:** Participants were mostly Caucasian (75%) men who have sex with men (80%). Median (interquartile range) age was 51 (47,56) years, duration of HIV infection 15 (8,21) years, CD4 count at enrolment 520 (392,719) cells/mm$^3$ and nadir CD4 count 142 (42,240) cells/mm$^3$. Valacyclovir was not associated with significant changes in any primary or secondary immunological outcomes in bivariate or multivariate models. Medication adherence was 97% by self-report, 96% by pill count and 84% by urine monitoring. Eight patients had adverse events deemed possibly related to study drug (5 placebo, 1 low-dose, 2 high-dose) and six patients reported at least one HSV outbreak (3 placebo, 3 low-dose, 0 high-dose).

**Conclusions:** Valacyclovir did not decrease systemic immune activation or inflammatory biomarkers in HIV-1, HSV-2 co-infected adults on suppressive ART.
5.2 Introduction

HIV infection is characterized by chronic immune activation and systemic inflammation, that are incompletely reversed despite virologically effective antiretroviral therapy (ART) [187-190]. This multifaceted inflammatory response involves immune activation, inflammatory cytokines, acute phase reactants and endothelial activation markers [47], and is felt to contribute not only to HIV disease progression, but also to mortality and emerging non-AIDS morbidity such as cardiovascular disease [47, 191-193]. Thus there is interest in identifying underlying drivers and amplifiers of HIV-related inflammation, and adjunctive therapeutic strategies for its attenuation.

Herpes simplex virus type 2 (HSV-2) may constitute one such driver, and co-infects 52-95% of people living with HIV [194, 195]. By altering genital immunology, HIV facilitates HSV-2 reactivation [128], which in turn triggers local increases in immune activation [129]. Furthermore, subclinical HSV-2 reactivations persist despite effective HIV suppression on ART [196], and previous studies have documented increased CD38 expression in both CD4+ (4.1% versus 2.9%, p=0.02) and CD8+ (7.9% versus 3.6%, p=0.002) T-cells in the setting of HSV-2 co-infection [197]. Taken together, these findings suggest that anti-HSV medications, such as valacyclovir [114], might reduce immune activation in co-infected individuals taking effective ART.

To test this hypothesis, we therefore conducted a double-blind, three-arm, placebo-controlled pilot study to assess the impact of valacyclovir on systemic immune activation and inflammation in HIV, HSV-2 co-infected adults on successful ART (VALacyclovir for Inflammation AttenuatioN Trial, VALIANT).
5.3 Methods

5.3.1 Study Participants

Participants were recruited from an academic clinic in Toronto, Canada. Inclusion criteria were age >18 years, HIV-1 and HSV-2 seropositivity, no recent (6 months) nor anticipated chronic anti-HSV therapy, sustained plasma HIV RNA<50 copies/mL on ART for ≥12 months, and no opportunistic infection for ≥12 months. HSV-2 status was determined by HerpeSelect® gG-1 and gG-2 ELISA (Focus Technologies, Cypress, CA), with primary analyses employing the manufacturer’s recommended threshold of 1.1 for defining seropositivity. Hepatitis B or C co-infection, cancer chemotherapy, estimated creatinine clearance <30mL/min, planned or active pregnancy, other terminal illness, or enrolment in any other clinical trial were exclusion criteria.

5.3.2 Study procedures

Enrolled participants were randomized 1:1:1 to receive placebo, 500mg valacyclovir twice daily, or 1g valacyclovir twice daily. Blinded study drug was administered for 12 weeks. Participants underwent study visits at baseline, 6-, 12- and 18-weeks, including clinical assessments, adherence assessments (pill count, self-report, and urine valacyclovir monitoring) and blood draws (CD4 cell count, plasma HIV RNA at 12 weeks, complete blood count, creatinine and immune/inflammatory markers). The six week post-treatment follow-up period was designed to assess for a rebound effect, and to observe for changes over time related to ART alone.
Co-primary outcome measures were the percentage of activated (CD38+HLADR+) CD8+ T-cells, highly sensitive C-reactive protein (hsCRP), interleukin-6 (IL-6) and soluble intercellular adhesion molecule 1 (sICAM-1), representing T cell immune activation, the acute phase response, pro-inflammatory cytokines, and endothelial activation markers, respectively. The primary hypothesis was that valacyclovir (both treatment arms pooled) would decrease marker levels among HIV-1, HSV-2 co-infected adults with ART-induced HIV virologic suppression; if any decreases were observed then a larger trial would be designed, informed by the results of this pilot study.

Secondary outcome measures included additional immune (CD8 exhaustion, CD4 exhaustion, regulatory T-cells), inflammatory cytokine (IL-1β, monocyte chemoattractant protein-1, MCP-1; tumour necrosis factor, TNF), and endothelial activation (soluble vascular cellular adhesion molecule-1, sVCAM-1; and the ratio of angiopoietin 1 to 2, Ang1/Ang2 ratio) markers. Episodes of symptomatic HSV reactivation, HIV virologic blips (single episodes of plasma HIV RNA >50 but <1000 copies/mL), changes in CD4 count, and adverse events were also recorded.

Informed consent was obtained from all participants before any study procedure. The study protocol was approved by the University Health Network Research Ethics Board. The trial was registered at [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov) (NCT01176409).

### 5.3.3 Study drug, assignment and blinding

The allocation schedule was developed using a computerized random number generator. Allocation concealment was achieved by having two research nurses, with no other roles in the
study, package study drug into sequentially numbered, opaque study packs. Valacyclovir 500 mg and matching odourless placebo tablets were purchased from Apotex Inc. (Toronto, Canada). The randomization code was held unbroken in a secure location throughout the trial by a single biostatistician; all other study personnel were blinded.

5.3.4 T Cell Activation and Regulatory T cells

Peripheral blood mononuclear cells (PBMCs) were initially cryopreserved at -150°C for batch testing. Samples were thawed incubated overnight at 37°C in 5% carbon dioxide before staining. Cells were then counted and 1 million PBMCs were stained for T cell activation, consisting of CD3-PerCP, CD4-APC, HLADR-APCCy7 (BD Biosciences), CD8-AlexaFluor700, CD38-PE, PD-1-PECy7, TCRαβ-FITC (eBioscience) and Live/Dead Aqua (Invitrogen). Another 1 million PBMCs were used to stain for Regulatory T cells (Tregs) with CD3-PerCP, CD4-APC (BD Biosciences), CD25-PE, CD127-AlexaFluor780 (eBioscience) and Live/Dead Aqua (Invitrogen), followed by a 30 minute incubation with Fixation/Permeabilization (eBioscience), washed and then stained for FoxP3. Activated T cells and Tregs were enumerated using LSR-2 flow cytometry (BD Biosciences) and analyzed using FlowJo 9.0 (Treestar).

5.3.5 Plasma biomarkers of inflammation

Aliquots of plasma were stored at -80°C prior to batch testing. Plasma concentrations (dilution factor in parentheses) of hsCRP(1:5000), IL-6 (1:2), IL-1β (1:2), MCP-1 (1:2), TNF
(1:2), sICAM (1:1000), sVCAM (1:2000), Ang-1 (1:5), and Ang-2 (1:5), were measured by ELISA (R&D Systems, Minneapolis MN) according to the manufacturers' instructions with the following changes: assays were performed in volumes of 50 µL/well; plasma samples were incubated overnight at 4°C; and ELISAs were developed using Extravidin®-Alkaline Phosphatase (Sigma, 1:1000 dilution, 45 min incubation) followed by addition of p-Nitrophenyl phosphate substrate (Sigma) and optical density readings at 405 nm.

Concentrations were interpolated from 4-parameter-fit standard curves. Background levels were determined from blank wells included on each plate (assay buffer added instead of sample), and subsequent optical densities were subtracted from all samples and standards prior to analysis. Samples with optical densities below the lowest detectable standard were assigned the value of that standard. The lower limits of detection for each assay were as follows: Ang-1 (19.53 pg/mL), Ang-2 (13.67 pg/mL), sICAM (7.81 pg/ml), sVCAM (3.91pg/ml), hsCRP(9.76 pg/mL), IL-1β (0.98 pg/ml), IL-6 (2.34 pg/ml), TNF (7.81 pg/ml) and MCP-1 (7.81 pg/ml).

5.3.6 Statistical Analysis

Baseline characteristics and outcomes were summarized by treatment group with frequencies and proportions for categorical variables and median and interquartile ranges (IQR) for continuous variables.

Pill counts at week 6 and 12 were combined to calculate total proportions of pills taken. Self-reported adherence scores were calculated at week 6 and 12 using the AIDS Clinical Trials Group adherence questionnaire as proposed by Reynolds et al [198], and averaged for each patient.
Generalized estimating equation (GEE) regression models using exchangeable correlation matrices were used to evaluate the effect of treatment (valacyclovir groups pooled) on each outcome over time [199]. Inflammatory markers were treated as continuous variables (with logarithmic transformations as appropriate) in linear regression models, or as binary variables (detectable versus undetectable) in logistic regression models. Time was treated as a four-level categorical variable: baseline, week 6, week 12, and week 18. The effect of treatment at each time point was estimated with treatment*time interaction terms, using multivariable models with main effects for treatment and time.

Change in CD4 count from baseline to week 12 was compared among treatment groups using the Wilcoxon rank sum test, while other clinical outcomes were compared using Fisher’s exact tests.

In post-hoc analyses of the four co-primary outcomes, valacyclovir ‘responders’ were defined as those with a negative slope on linear regression of marker levels from baseline to week 12 for continuous outcomes, or a transition from detectable to non-detectable marker levels at week 12 for binary outcomes. Baseline characteristics among responders and non-responders were compared using the Wilcoxon rank sum test for continuous variables and the chi-square or Fisher’s exact test as appropriate for categorical variables.

Because some studies have recommended higher laboratory thresholds for defining HSV-2 seropositivity among HIV patients using the HerpeSelect® 2 ELISA [200-202], sensitivity analyses were conducted using a cutoff of 3.5; participants with results equal to or less than 3.5 were excluded from this analysis.
All statistical analyses were performed using SAS Version 9.3 (SAS Institute Inc., Cary, NC, USA).

### 5.3.7 Sample size considerations

The sample size of 60 participants was determined primarily based on feasibility considerations. Using equations that account for correlations between repeated measures and unequal group sizes [203], this sample size had 80% power to detect a minimum difference in the percentage of activated CD8+ T-cells of 2.4% between those receiving valacyclovir and those receiving placebo using a two-sided alpha threshold of 0.05. Estimates for the variance in the percentage of activated CD8+ T-cells among HIV-infected adults on suppressive ART were based on published data from patients achieving HIV viral loads <75 copies/mL, in which the median (IQR) percentage of activated CD8+ T-cells was roughly 11 (7, 15) % [190, 204].
5.4 Results

5.4.1 Participant characteristics

Of 112 patients screened for study eligibility, 52 were excluded (11 withdrew consent, 38 were HSV-2 seronegative, 2 were not suppressed on ART, 1 had used anti-HSV therapy) and 60 participants were enrolled, including 20 in each treatment arm (Figure 1). Most (88%) were men, and median (IQR) age was 51 (47, 56) years. Median nadir CD4 count was 142 (42, 240) cells/mm$^3$ while current CD4 count was 520 (392, 719) cells/mm$^3$. Participants had been on ART for a median of 9 (5, 13) years. Overall, baseline characteristics including demographics, active comorbidities, years on ART and smoking status were balanced between study arms (Table 1). The proportions of participants reporting a history of prior HSV symptoms were 55%, 67% and 74% for the placebo, low-dose and high-dose valacyclovir arms, respectively. All enrolled participants completed all four study visits.

5.4.2 Medication adherence

Overall median (IQR) adherence levels by pill count for the placebo, low-dose valacyclovir and high-dose valacyclovir arms were high and similar across study arms, at 98 (87,100)%%, 96 (86,100)% and 93 (82,99)% respectively. Similarly, median (IQR) adherence by self-report was 98 (91,100)%, 96 (91,100)% and 96 (90,100)% respectively. Of the 40 participants randomized to receive valacyclovir, 38 (19 low-dose, 19 high-dose) and 37 (19 low-dose, 18 high-dose) had detectable urine valacyclovir levels at the 6- and 12-week visits respectively; no placebo participants had detectable drug levels at any time point.
5.4.3 Impact of valacyclovir on immune and inflammatory markers

Figure 2 illustrates the flow cytometry gating strategy used to identify CD38+HLADR+CD8 T cells. Compared to placebo, participants taking valacyclovir had no significant change in T cell activation at any time point, with model estimates of -0.08 (95% confidence interval, 95%CI=-0.19,0.02) at week 6 of treatment, -0.01 (95%CI=-0.12,0.11) at week 12 of treatment, and -0.06 (95%CI=-0.18,0.06) at week 18, six weeks after finishing study drug (Table 2). No changes were seen in multivariable analysis, controlling individually for nadir CD4 count, age, duration of ART use and current CD4 count (data not shown).

Results for hsCRP, IL-6 and sICAM-1 also showed no effect of valacyclovir at any time point, as shown in Table 2. Similarly, no effect was seen for any secondary immune or inflammatory markers at any time point. There was no apparent evidence for a valacyclovir dose-response relationship for any marker examined (data not shown). Results did not qualitatively change in sensitivity analyses restricted to the 49 participants with high HSV-2 index values.

5.4.4 Post-hoc analyses

In exploratory analyses of the four co-primary outcome measures, participants who responded to valacyclovir were no more likely to report a prior history of HSV symptoms than non-responders. In fact, valacyclovir responders were less likely to report prior HSV symptoms, with respect to T cell activation (89% versus 53%, p=0.02); numerically similar results were seen
for hsCRP (76% versus 63%, p=0.37), sICAM-1 (83% versus 58%, p=0.09), and IL-6 (72% versus 0%, p=0.12), although these findings did not reach statistical significance. No other baseline characteristics differentiated primary outcome responders and non-responders.

5.4.5 Clinical outcomes and adverse events

Ten symptomatic HSV reactivations were documented among six participants, seven among three placebo participants and three in low-dose valacyclovir participants (Table 3). All episodes among low-dose participants occurred after week 12, when valacyclovir was discontinued. Only one participant (in the placebo group) experienced an HIV viremia blip. Changes in CD4 count from baseline to week 12 were similar between arms, with median (IQR) changes of 16 (-23,41), 17 (-68,42), and -11 (-53,31) cells/mm3 for placebo, low-dose, and high-dose respectively (p=0.71). Eight participants experienced ten adverse events possibly related to study drug. Of these, three participants receiving valacyclovir had four events (two of grade 1 nausea, one of grade 1 diarrhea, one of grade 1 headache). Five patients had a total of 9 adverse events leading to temporary drug interruptions lasting 1-21 days; no participants prematurely discontinued study drug due to adverse events.
5.5 Discussion

Valacyclovir therapy reduces the plasma and genital HIV RNA viral load in HIV-HSV co-infected individuals and delays HIV progression [205, 206]. It has been hypothesized that this may relate to reduced systemic immune activation during herpes suppression or to direct antiviral effects on HIV [169]. This was the first randomized controlled trial assessing the impact of valacyclovir on systemic immune activation and inflammation in ART-treated, HIV-1, HSV-2 co-infected individuals. Over a 12 week treatment period, we observed no significant impact of valacyclovir 500 mg or 1g twice daily on the percentage of activated CD8+ T-cells, hsCRP, IL-6 and sICAM-1, nor on a range of secondary immune and inflammatory markers. CD4 counts were also similar between treatment arms, and only one participant in the placebo arm experienced an HIV viral blip during the treatment period. Adherence to study drug was good throughout the trial.

We had hypothesized that valacyclovir would benefit this population because HSV-2 co-infection has been associated with increased T-cell activation in prior work [197]. T-cell activation has in turn been linked to impaired CD4 count reconstitution on ART [190], and in combination with other aspects of the systemic inflammatory response, has been postulated to be a driver of non-AIDS comorbidities such as cardiovascular disease [191]. HSV-2 reactivation has also been associated with clinically significant increases in HIV plasma viral load [207], and with some measures of accelerated HIV disease progression in ART-untreated individuals [208, 209]. To the extent that HSV-2 might analogously contribute to low-level HIV viremia below the limit of detection of modern assays or drive ongoing HIV replication in other anatomic
compartments, valacyclovir might also have been expected to decrease the inflammatory response related to these pathways.

There are several potential explanations for our negative results. First, our 12-week treatment period may have been insufficiently long to decrease systemic inflammation. HSV is known to trigger virus-specific immune responses that persist in genital mucosal tissues for up to 20 weeks after healing [129]; pharmacologic reversal of HSV-induced immune perturbations may thus be expected to require several months or more. However, another study among ART-untreated, co-infected Kenyan women observed that one year of valacyclovir 500 mg twice daily had no impact on T cell activation when compared to placebo, although the study was limited by its cross-sectional design [210]. Furthermore, since asymptomatic genital HSV reactivation occurs frequently even in individuals taking high-dose valacyclovir [211], prolonging therapy beyond 20 weeks is unlikely to have further impacted inflammation of mucosal origin.

Second, because the natural history of HSV-2 is characterized by a decreasing frequency of clinical reactivation over time [212-215], it is also possible that our study participants had relatively inactive HSV-2 disease. If valacyclovir can attenuate systemic inflammation in ART-treated, co-infected patients by suppressing HSV-2, its effects might be most readily observed in persons with frequent subclinical or clinical reactivations. Arguing against this hypothesis is our observation that the 26 participants in the treatment arms reporting prior symptomatic herpes were, if anything, less likely to demonstrate reduced systemic inflammation on valacyclovir than their asymptomatic treatment arm counterparts, and that both genital and systemic biomarkers of inflammation are increased in individuals with asymptomatic HSV infection [128]. We did not measure asymptomatic HSV shedding in our cohort.
Perhaps a more likely possibility is that valacyclovir simply does not suppress HSV potently enough to result in decreased systemic inflammation. Indeed, clinical trial data show that even dosed at 1g three times daily, valacyclovir is insufficient to prevent breakthrough HSV shedding at an annualized rate of 16.5 episodes per person-year [211]. That we observed no dose-response effect of valacyclovir on inflammatory markers in this trial is consistent with this notion.

Finally, it is possible that suppression of HSV-2 is simply not a viable strategy for attenuating systemic inflammation in ART-treated HIV-infected persons, perhaps because HSV-2 reactivations are too anatomically localized. No prior study has formally assessed the impact of HSV-2 on systemic immune activation and inflammation in the setting of ART; further work in this area could help elucidate explanations for our findings, and better define the relative contributions of HSV-2 reactivations in driving systemic effects.

In contrast to our results, a randomized study of 30 HIV-infected adults with persistently low CD4 counts despite ART previously showed that valganciclovir is associated with significant reductions in CD8+ T-cell activation [216]. The authors proposed that benefits were mediated through cytomegalovirus (CMV) suppression, but could not definitively rule out a contribution from anti-HSV-2 effects. Because valacyclovir has minimal anti-CMV activity, our results support their hypothesis that the anti-CMV effects of valganciclovir were responsible for those observations.

This study has limitations that warrant consideration. Our sample size was relatively small, because the primary purpose of this pilot study was to assess the feasibility and appropriateness of proceeding to a larger, definitive study. As such, we cannot exclude the possibility of a small benefit of valacyclovir on some of the markers tested. In addition, although
we selected a range of cellular and soluble markers representing various pathways relevant to HIV pathogenesis, it is possible that valacyclovir may have salutary effects on inflammatory pathways other than those we studied. As the literature on inflammatory markers evolves, it may be important to revisit the potential benefits of this intervention.

In summary, we observed no significant impact of valacyclovir on systemic immune activation or inflammation in ART-treated, HIV and HSV-2 co-infected adults. Further work is needed to elucidate the mechanisms by which HIV and herpes viruses interact, and to identify novel strategies for attenuating the untoward clinical effects of inflammation in HIV-infected persons.
Figure 5.1 Trial profile

112 assessed for eligibility

52 ineligible:
11 withdrew consent
38 HSV-2 negative
2 not on HAART
1 used acyclovir within 12 months

60 randomized

20 assigned to placebo and included in the analysis
20 assigned to 500mg valacyclovir and included in the analysis
20 assigned to 1g valacyclovir and included in the analysis
Table 5.1 Demographic and clinical characteristics at baseline visit by treatment group\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Low Dose Valacyclovir</th>
<th>High Dose Valacyclovir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=20</td>
<td>n=20</td>
<td>n=20</td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>51 (45-59)</td>
<td>52 (46-55)</td>
<td>50 (49-54)</td>
</tr>
<tr>
<td>Male</td>
<td>18 (90%)</td>
<td>16 (80%)</td>
<td>19 (95%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>14 (70%)</td>
<td>14 (70%)</td>
<td>17 (85%)</td>
</tr>
<tr>
<td>Black</td>
<td>3 (15%)</td>
<td>4 (20%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Asian</td>
<td>3 (15%)</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Other</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>HIV Risk Factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSM</td>
<td>16 (80%)</td>
<td>15 (75%)</td>
<td>17 (85%)</td>
</tr>
<tr>
<td>IDU</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>5 (25%)</td>
<td>5 (25%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Blood product</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Active Comorbidities (Controlled or Uncontrolled)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>5 (25%)</td>
<td>4 (20%)</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>10 (50%)</td>
<td>13 (65%)</td>
<td>12 (60%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>0 (0%)</td>
<td>2 (10%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Asthma</td>
<td>2 (10%)</td>
<td>1 (5%)</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Other(^b)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td><strong>HSV Related</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV-1 Seropositive (n=59)</td>
<td>15 (75%)</td>
<td>14 (74%)</td>
<td>17 (85%)</td>
</tr>
<tr>
<td>Prior Hx of HSV Symptoms (n=57)</td>
<td>11 (55%)</td>
<td>12 (67%)</td>
<td>14 (74%)</td>
</tr>
<tr>
<td>Symptomatic HSV</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Oral Sites Typically Involved

<table>
<thead>
<tr>
<th></th>
<th>6 (30%)</th>
<th>8 (40%)</th>
<th>11 (55%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average number of oral episodes per year (n=25)</td>
<td>1 (0-1)</td>
<td>1 (0-2)</td>
<td>1 (0-1)</td>
</tr>
</tbody>
</table>

### Genital Sites Typically Involved

<table>
<thead>
<tr>
<th></th>
<th>3 (15%)</th>
<th>5 (25%)</th>
<th>2 (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average number of genital episodes per year (n=10)</td>
<td>1 (0-2)</td>
<td>0 (0-1)</td>
<td>1 (0-2)</td>
</tr>
</tbody>
</table>

### Perianal Sites Typically Involved

<table>
<thead>
<tr>
<th></th>
<th>4 (20%)</th>
<th>1 (5%)</th>
<th>5 (25%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average number of perianal episodes per year (n=10)</td>
<td>1 (0-1)</td>
<td>0 (0-0)</td>
<td>2 (1-2)</td>
</tr>
</tbody>
</table>

### HIV Related

<table>
<thead>
<tr>
<th></th>
<th>15 (7-21)</th>
<th>16 (9-19)</th>
<th>14 (8-20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Years since HIV Diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years since 1st ART</td>
<td>10 (4-13)</td>
<td>10 (8-13)</td>
<td>7 (4-12)</td>
</tr>
<tr>
<td>HLA-B*5701 Positive (n=35)</td>
<td>2 (15%)</td>
<td>0 (0%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>HIV B Clade (n=24)</td>
<td>10 (100%)</td>
<td>7 (100%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Nadir CD4 (cells/mm³)</td>
<td>148 (62-214)</td>
<td>119 (22-211)</td>
<td>212 (78-313)</td>
</tr>
<tr>
<td>Current CD4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells/mm³</td>
<td>506 (337-627)</td>
<td>682 (398-819)</td>
<td>485 (408-604)</td>
</tr>
<tr>
<td>Percentage</td>
<td>29 (23-34)</td>
<td>31 (26-34)</td>
<td>29 (26-33)</td>
</tr>
<tr>
<td>Current Regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 NRTIs + NNRTI</td>
<td>4 (20%)</td>
<td>5 (25%)</td>
<td>8 (40%)</td>
</tr>
<tr>
<td>2 NRTIs + boosted PI (ritonavir)</td>
<td>8 (40%)</td>
<td>8 (40%)</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>Other</td>
<td>8 (40%)</td>
<td>7 (35%)</td>
<td>7 (35%)</td>
</tr>
</tbody>
</table>

### Other

|                                      | 7 (35%)   | 8 (40%)   | 11 (55%)  |
|                                      | 6 (30%)   | 6 (30%)   | 7 (35%)   |
|                                      | 7 (35%)   | 6 (30%)   | 2 (10%)   |

### Smoking Status

|                                      | 7 (35%)   | 8 (40%)   | 11 (55%)  |
|                                      | 6 (30%)   | 6 (30%)   | 7 (35%)   |
|                                      | 7 (35%)   | 6 (30%)   | 2 (10%)   |

### Baseline Outcome Values

<table>
<thead>
<tr>
<th></th>
<th>4.39 (3.24-7.97)</th>
<th>4.57 (3.50-6.93)</th>
<th>5.10 (3.68-11.00)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 Activation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP</td>
<td>0.94 (0.48-1.32)</td>
<td>1.33 (0.78-2.02)</td>
<td>0.92 (0.51-2.38)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>122 (100-158)</td>
<td>120 (91-162)</td>
<td>112 (87-146)</td>
</tr>
</tbody>
</table>

<p>| | | | |
|                                  |                   |                  |                   |
|                                  |                   |                  |                   |
|                                  |                   |                  |                   |</p>
<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 Undetectable</td>
<td>17 (85%)</td>
<td>19 (95%)</td>
<td>16 (80%)</td>
</tr>
<tr>
<td>IL-1β Undetectable</td>
<td>19 (95%)</td>
<td>20 (100%)</td>
<td>19 (95%)</td>
</tr>
<tr>
<td>MCP-1 Undetectable</td>
<td>14 (70%)</td>
<td>13 (65%)</td>
<td>15 (75%)</td>
</tr>
<tr>
<td>TNF Undetectable</td>
<td>17 (85%)</td>
<td>18 (90%)</td>
<td>16 (80%)</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>317 (262-392)</td>
<td>298 (274-399)</td>
<td>375 (289-400)</td>
</tr>
<tr>
<td>ANG1/ANG2</td>
<td>9.29 (5.49-25.28)</td>
<td>8.73 (3.34-15.74)</td>
<td>6.28 (1.64-13.18)</td>
</tr>
<tr>
<td>CD4 Exhaustion</td>
<td>5.80 (2.27-9.14)</td>
<td>5.15 (2.29-7.27)</td>
<td>5.27 (2.28-10.00)</td>
</tr>
<tr>
<td>CD8 Exhaustion</td>
<td>6.85 (4.35-9.84)</td>
<td>8.04 (5.67-10.15)</td>
<td>9.09 (3.86-12.60)</td>
</tr>
<tr>
<td>Regulatory T Cells</td>
<td>2.48 (1.41-3.31)</td>
<td>1.69 (1.11-2.70)</td>
<td>2.49 (2.12-3.13)</td>
</tr>
</tbody>
</table>


*b Includes osteoarthritis in one low-dose participant, and treated squamous cell rectal carcinoma in one high-dose participant.
Table 5.2 Effect of Valacyclovir on the levels of systemic immune activation and inflammatory markers\textsuperscript{ab}

<table>
<thead>
<tr>
<th></th>
<th>Estimated Effect of Treatment at 6 Weeks</th>
<th>Estimated Effect of Treatment at 12 Weeks</th>
<th>Estimated Effect of Treatment at 18 Weeks</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate (95% CI)</td>
<td>Estimate (95% CI)</td>
<td>Estimate (95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>Primary outcome measures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8 Activation Log\textsubscript{10}</td>
<td>-0.08 (-0.19,0.02)</td>
<td>0.10</td>
<td>-0.01 (-0.12,0.11)</td>
<td>0.93</td>
</tr>
<tr>
<td>hsCRP Log\textsubscript{10}</td>
<td>0.06 (-0.13,0.26)</td>
<td>0.53</td>
<td>-0.16 (-0.39,0.07)</td>
<td>0.17</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>1.54 (-26.4,29.5)</td>
<td>0.91</td>
<td>0.23 (-26.2,26.7)</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-6\textsuperscript{d}</td>
<td>1.26 (0.46,3.47)</td>
<td>0.65</td>
<td>1.82 (0.80,4.17)</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Secondary outcome measures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1\textsubscript{β}\textsuperscript{d}</td>
<td>0.60 (0.05,7.08)</td>
<td>0.68</td>
<td>0.60 (0.05,7.11)</td>
<td>0.69</td>
</tr>
<tr>
<td>MCP-1\textsuperscript{d}</td>
<td>1.23 (0.55,2.75)</td>
<td>0.62</td>
<td>1.26 (0.66,2.38)</td>
<td>0.48</td>
</tr>
<tr>
<td>TNF\textsuperscript{b}</td>
<td>0.78 (0.29,2.08)</td>
<td>0.62</td>
<td>1.24 (0.82,1.86)</td>
<td>0.31</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>-9.20 (-79.1,60.7)</td>
<td>0.80</td>
<td>-10.3 (-66.3,45.7)</td>
<td>0.72</td>
</tr>
<tr>
<td>Ang1/Ang2 Log\textsubscript{10}</td>
<td>0.14 (-0.16,0.44)</td>
<td>0.37</td>
<td>0.07 (-0.18,0.32)</td>
<td>0.60</td>
</tr>
<tr>
<td>CD4 Exhaustion Log\textsubscript{10}</td>
<td>-0.03 (-0.15,0.08)</td>
<td>0.58</td>
<td>-0.02 (-0.13,0.10)</td>
<td>0.78</td>
</tr>
<tr>
<td>CD8 Exhaustion Log\textsubscript{10}</td>
<td>-0.01 (-0.10,0.08)</td>
<td>0.82</td>
<td>-0.02 (-0.12,0.09)</td>
<td>0.78</td>
</tr>
<tr>
<td>Regulatory T Cells Log\textsubscript{10}</td>
<td>0.04 (-0.09,0.17)</td>
<td>0.57</td>
<td>0.07 (-0.07,0.21)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

\textsuperscript{a}hsCRP: Highly Sensitive C-Reactive Protein, sICAM-1: Soluble Intercellular Adhesion Molecule-1, IL-6: interleukin-6, IL-1\textbeta: interleukin-1\beta, MCP-1: Monocyte Chemoattractant Protein 1, TNF: Tumour Necrosis Factor, , VCAM-1: Soluble Vascular Cellular Adhesion Molecule-1, Ang1/Ang2: the ratio of angiopoi etin 1 to 2.
The effect of treatment was estimated with an interaction term between time and treatment in a generalized estimating equation linear or logistic regression model adjusting for the main effects of time and treatment. The effect of valacyclovir was estimated by comparing patients on both doses of valacyclovir to placebo.

Participants were taking study drug between baseline and week 12 only.

Odds ratio of having an undetectable value is presented.
<table>
<thead>
<tr>
<th>Clinical Outcomes</th>
<th>Placebo</th>
<th>Low Dose Valacyclovir</th>
<th>High Dose Valacyclovir</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virologic Blip</td>
<td>n=20</td>
<td>n=20</td>
<td>n=20</td>
<td></td>
</tr>
<tr>
<td>1 adverse event at least possibly</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0.99</td>
</tr>
<tr>
<td>related to the study drug</td>
<td>5 (25%)</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Headache</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Intermittent bloating</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Itching</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Finger joint pain</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Flatulence</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Nausea</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>1 (5%)</td>
<td>0.99</td>
</tr>
<tr>
<td>≥ 1 HSV Flare</td>
<td>3 (15%)</td>
<td>3 (15%)</td>
<td>0 (0%)</td>
<td>0.23</td>
</tr>
<tr>
<td>CD4 Change (cells/mm3)</td>
<td>16 (-23, 41)</td>
<td>17 (-68, 42)</td>
<td>-11 (-53, 31)</td>
<td>0.71</td>
</tr>
</tbody>
</table>
Figure 5.2 Gating strategy for co-expression of HLADR and CD38 by CD4+ and CD8+ T cells.

Gating was done in the following sequential order: a) lymphocytes, b) singlets, c) live cells, d) CD3+ and e) CD4+ and CD8+ cells were gated for co-expression of HLADR+ and CD38+.

a) [Image of FSC-A with SSC-A and SSC-W plots showing 66.5% and 97.6% gated cells]

b) [Image of FSC-A with SSC-W plot]
e)

[Diagrams showing flow cytometry analysis of CD4, CD8, HLA-DR, and CD38 expression levels.]
Chapter 6

6 Conclusion and Discussion
6.1 Conclusion

The epidemiological negative synergy between HSV-2 and HIV has been well characterized; HSV-2 infection is associated with increased HIV susceptibility in HIV uninfected, HSV-2 infected individuals, and an increased plasma viral load in HIV and HSV-2 co-infected individuals increases secondary transmission. Several clinical trials have been conducted to explore the impact of HSV-2 therapy on these parameters [165]. While HSV-2 treatment in HSV-2 mono-infected individuals had no impact on HIV susceptibility [130, 131], HSV-2 treatment in co-infected people led to a moderate decrease in plasma HIV VL and slowed disease progression but did not reduce HIV transmission [165, 166]. The reason for these negative results is unclear. In an attempt to elucidate the immunological basis of these outcomes, my Ph.D focused on the immune impact of herpes therapy in the genital tract and blood of HIV uninfected women, and on the systemic immune impact in HIV co-infected individuals. Two formal clinical trials were conducted to assess the immune impact of valacyclovir in these contexts (ClinicalTrials.gov identifier: NCT00946556 and NCT01176409).

One possible reason for increased HIV susceptibility in HSV-2 infected individuals is the increased numbers of HIV target cells seen in the endocervix of women with HSV-2 infection [128]. Thus, to examine whether herpes therapy can reverse these changes, a randomized, placebo-controlled, cross-over trial of 1g valacyclovir once daily in HSV-2 infected women was conducted. The primary endpoint was the change in the absolute number of CD4+ T cells on a cytobrush. The secondary endpoints focused on changes in the subsets of cervical CD4+ T cells and DCs. Despite a relatively high adherence rate of 89%, the absolute numbers of CD4+ T cells or DCs did not differ between valacyclovir and placebo phases. Indeed, there was a trend to an
increase frequency of CD4+ T cells that expressed CD69 and CCR5 in the endocervix on valacyclovir treatment. This trend to increased expression of CCR5 and CD69 on CD4+ T cells suggests that valacyclovir treatment might actually promote HIV acquisition. Interestingly, though it was not significant, the two large clinical trials that examined the impact of herpes therapy in reducing HIV acquisition demonstrated slightly higher HIV incidence in the acyclovir treatment group (RR of 1.08 and 1.16) [130, 131]. Overall, my study concluded that valacyclovir treatment had no impact on the number of HIV target cells in the cervix of HSV-2 infected women.

Our lab has previously shown the association between HSV-2 infection and increased frequencies of activated CD4+ and CD4- T cells in blood, possibly due to the nature of HSV-2 being a life-long infection and leading to persistent HSV-2 reactivation and shedding, even in asymptomatic individuals. Although one study examined the effect of herpes therapy on systemic T cell activation in HIV and HSV-2 co-infected individuals [177], studies focusing on HSV-2 mono-infected individuals are lacking. Therefore, I performed a secondary analysis to define the impact of valacyclovir on reversing the increased CD4+ and CD4- T cells in blood of HSV-2 mono-infected individuals. After examining T cell subsets in blood, valacyclovir treatment had no impact on these parameters.

The last part of my Ph.D focused on herpes treatment in HIV and HSV-2 co-infected individuals. HIV infected individuals display increased systemic immune activation, which has been associated with not only HIV disease progreassion, but also non-AIDS morbidity, such as cardiovascular disease [47, 191]. Although ART is associated with a decrease in this activation, the activation level remains above that of HIV uninfected individuals [190]. Since HSV-2 co-infection is associated with systemic immune activation and is a common co-infection in HIV
infected individuals, the Valacyclovir for Inflammation Attenuation Trial (VALIANT) was designed to determine if herpes therapy, in addition to ART, could further reduce systemic immune activation in co-infected individuals. VALIANT was a randomized, placebo-controlled trial with two treatment arms: 500mg valacyclovir daily, 1g valacyclovir daily. Participants were treated for 12 weeks and despite the fact that adherence by pill count (96%) and urine samples (84%) was excellent, it was determined that herpes therapy, in addition to ART, had no effect on the systemic immune activation in HIV and HSV-2 co-infected individuals. Therefore, it is likely that valacyclovir does not suppress HSV-2 potently enough to decrease systemic immune activation and is not a suitable strategy to reduce persistent systemic immune activation on ART suppressed HIV infected individuals.

### 6.2 Discussion

Overall, herpes suppressive therapy with valacyclovir had no effect on the genital or systemic immunology of HSV-2 mono-infected women, or on the systemic immunology of HIV, HSV-2 co-infected individuals on ART. Taken together, these findings contribute to highlight the possible limitations of current HSV-2 therapy and its lack of impact on genital and systemic immunology.
6.2.1 Herpes therapy cannot reverse HSV-2 associated mucosal alterations

Two months of valacyclovir therapy was not able to reduce HIV target cells in the endocervix compared to placebo, and may even enhance T cell activation and CCR5 expression by CD4+ T cells. This finding supports results from a study of biopsies of HSV-2 ulcerative sites. Zhu et al. previously demonstrated that biopsies at the site of an actual HSV-2 ulcer had increased number of CCR5+ CD4+ T cells and DCSIGN expressing DCs compared to a control site (a biopsy from the opposite anatomic site of the ulcer) and that these increases persisted even after >20 weeks of acyclovir therapy [129]. Although the findings are similar, the participants in our study were mostly asymptomatic, which may be more relevant to the general population of HSV-2 infected individuals, as >80% are asymptomatic. In addition to the obvious clinical difference in the participants and sampling sites between our trial and that of Zhu et al., we used valacyclovir – a prodrug of acyclovir – rather than acyclovir [129]. This is relevant because all the prior clinical trials examining the effect of HSV-2 suppression on HIV acquisition used acyclovir, and some investigators suggested the possible need for repeating these trials using a more potent anti-herpes drug, such as valacyclovir [217]. However, our study indicates that these mucosal alterations associated with HSV-2 are not reversed even by the use of valacyclovir – at least in the endocervix. To support this, a recent study by Johnston et al. concluded that on average, 16.5 HSV-2 shedding episodes occurred per year even in participants who took a very high-dose of valacyclovir, 1g three times daily, indicating that currently available therapy may not be potent enough to fully suppress HSV-2 [125].
6.2.2 Herpes therapy has no effect on systemic immunology in both HSV-2 mono-infected and HIV, HSV-2 co-infected individuals

The increase in systemic T cell immune activation and α4β7 expression by CD4+ T cells – which correlated with the number of activated CD4+ T cells in the cervix – remained unchanged in HIV uninfected, HSV-2 infected individuals after valacyclovir treatment. Herpes therapy has been associated with a decrease in plasma HIV VL and delayed HIV disease progression by 16% [166]. Yet whether this is due to the direct anti-HIV properties of acyclovir or achieved through a reduction in HSV-2-associated immune activation remains to be elucidated. Although no conclusive statement can be drawn from our study regarding the mechanism of acyclovir in reducing plasma HIV VL and delayed disease progression in HIV and HSV-2 co-infected individuals, the lack of association between valacyclovir and systemic immune activation from our trial indicates that the benefits may be due to inherent anti-HIV properties of herpes therapy. Indeed, in vitro studies demonstrate that acyclovir can suppress HIV replication [168, 169].

6.2.3 Valacyclovir has no impact on reducing systemic immune activation in HIV and HSV-2 co-infected individuals on ART

Increased systemic immune activation has been associated with disease progression and cardiovascular disease in HIV infected individuals and remains elevated in HIV infected individuals even after ART [190]. Our study concluded that 12-week of valacyclovir therapy was not associated with any changes in systemic immune activation in HIV and HSV-2 co-infected individuals who were on ART. Although is possible that longer therapy duration may be required
to see changes in systemic immune activation, a recent study demonstrated that one year of valacyclovir 500mg twice daily in ART-naïve, co-infected women had no impact on systemic immune activation [177]. This finding and our systemic immune activation results from both HSV-2 mono-infected and HIV, HSV-2 co-infected individuals illustrate the lack of impact on systemic immune activation by valacyclovir – once again hinting that herpes therapy related benefits in ART naïve, co-infected individuals are likely due to antiviral activity against HIV.

6.2.4 Future directions

In the three clinical studies conducted during my Ph.D, my work demonstrated that current herpes therapy had no effect on systemic or mucosal immunology. Specifically, did not reverse the cervical and systemic immune changes associated with HSV-2 despite the critical role HSV-2 plays in exacerbating HIV transmission. Thus, with the results generated from my thesis and a similar recent study demonstrating that even high-dose valacyclovir therapy cannot suppress HSV-2, new and upcoming studies should instead focus on HSV-2 prevention and developing a novel strategy to reduce systemic immune activation in HSV-2 infected individuals.

Recent studies show that certain anti-HIV or antiretroviral medication, including nucleoside reverse transcriptase inhibitor (NRTI), tenofovir (TFV) reduced HSV-2 transmission. The use of a vaginal gel containing 1% TFV was associated with a 51% decrease in HSV-2 incidence in women [218] and oral administration of tenofovir disoproxyl fumarate (TDF) was associated with reduced HSV-2 incidence. One study reported that a pre-exposure prophylaxis (PrEP) regimen consisting of daily oral TDF reduced HSV-2 incidence by 21% and the addition of another NRTI drug to this regimen, emtricitabine, further reduced HSV-2 incidence by 35%
However, another study concluded that daily oral TDF was not associated with a reduction in HSV-2 transmission [220]. Although the anti-HSV-2 properties of TFV have been confirmed in vitro [221], these studies show that the use of NRTIs is not effective enough to fully prevent HSV-2 infection. Given that valacyclovir (500mg daily) treatment in HSV-2 infected individuals has been associated with a 48% reduction in HSV-2 transmission [124], I hypothesize that valacyclovir as PrEP would reduce HSV-2 transmission.

To determine the role of oral valacyclovir PrEP in preventing HSV-2, I propose a randomized, double-blinded, placebo-controlled, clinical trial to recruit young women in Africa. Approximately 50% of young women in urban centres of Africa are infected with HSV-2 [99] and strikingly, young women are more likely to be infected with HIV than men by 8-fold [53]. As HSV-2 infection has been associated with a three-fold increase in HIV acquisition, young women (15-24 years old) are an ideal population to study the potential role of valacyclovir in preventing HSV-2 infection. Recruited participants will be administered either 1g valacyclovir or placebo once daily for 12 months. The primary endpoint of the study is to compare the number of HSV-2 infection between valacyclovir and placebo arm. The secondary endpoint of the study will be to measure the level of valacyclovir in the genital tract between those who acquire HSV-2 and those who remain seronegative in the treatment group. This study will be able to conclude whether HSV-2 therapy as PrEP can prevent HSV-2 infection and if so, possibly determine the level of valacyclovir required in the genital tract to prevent new infection.

Similar to a vaginal gel containing 1% TFV, which showed 51% reduction in HSV-2 incidence, a microbicide containing acyclovir warrants future work. Acyclovir is much more potent/efficient at suppressing herpes activity than TFV [221]; therefore 1% acyclovir gel may be more effective at reducing HSV-2 infection than 1% TFV. After conducting a small trial to
determine the safety of acyclovir vaginal gel, a larger trial should be conducted to observe its efficacy. If indeed the acyclovir vaginal gel prevents HSV-2 infection, the next step would be to observe for any possible drug interaction between TFV and acyclovir gel. This would ideally lead into developing a vaginal gel that contains both TFV and acyclovir to reduce HIV and HSV-2 infection.

A second set of studies is required to address the population who are already infected with HSV-2 since HSV-2 infection is associated with increased systemic immune activation irrespective of HIV infection [159, 175]. However, treating for HSV-2 in my study did not reverse HSV-2 related systemic immune activation and a more generalized method of reducing systemic immune activation may be required. Therefore, I propose a randomized, placebo-controlled, cross-over pilot trial to observe the impact of non-steroidal anti-inflammatory drugs (NSAIDs) in reducing systemic immune activation in HSV-2 infected, HIV uninfected individuals. Participants who are HSV-2 seropositive will be recruited and randomized into taking either NSAIDs or placebo for 3 months, followed by a one-month wash-out period and switched over to the alternative regimen for 3 months. The primary endpoint of the trial would be the change in the proportion of CD4 and CD8 T cells co-expressing CD38 and HLADR in blood. The secondary endpoint would be the change in systemic levels of regulatory T cells and expression of α4β7 by CD4 T cells, which are also increased in HSV-2 infected individuals. The results of the study would determine whether NSAIDs are effective in reducing systemic immune activation in HSV-2 infected individuals and if so, similar trial can be conducted in HIV co-infected individuals.

Overall, current herpes therapy cannot suppress HSV-2 reactivation or reduce HSV-2 associated systemic immune activation adequately. Therefore, until a successful vaccine or an
effective treatment is readily available for HSV-2 infection, future studies are needed on HSV-2 prevention and novel methods of reducing systemic immune activation.
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Appendix:

Questionnaire for the trial described in Chapter 2
Appendix 1: Questionnaire for the trial described in Chapter 2

Research Study:
Examining the ability of herpes therapy to reduce HIV target cell numbers in the cervix.

Principal Investigator: Dr. Rupert Kaul
Contact information: 416-978-8607 (office), 416-946-7054

Subject Study ID:          Visit #:          Date:          
How old are you? ___________          
When was the last day of your last period? ____________________________
When did you last have vaginal sex? Within: 1day [ ] 3 days [ ] 7 days [ ]
Greater than 7 days [ ] What time? AM [ ] PM [ ]
Did you use a condom? Yes [ ] No [ ]
Are you currently using any hormonal contraceptive? Yes [ ] No [ ]
If yes, is it: a vaginal ring [ ] an injection [ ] a pill [ ]
What is the name of the contraceptive (if you remember)? __________________________
Do you take any other hormonal therapy? Yes [ ] No [ ]
If yes, please provide the name of the medication: _______________________________
Please list ANY other medications/supplements that you have taken in the past week:
______________________________________________________________________________
Do you now have symptoms of the following? (please check all that apply)
Vaginal: irritation [ ] discharge [ ] Genital: ulcers [ ] swelling [ ]
Do you practice vaginal douching? By this we mean washing or cleaning inside the vagina (do not include regular bathing): Yes [ ] No [ ]
If yes, answer the following question; if no, please stop.
Do you insert fluid inside vagina? Yes [ ] No [ ]
What do you use for douching? (please check all that apply)
Water [ ] Soap [ ] Detergent [ ] Antiseptic [ ] Other________________
How often do you douche? More than once per day [ ] Once per day [ ]
1-6 days per week [ ] Once a week [ ] Less than once a week [ ]
When was the last day and time that you douched, including today?
Within: 1 day [ ] 3 days [ ] 7 days [ ] Greater than 7 days [ ]