INVESTIGATING THE ROLES OF CO-INFECTION AND FEMALE SEX HORMONES ON HIV-1 INFECTION AND REPLICATION IN THE FEMALE GENITAL TRACT

By

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TITLE: Investigating the roles of co-infection and female sex hormones on HIV-1 infection and replication in the female genital tract.

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ABSTRACT

Although women constitute more than half of the estimated 34-40 million people living with HIV/AIDS worldwide, little is known about the early events of HIV-1 infection in the female genital tract (FGT). Genital epithelial cells (GECs) line the FGT and act as intrinsic barriers providing mechanical protection against foreign microbes. GECs are also sentient and are capable of sensing and immunologically responding to various types of pathogens including sexually transmitted infections (STIs). These responses play a central role in preventing disease and also help mobilize both innate and adaptive immune cells against invading threats. While it is well understood that GECs exert important physical and immunological protective roles in the FGT, little is known regarding the role of GECs and GEC inflammatory responses in HIV infection.

It is estimated that 40% of all new HIV infections are established each year in the FGT. Our understanding of the early events following HIV transmission in the FGT remains particularly elusive in the context of endogenous or exogenous factors found in the genital microenvironment that may influence susceptibility to HIV-1. Inflammation is known to play a critical role in increasing HIV susceptibility, replication as well as initiating and maintaining chronic immune activation, a hallmark of disease progression. Among the key factors in the FGT that are known to or that could influence inflammation are sexually transmitted co-infections and female sex hormones.
The work summarized in this thesis examines how GEC innate immune responses to co-infecting microbes or female sex hormones impact HIV infection and replication in the FGT. Our results show that GEC innate immune response against herpes simplex virus type 2 (HSV-2), a common HIV co-infecting agent, consists of elevated proinflammatory cytokines and chemokines in addition to biologically active interferon-β. Furthermore, our results show that these responses require potent viral HSV-2 replication and that proinflammatory cytokine and chemokine responses are enhanced in the absence of the HSV-2 virus host shutoff protein. Based on this work, we decided to investigate whether GEC inflammatory responses to common STIs played a role in regulating HIV replication in T-cells. We found that HIV co-infecting microbes, specifically HSV-1, HSV-2 and *Neisseria gonorrhoeae*, directly induced HIV replication in T-cells, and caused primary GECs to upregulate inflammatory responses that indirectly increased HIV replication in T-cells.

Next we examined a translational aspect of the aforementioned studies by examining whether blocking inflammatory pathways, using the broad anti-inflammatory compound curcumin, could provide prophylactic or therapeutic protection against HIV. We found that curcumin pre-treatment a) protected the genital epithelial barrier against HIV-1-mediated disruption and inflammation, b) prevented the gp120-mediated upregulation of chemokines by GECs that recruit HIV target cells to the FGT, c) blocked GEC innate inflammatory responses to co-infecting microbes and indirect activation of the HIV promoter in T-cells, d) decreased HIV
amplification in chronically infected T-cells and e) blocked HSV-2 viral replication in GECs by a mechanism that likely involves NFκB.

Lastly, it has long been speculated that female sex hormones can regulate inflammatory responses, and numerous lines of evidence suggest that they may also regulate susceptibility to HIV-1. Thus, we investigated how female sex hormones and the hormonal contraceptive medroxyprogesterone acetate (MPA), used by more than 100 million women worldwide, regulated HIV infection and replication in GECs and whether inflammation played an important role in this regulation. Our results showed that progesterone and in particular MPA increased uptake of HIV-1 and transcytosis, but not replication, across GECs – in the absence of a proinflammatory milieu - and that this enhanced transcytosis resulted in increased infection of HIV target cells.

These results demonstrate that sex hormones and co-infection both play an important role in regulating HIV-1 infection and replication in the FGT. Furthermore, our results suggest that anti-inflammatory compounds such as curcumin may offer paradigm shifting prophylactic or therapeutic strategies against HIV-1 infection and future research should investigate its potential benefit in vivo.
ACKNOWLEDGEMENTS

I saved this section for last because honestly it was the part that I was most excited to write. Not only does it provide me with the most creative freedom (I promise there will be no use of the words “thus”, “therefore” or “suggested”), but it also provides me the opportunity to thank all the people who have helped me get to this place in my life.

I’d like to begin by thanking my supervisor Dr. Charu Kaushic. I’d like to come up with some sort of witty metaphor to describe my time in your lab, but I feel like that would be encroaching on your territory. I’d probably just end up opening a can of worms doing that and at this stage in the game, I can’t afford to burn my bridges – there’s too much meat and potatoes on my plate, for Pete’s sake! Jokingly aside, you have provided me many amazing opportunities, which have allowed me to become the researcher I am today. I am beyond thankful for your supervision. Next, I’d like to thank Dr. Aisha Nazli. Aisha, I like to consider you my back-up supervisor: you have been a great leader in the lab; have given me great ideas; and watched over me with tremendous kindness. To Charu and Aisha, I just want to say that you are tremendous role models for your children as well as all women in science. You should be immensely proud of yourselves.

To all the past and current members of the Kaushic lab, your friendships have made the past 6 years incredibly rewarding. Kristin, you are the most organized person I have ever met. You run the lab with tremendous efficiency and if it wasn’t for you, I’d probably still be waiting to get my BSL3 certification. To Varun and Jess (a.k.a. Feenis), you have been here with me the longest. You have been my
confidantes when things go bad. You guys have been so kind and helpful throughout these years. I am excited to see what the future holds for you because I know you have tremendous potential and will be very successful. Lastly, I wanted to thank Sara Dizzell. You have only been with the lab for a short amount of time, but in that time, you have made tremendous strides. You are always willing to lend a hand and if it weren't for you, I don't think I would have finished my thesis work. I wish you great luck in your Masters and in the future, even though I’m pretty sure you won't need it since you have a “great pair of hands.”

To my committee members, Drs. Karen Mossman and Ali Ashkar, thank you for challenging me. You never went easy on me and although at times it caused a great deal of unwanted stress, it’s made me a stronger person and a better researcher. To the members of the CGT/MIRC, thank you for being wonderful neighbours and providing me with assistance whenever it was needed. I’d also like to take this moment to thank all the McMaster staff members that have helped me along the way, including the administrative assistants at MIRC. In particular, I want to thank Debra Vanderar, who, from day one, has been supportive and incredibly kind to me. Furthermore, I wanted to thank all the pre-op nurses, Pathology physicians and technicians who have helped me complete my experiments over the past six years, especially Scott and Janice in Pathology and Marnie in the microscopy facility. Also, thank you to all the women who donated their tissues to my study; I am truly grateful for your contributions.

To all the teachers I have had over the past 24 years of my life, I cannot begin to explain the impact you have had on my life. You have taught me to love the
written word. You have opened my mind to the mysteries of science and the universe. You have filled my mind with knowledge and believed in me, and even continue to believe in me, even when I struggle to believe in myself. “One looks back with appreciation to the brilliant teachers, but with gratitude to those who touched our human feelings. The curriculum is so much necessary raw material, but warmth is the vital element for the growing plant and for the soul of the child.” – Carl Jung.

To my friends - my chosen family – you have filled my life with joy, love and provided me with unwavering support. I’m not the smartest, or tallest, or best-looking, or funniest, or tallest, or richest, or most interesting or tallest person in the world, and yet you choose to stick by my side and in times of great struggle, and believe me there have been some excruciatingly difficult moments, you’ve always been there to keep me afloat. I take back that last comment about not being the richest person in the world, because with you guys, I am. I know that line was beyond cheesy and saccharine-sweet, to the point it would make even Nicholas Sparks roll his eyes and gag, but I mean it with the upmost sincerity. To Sameer Rawal and Amanda Ramdyal: I know I already have a pretty big family, but I like to think of you guys as my little brother and my little sister (even though you’re technically a few days older, Amanda). You care for me like family, and no matter what you are always, ALWAYS there for me. And we know that’s not an exaggeration, either! I love you guys and am so unbelievably grateful your parents made you! To Amanda Schartinger (a.k.a. “My Amanda”), I am so lucky to call you my partner. Your support and love mean everything to me.
I come from an unusual circumstance where I am the first person in my family to not only get a PhD, but also, to even go to university. My parents never finished elementary school in Portugal because they had to support their families when they were young. And although most of my siblings have finished high school and gone on to complete some college courses, they never had the opportunity to study right out of school because they had to help support our family when we first immigrated to Canada. The reason I am here writing this, and getting a chance to do something I love, is because of all of the incredible sacrifices my family has made for me. I joke around and tell people that I was a mistake-baby because all my siblings are much older than I am, and my parents had me so late in life. It’s probably true, yet they (mostly) never treated me like a mistake. To my mom and dad, eu tenho a sorte de ser seu filho e ser amado por você. Obrigado por todo que vosso fizeram. Tenho orgulho de chamá você o meus pais. To Natercia & Serge, Rui & Claudia and Marco, my nieces Sarah, Juliana, Leah, my nephew Lucas, my entire extended family, and to all those who have supported me over the years, thanks for believing in me. I could not have done it without you.

Lastly, I want to thank the Government of Ontario, Wendy’s (yes, the fast food restaurant), the Canadian Institutes of Health Research, the Ontario HIV Treatment Network and McMaster University for their financial support over the past 10 years. Had it not been for you, I would have never gotten the chance to get my PhD and even step onto a university campus. To Hamilton and McMaster University: I have called you home for the past 10 years. I don’t know if this is Stockholm syndrome speaking, but I will miss you.
And so, the time has come for some actual science, and as such, I leave you with this final thought that encapsulates how I feel about the past 10 years of my life: “The problem, often not discovered until late in life, is that when you look for things in life like love, meaning, motivation, it implies they are sitting behind a tree or under a rock. The most successful people in life recognize, that in life they create their own love, they manufacture their own meaning, they generate their own motivation. For me, I am driven by two main philosophies, know more today about the world than I knew yesterday, and lessen the suffering of others. You’d be surprised how far that gets you.” - Neil deGrasse Tyson.
PREFACE

This thesis is prepared in the “sandwich” format as outlined in the “Guide for the preparation of Master’s and Doctoral Theses” available through the School of Graduate Studies at McMaster University. Chapter 1 of this thesis serves as a general introduction. The body of this thesis consists of 4 chapters (Chapter 2-5), each one an independent study, two of which are published and the others submitted for publication at the time of the thesis submission. All submitted and published studies and manuscripts included in this thesis were written by the author of this thesis, who is also the first author on all included works. The preamble section preceding each chapter describes the contributions of other authors to the multi-authored works. Finally, the discussion section (Chapter 6) summarizes the conclusions of this thesis and draws out the overall implications.
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C: Permission to use Ferreira et al., 2013; AJRI
D: Permission to use Ferreira et al., 2011; J Infect Dis
LIST OF ABBREVIATIONS

7TM Seven transmembrane
AIDS Acquired immune deficiency syndrome
AMPs Antimicrobial peptides
ANOVA Analysis of variance
AP-1 Activator protein-1
APOBEC3G Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
AR Androgen receptor
BST2 Bone marrow stromal antigen 2
CDC Centers for Disease Control and Prevention
cDNA Complimentary DNA
CFUs Colony forming units
CVL Cervico-vaginal lavage
DC Dendritic cell
DMEM Dulbecco’s modified essential media
DMPA Depot medroxyprogesterone acetate
dsRNA Double stranded RNA
E2 (17)β-estradiol
EM Electron microscopy
Env HIV envelope gene
ERS Estrogen receptors
FBS Fetal bovine serum
FGT Female genital tract
Gag Group-specific antigen
G-CSF Granulocyte colony-stimulating factor
GalCer Galactosylceramide
GECs Genital epithelial cells
G-CSF Granulocyte-colony stimulating factor
GM-CSF Granulocyte-macrophage colony-stimulating factor
gp120 HIV glycoprotein 120
GPCR G-protein coupled receptor
GPR-30 G-protein receptor-30
GR Glucocorticoid receptor
HAART Highly active antiretroviral therapy
HADs Human alpha defensins
HBDs Human beta defensins
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESN Highly exposed persistently seronegative
HIV-1 Human immunodeficiency virus -1
HS Heparan sulphate
HSPGs Heparan sulphate proteoglycans
HSV-1 Herpes simplex virus type 1
HSV-2 Herpes simplex virus type 2
HREs Hormone response elements
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ICP0</td>
<td>Infected cell protein-0</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>IP-10</td>
<td>Inflammatory protein-10</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor-3</td>
</tr>
<tr>
<td>ISGs</td>
<td>Interferon stimulated genes</td>
</tr>
<tr>
<td>IVUs</td>
<td>Infectious viral units</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Junk N-terminal kinase</td>
</tr>
<tr>
<td>LCs</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>L-glu</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>LGT</td>
<td>Lower genital tract</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MDDCs</td>
<td>Monocyte-derived dendritic cells</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MPA</td>
<td>Medroxyprogesterone acetate</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>MX2</td>
<td>Myxovirus resistance 2</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative factor</td>
</tr>
<tr>
<td>NET-A</td>
<td>Norethisterone acetate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NK</td>
<td>Natural kill cells</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>OCPs</td>
<td>Oral contraceptive pills</td>
</tr>
<tr>
<td>P4</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCP</td>
<td>Pneumocystis pneumonia</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDTC</td>
<td>Pyrrolidine dithiocarbamate</td>
</tr>
<tr>
<td>pen/strep</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFUs</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>Polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PRs</td>
<td>Progesterone receptors</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of virion proteins</td>
</tr>
<tr>
<td>RPMI</td>
<td>Royal Park Memorial Institute media</td>
</tr>
<tr>
<td>RRE</td>
<td>HIV Rev response element</td>
</tr>
<tr>
<td>RTase</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAMHD1</td>
<td>SAM domain and HD domain 1</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SEVI</td>
<td>Semen-derived enhancer of virus infection</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>STIs</td>
<td>Sexually transmitted infections</td>
</tr>
<tr>
<td>Tat</td>
<td>HIV trans-activating protein</td>
</tr>
<tr>
<td>TER</td>
<td>Trans-epithelial resistance</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumor growth factor-β</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRIM5α</td>
<td>Tripartite motif 5α</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>United Nations joint program on HIV/AIDS</td>
</tr>
<tr>
<td>UGT</td>
<td>Upper genital tract</td>
</tr>
<tr>
<td>VHS/vhs</td>
<td>Virus host shutoff protein</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>Vpx</td>
<td>Viral protein X</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
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CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES
1.1 – The Human Immunodeficiency Virus (HIV)

1.1.1 – Epidemiology

HIV/AIDS is a global pandemic (M. S. Cohen, Hellmann, Levy, DeCock, & Lange, 2008). As of 2011 approximately 34-40 million people are living with HIV/AIDS worldwide, and of these, approximately half are women. The exact number of people living with HIV is unknown as it is estimated that many people with HIV do not known they are infected and may never know (UNAIDS, 2011b). Since AIDS was first recognized in 1981, it has led to nearly 36 million deaths, but the advent of highly active antiretroviral therapy (HAART) in the mid to late 1990s, has led to a 60-80% decline in rates of AIDS, death, and hospitalizations due to HIV-1 (UNAIDS, 2011b). The region most heavily affected by HIV-1 is Sub-Saharan Africa. In 2010, an estimated 68% of all HIV cases and 66% of all deaths occurred in this region. In 2008, approximately 1.2 million people in the United States were living with HIV. In Canada, close to 60,000 people are living with HIV and since the early 1990s, the incidence of infection among women has continued to increase. In fact, between 1999 and 2009, the number of women living with HIV increased from 12% to 27% of the Canadian national total (PHAC, 2010).

1.1.2 – Women and HIV

According to the WHO and the United Nations Joint Program on HIV/AIDS (UNAIDS) (UNAIDS, 2011b), women comprise 50% of all people living with HIV-1.
In sub-Saharan Africa, women constitute 60% of people living with HIV. The proportion of women living with HIV has been increasing in the last 10 years. Women aged 15–24 years represent the most at-risk demographic for acquiring HIV-1, accounting for approximately 22% of all new infections worldwide (UNAIDS, 2011b). Sexual transmission remains the most common mode of HIV-1 transmission (Haase, 2005), and it is estimated that 40% of annual global infections occur through HIV invasion of the female genital tract (FGT) via exposure to virus-containing semen (Hladik & McElrath, 2008).

1.1.3 - The origin and identification of HIV/AIDS

Current evidence indicates that the human immunodeficiency viruses (HIV-1 and HIV-2) entered the human population through multiple zoonotic infections from simian immunodeficiency virus (SIV)–infected nonhuman primates in Africa, however the means by which this occurred still remain highly controversial and unknown (Hahn, Shaw, De Cock, & Sharp, 2000).

The AIDS epidemic officially began on June 5, 1981, when the U.S. Centers for Disease Control and Prevention (CDC) reported unusual clusters of pneumocystis pneumonia (PCP) caused by Pneumocystis jirovecii in five homosexual men in Los Angeles (M. T. Gilbert et al., 2007). Over the next 18 months, more PCP clusters were discovered among otherwise healthy men in cities throughout the country, along with other opportunistic diseases, such as Kaposi’s sarcoma (M. T. Gilbert et al., 2007). In June 1982, a report of a group of cases amongst homosexual men in Southern California suggested that a sexually transmitted infectious agent might be
the etiological agent and the syndrome was initially termed "GRID", or gay-related immune deficiency. (M. T. Gilbert et al., 2007)

Health authorities soon realized that nearly half of the people identified with the syndrome were not homosexual males. The same opportunistic infections were also reported among hemophiliacs, heterosexual intravenous drug users, and Haitian immigrants—leading some researchers to call it the "4H" disease (J. Cohen, 2006). By August 1982, the disease was being referred to by its new CDC-coined name, Acquired Immune Deficiency Syndrome (AIDS) (J. L. Marx, 1982). In May 1983, doctors from Dr. Luc Montagnier's team at the Pasteur Institute in France reported that they had isolated a new retrovirus from lymphoid ganglions that they believed was the cause of AIDS (Barre-Sinoussi et al., 1983). The virus was later named lymphadenopathy-associated virus. In May 1984 a team led by Robert Gallo of the United States confirmed the discovery of the virus, but they renamed it human T lymphotropic virus type III (Popovic, Sarngadharan, Read, & Gallo, 1984). By March of 1985 it was clear that the viruses were the same, and were the etiological agent of AIDS (S. Y. Chang, Bowman, Weiss, Garcia, & White, 1993; J. L. Marx, 1985). In May 1986, the International Committee on Taxonomy of Viruses ruled that both names should be dropped and a new name, human immunodeficiency virus, HIV, be used (J. Coffin et al., 1986).

1.1.4 – HIV Structure and Genome

HIV is a member of the genus Lentivirus, part of the family Retroviridae. The bilayer envelope holds multiple copies of 12 different glycoproteins that protrude
from the membrane (Cochrane, 2011). HIV is transmitted as single-stranded, positive-sense (+), enveloped RNA virus. Upon entry into the target cell, the viral RNA genome is reverse transcribed into double-stranded DNA by a virally encoded reverse transcriptase that is transported along with the viral genome in the virus particle (di Marzo Veronese et al., 1986). The resulting viral DNA is then imported into the cell nucleus and integrated into the cellular DNA by a virally encoded integrase and host co-factors. Once integrated, the virus may become latent, allowing the virus and its host cell to avoid detection by the immune system (Craigie & Bushman, 2012; M. D. Miller, Farnet, & Bushman, 1997; Pomerantz, Bagasra, & Baltimore, 1992). Alternatively, the virus may be transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus particles that begin the replication cycle anew (Cochrane, 2011; Freed, 2001; Gomez & Hope, 2005).

Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is more virulent than HIV-2 (P. B. Gilbert et al., 2003) and is the cause of the majority of HIV infections globally. Because of its relatively poor capacity for transmission, HIV-2 is largely confined to West Africa (Reeves & Doms, 2002).

HIV is different in structure from other retroviruses. It is roughly spherical with a diameter of about 120 nm, around 60 times smaller than a red blood cell, yet large for a virus (McGovern, Caselli, Grigorieff, & Shoichet, 2002). It is composed of two copies of single-stranded RNA that codes for the virus's nine genes. It is enclosed by a conical capsid composed of 2,000 copies of the viral protein, p24 (Gitti et al., 1996). The single-stranded RNA is also tightly bound to a nucleocapsid
composed of several p7 proteins and is associated with various enzymes that are needed for a productive infection such as reverse transcriptase, proteases and integrase (Aldovini & Young, 1990; Aronoff, Hajjar, & Linial, 1993; Barklis et al., 1997). A matrix composed of the viral protein p17 surrounds the capsid, ensuring the integrity of the virion particle (Hill, Worthylake, Bancroft, Christensen, & Sundquist, 1996). The virus envelope surrounds the outer portion of the particle and is composed of two layers of phospholipids taken from the host cell which it infects (Arthur et al., 1992; Cochrane, 2011; Hockley, Wood, Jacobs, & Garrett, 1988; Turner & Summers, 1999).

Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle. This protein, encoded by the Env gene, consists of a cap made of three molecules of glycoprotein 120 (gp120), and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle (Arthur et al., 1992; D. C. Chan, Fass, Berger, & Kim, 1997; Lu, Blacklow, & Kim, 1995).

The RNA genome consists of nine genes (Gag, Pol, Env, Tat, Rev, Nef, Vif, Vpr and Vpu, or Vpx, in lieu of Vpu for HIV-2), which encode 19 proteins (Turner & Summers, 1999). Three of these genes, Gag, Pol, and Env, contain information needed to make the structural proteins for new virus particles. For example, Env codes for a protein called gp160 that is broken down by a cellular protease to form gp120 and gp41. The six remaining genes are regulatory genes for proteins that
control the efficiency of the infection cycle or cause disease (Cochrane, 2011; Freed, 2001; Turner & Summers, 1999).

HIV-1 negative factor (Nef) is a 27 kDa, N-terminally myristoylated regulatory factor of 206 amino acid residues that is expressed in high concentrations shortly after viral infection. This accessory protein is important for achieving and maintaining high viral loads in vivo. Nef has at least two distinct roles: it enhances viral replication and stimulates a reduction in the number of CD4 receptors on the surface of the infected cell (Goldsmith, Warmerdam, Atchison, Miller, & Greene, 1995). Evidence also suggests that HIV-1 Nef is capable of negatively regulating cytotoxic T lymphocyte immune responses by mislocalizing and degrading MHC-I proteins (K. L. Collins, Chen, Kalams, Walker, & Baltimore, 1998).

Rev participates in the sequence-specific transport of unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm. The 116-residue protein binds specifically to the Rev-response element (RRE) RNA target sequence located within the unspliced viral transcripts of the env gene (Daly, Cook, Gray, Maione, & Rusche, 1989; Malim, Bohnlein, Fenrick, et al., 1989; Malim, Bohnlein, Hauber, & Cullen, 1989). In contrast, Tat functions to enhance transcriptional elongation by binding to the trans-activating response element (TAR) stem-loop site on the nascent RNA transcript (Herrmann & Rice, 1995; Reines, Conaway, & Conaway, 1996).

HIV Vif is important for synthesis of infectious viruses in T-cells and macrophages, while Vpr plays a role in the nuclear import of the pre-integration
complex and arresting the cell cycle, and Vpu plays a role in CD4 degradation via ubiquitin proteasome and plays a role in releasing virions from infected cells (Turner & Summers, 1999). HIV-1 Vif, Vpu, and Vpr also adapt cellular ubiquitin ligase adaptors to counteract host antiviral responses. For example, Vif counteracts the antiviral activities of APOBEC3, or A3 proteins, especially APOBEC3G (Sheehy, Gaddis, Choi, & Malim, 2002).

1.1.5 - The HIV Replication Cycle

One of the first interactions between HIV and susceptible cells is viral attachment to host target cells, also known as viral adsorption. Attachment to host cells is mediated by HIV gp120 (Gibson & Arts, 2012). Binding typically leads to subsequent viral entry into the cell or it can trigger cellular signaling pathways. Canonical HIV-1 entry follows the initial binding of the viral surface gp120 to a CD4-molecule on the surface of target cells (D. C. Chan & Kim, 1998) (Ho et al., 1995; Perelson, Neumann, Markowitz, Leonard, & Ho, 1996; Wei et al., 1995). CD4 is a glycoprotein expressed on immune cells, including helper T-cells, macrophages and dendritic cells (DCs), and acts as a co-receptor during helper T-cell activation (K. Murphy, 2011). This interaction promotes attachment of gp120 to one of two chemokine co-receptors, CCR5 (for macrophages) or CXCR4 (for CD4+ T-cells); DCs may use either (Rubbert et al., 1998). Co-receptor binding promotes a conformational change in gp120, allowing exposure of gp41, which inserts its hydrophobic terminus into the target cell forming a transient intermediate that spans both the viral and target cell membranes (Clapham & Weiss, 1997; Doranz et
The gp41 then folds back on itself, drawing the virus toward the cell, facilitating the fusion of their membranes (Caffrey et al., 1998). The viral nucleocapsid then enters the host cell and releases the viral genome as well as the enzymes required to establish a productive infection (Freed, 2001; Freed & Mouland, 2006; Gomez & Hope, 2005).

Following fusion, the viral core enters the host cytoplasm. There, reverse transcription begins with the reverse transcriptase (RTase)-catalyzed synthesis of HIV DNA with duplicated long terminal repeat (LTR) ends (J. M. Coffin, Hughes, & Varmus, 1997; Schwartz, Marechal, Danos, & Heard, 1995). This DNA can then be integrated into the host cell genome as proviral DNA, where it can be transcribed by cellular machinery into several RNA copies (Arts et al., 1996; Arts & Wainberg, 1996; Basu et al., 2008; Doitsh et al., 2010; Freed, 2001). The RNA transcripts can then be left unspliced or can be spliced to generate all the proteins necessary to have a productive infection (D. D. Chang & Sharp, 1989; Purcell & Martin, 1993). DNA integration and RNA splicing are both steps of the HIV-1 infection cycle that are critical to creating progeny virus and can therefore be measured to assess productive infection (Freed, 2001; Gomez & Hope, 2005). A schematic illustration of the HIV replication cycle can be found in Appendix A.

1.1.6 – HIV Pathogenesis & Treatment

Sexual transmission of HIV following exposure to cell-free or cell-associated infectious virus in semen or mucosal surfaces represents the most common route of
HIV transmission globally. Less frequent routes include transmission via injection drug use, exposure to tainted blood products via transfusions or vertical transmission from mother to child (Moir, Chun, & Fauci, 2011). Following transmission, HIV disseminates rapidly in the absence of preexisting immune pressures. Several recent studies suggest that a single “founder” virus (or infected cell) is transmitted in a majority of individuals and that HIV begins to evolve or diverge from the founder virus only once a cellular immune response arises several weeks after exposure (Goonetilleke et al., 2009; Little, McLean, Spina, Richman, & Havlir, 1999; Salazar-Gonzalez et al., 2009).

Approximately two to four weeks following the transmission of the virus, a majority of HIV-infected individuals experience an acute HIV syndrome, defined by flu-like clinical manifestations associated with high plasma viremia and often fever and lymphadenopathy (Gurunathan et al., 2009). During this early phase, HIV often replicates extremely aggressively in the absence of an immune response, reaching levels of plasma viremia as high as 10 million copies/mL (Little et al., 1999; Piatak et al., 1993). In the absence of antiretroviral therapy, plasma viremia typically peaks at three to four weeks post-exposure (Fiebig et al., 2003; Little et al., 1999), then declines spontaneously for several months before reaching a steady state. The acute phase of HIV infection is usually accompanied by a dramatic depletion of CD4+ T cells in the peripheral blood, which may rebound somewhat after the initial burst of viremia decreases. The early and high level of replication is followed by dissemination of the virus to peripheral lymphoid tissue and the establishment of persistent lymphoid tissue viral reservoirs (Moir et al., 2011).
In the absence of treatment, most patients will eventually progress to AIDS due to persistent replication of virus and depletion of CD4\(^+\) T-cells. However, a small proportion (<5%) retains high levels of CD4\(^+\) T cells without antiretroviral therapy. These individuals are classified as elite controllers or long-term non-progressors (Blankson, 2010). AIDS is typically defined in terms of either a CD4\(^+\) T cell count below 200 cells/\(\mu\)L. In the absence of specific treatment, around half of people infected with HIV develop AIDS within ten years (Moir et al., 2011). During AIDS, patients are at perilous risk for acquiring opportunistic infections or cancers that usually result in death.

Current HAART options are combinations (or cocktails) consisting of at least three medications belonging to at least two classes of antiretroviral agents. Initially treatment is typically a non-nucleoside reverse transcriptase inhibitor (NNRTI) (i.e. Nevirapine or Efavirenz) plus two nucleoside analogue reverse transcriptase inhibitors (NRTIs). Typical NRTIs include: Zidovudine, Tenofovir, or Emtricitabine. HAART regimens consisting of protease inhibitors such as Lopinavir or integrase inhibitor such as Raltegravir are used if the above regimen loses effectiveness (Maartens, Celum, & Lewin, 2014). When to start antiretroviral therapy is subject to debate. The World Health Organization recommends antiretrovirals in all adolescents, adults and pregnant women with a CD4 count less than 500/\(\mu\)L. The desired outcome of treatment is a long term plasma HIV-RNA count below 50 copies/mL (Maartens et al., 2014). Benefits of treatment include a decreased risk of progression to AIDS, death, transmission to uninfected sexual partners and mother-to-child transmission (When To Start et al., 2009).
1.2. The Female Genital Tract

1.2.1 – Anatomy of the Human Female Genital Tract

The human FGT can be divided into two main components: the lower genital tract (LGT) and the upper genital tract (UGT). The LGT consists of the vagina and the ectocervix, whereas the UGT is composed of the endocervix, uterus, Fallopian tubes and ovaries. The vagina meets the external organs at the vulva, which includes the labia, clitoris and urethra. The vagina is attached to the uterus through the cervix, while the uterus is attached to the ovaries via the Fallopian tubes (Marieb, 2012).

1.2.2. - Mucosal Immunity in the Genital Tract

The immune system of the FGT is part of the mucosal immune system. As such, it has many characteristics that are defining features unique to mucosal immune systems, including mucosal homing markers, secretory immunoglobulins, and tissue resident innate lymphocytes (Mestecky, Moldoveanu, & Russell, 2005). In addition to the common features shared with other mucosal surfaces, the FGT has unique adaptations that have evolved primarily to provide protection to the developing fetus from being recognized and attacked by immune cells. Sex hormones have been shown to be key regulators of immune cells and responses in the FGT and play an important role in cyclical changes in immunity (Kaushic, Roth, Anipindi, & Xiu, 2011; C.R. Wira, Richardson, & Prabhala, 1994). Besides sex
hormones, there is increasing evidence that the presence of a microbiome dominated by specific bacterial species, such as *Lactobacilli*, plays a key role in shaping genital tract innate and adaptive immune responses (Brotman, Ravel, Bavoil, Gravitt, & Ghanem, 2014; Mirmonsef et al., 2011).

1.2.3 – *Dendritic Cells and Macrophages in the Female Genital Tract*

Numerous resident or recruited innate immune cells populate the FGT (Nguyen, Kafka, Ferreira, Roth, & Kaushic, 2014). Of these, two of the most important cells with respect to HIV infection or transmission are dendritic cells (DCs) and macrophages. Both DCs and macrophages are sentinels of the mucosal immune system, constantly surveying and processing antigens from the external environment and providing important information and signals to the host immune system (Banchereau & Steinman, 1998). DCs in particular serve the important function of bridging the innate responses with initiation of adaptive immunity. Mucosal DCs are recognized for their unique ability to recognize and respond to antigens by induction of host immune responses that can range from tolerogenic to induction of antigen-specific adaptive immunity (Iwasaki, 2007). Macrophages and DCs have been identified throughout the FGT, especially in the ecto- and endocervix (Pudney, Quayle, & Anderson, 2005; Trifonova, Lieberman, & van Baarle, 2014; C. R. Wira, Fahey, Sentman, Pioli, & Shen, 2005). CD1a+ Langerhans cells (LCs) and other DC subsets are also localized within the squamous epithelial layers and at the stromal-epithelial interface of the vagina and ectocervix (Pudney et al., 2005).
Genital tract DCs and macrophages have been implicated in HIV transmission. For many years, HIV-1 acquisition in the LGT was assumed to occur through internalization of HIV-1 by LCs. This view was supported by evidence that skin LCs are susceptible to infection by HIV-1 (Kawamura et al., 2000; Tschachler et al., 1987) and that genital mucosal LCs harbour SIV virions within 24 hours of intravaginal inoculation of macaques (J. Hu, Gardner, & Miller, 2000). LCs have dendrites that extend and retract through the intercellular spaces (Nishibu et al., 2006), and even reach up to the epithelium surface where HIV can bind directly to these cells. Based on observations of gut DCs (Niess et al., 2005; Rescigno et al., 2001), this could be particularly true for DCs that are located just beneath the endocervical columnar epithelium. However, direct sampling of luminal pathogens by endocervical DCs or vaginal LCs, which could be exploited by HIV to bypass the epithelial cell barrier, has not yet been formally demonstrated. Furthermore, In situ explant models have failed to identify DCs in the cervicovaginal stroma as foci for productive HIV infection (K. B. Collins, Patterson, Naus, Landers, & Gupta, 2000; Greenhead et al., 2000). By contrast, SIV-infected DCs were present in the lamina propria of the cervicovaginal mucosa of macaques shortly after intravaginal SIV challenge, as well as in chronically infected animals (J. Hu et al., 2000; J. Hu, Pope, Brown, O’Doherty, & Miller, 1998; Spira et al., 1996).

Macrophages in the female genital mucosa are also susceptible targets for early HIV-1 infection, as demonstrated in studies using human explant models (K. B. Collins et al., 2000; Cummins et al., 2007; Greenhead et al., 2000), and in two reports were the major cell type infected by R5-tropic HIV-1 (Cummins et al., 2007;
Greenhead et al., 2000). Once captured, HIV-1 can be sequestered for several days and then transmitted to T cells in trans (Groot, Welsch, & Sattentau, 2008; Sharova, Swingler, Sharkey, & Stevenson, 2005). If genital macrophages similarly archive infectious virions, their role in viral propagation once HIV-1 invades the stroma may be significant.

1.2.4 – Genital Tract T-cells

CD4 and CD8+ T-cells are found throughout the FGT. In the UGT CD8+ T-cells are present in higher proportion, compared to CD4 T-cells. Yeaman et al showed that uterine endometrium contains organized lymphoid aggregates (Yeaman et al., 1997). These structures have a B-cell core surrounded by CD8+ T-cells, with an outer halo of macrophages. Pudney et al did extensive immunohistological characterization to show that CD4+ T-cells were most prevalent in the cervical transformation zone and surrounding tissue, while the vaginal tissue contained few T-cells (Pudney et al., 2005). The majority of the T-cells in the LGT were localized to the stromal-epithelial interface. Furthermore, significant numbers of CD8+ intraepithelial T-cells were also present interspersed in the vaginal and ectocervical squamous epithelium. In comparison, only the ectocervix contained significant numbers of intraepithelial CD4+ T-cells. Recent studies have also shown that regulatory T-cells and Th17 cells are present in the FGT. Typically the presence of these cells was noted following exposure to inflammatory or immunoregulatory conditions (McKinnon et al., 2011; Robertson, Prins, Sharkey, & Moldenhauer, 2013).
On day one post-HIV inoculation of vaginal, ectocervical and endocervical tissue cultures, infected CD4+ T-cells were shown to be confined to the mucosal stroma (Greenhead et al., 2000; P. Gupta et al., 2002; Q. Hu et al., 2004; Maher, Wu, Schacker, Horbul, & Southern, 2005). Analysis of the fate of fluorescence-tagged virions also found that R5-tropic HIV-1 bound to intraepithelial vaginal CD4+ T-cells very efficiently, followed by fusion and productive infection (Hladik et al., 2007). The central role for genital CD4+ T-cells in early infection and propagation is also evident from SIV challenge experiments in macaques (Spira et al., 1996; Veazey, Marx, & Lackner, 2003; Z. Zhang et al., 1999). Interestingly, not only does SIV productively infect activated T cells, but also resting state T-cells (Z. Zhang et al., 1999). The fact that vaginal CD4+ T-cells are rapidly depleted following intravenous SIV inoculation of macaques (Joag et al., 1997; Veazey, Marx, et al., 2003), similar to that observed in CD4+ T-cells of the gut during acute SIV infection (Picker & Watkins, 2005), further illustrates their high susceptibility to infection in vivo. Evidence also suggests that a subset of Th17 CD4+ T cells in the cervical mucosa co-expressing multiple HIV susceptibility markers was dramatically depleted after HIV infection, suggesting that these may serve as key target cells during HIV transmission (McKinnon et al., 2011).

1.2.5 – Genital epithelial cells

The FGT forms the first line of defense against HIV. Genital epithelial cells (GECs) play an important role in mediating that protection. Epithelium is one of the
four basic types of animal tissue, along with connective, muscle and nervous tissue. Epithelial tissues line the cavities and surfaces of structures throughout the body, and also form many glands. Functions of epithelial cells include secretion, selective absorption, protection, transcellular transport and detection of sensation. In general, epithelia are classified by the morphology of their cells, and the number of layers of which they are composed. Epithelial tissue that is only one cell thick is known as simple epithelium. If it is two or more cells thick, it is known as stratified epithelium. There are three principal morphologies associated with epithelial cells: squamous epithelium has cells that are wider than they are tall, thus they appear flat and scale-like; cuboidal epithelium has cells whose height and width are approximately the same and are thus said to be cube shaped; and columnar epithelium has cells taller than they are wide (Marieb, 2012).

The LGT consisting of the vagina and ectocervix is lined by stratified squamous epithelium, and the UGT consisting of the endocervix, endometrium and Fallopian tubes, is lined by a simple columnar epithelium. The LGT relies primarily on the presence of multiple layers to provide a protective barrier against entry of organisms (Blaskewicz, Pudney, & Anderson, 2011). The superficial layers of the LGT are terminally differentiated and lack most intracellular organelles including nuclei, while the basal layers are metabolically active and undergo active proliferation (Anderson, Marathe, & Pudney, 2014). Consequently, the superficial layers of the LGT are said to be “leaky”, allowing penetration by endogenous and pathogenic microbes and other mediators. In contrast, although they are only a single layer of cells thick, the tight junction (TJ) proteins (such as ZO-1 and
occludin) between columnar epithelial cells of the UGT form a tight mechanical barrier, preventing pathogens from breaching the internal milieu (Ferreira, Kafka, & Kaushic, 2014; Kaushic, Ferreira, Kafka, & Nazli, 2010; Nguyen et al., 2014; C. R. Wira, Fahey, et al., 2005).

1.3 - HIV in the Female Genital Tract

1.3.1 – Sites of HIV Transmission in the Female Genital Tract

It has been proposed that HIV crosses the mucosal epithelial barrier to establish a small founder population that then expands systemically (Haase, 2011). These early events represent a window of maximum opportunity for interventions to prevent disseminated infection. Despite much debate on the topic, there is no clear consensus regarding the primary site of transmission of HIV-1 in the FGT.

The multilayered squamous epithelium, when intact, may likely provide better mechanical protection against HIV invasion than the single layer columnar epithelium that lines the UGT. However, the greater surface area of the vaginal wall and ectocervix, arguably allows more access sites for HIV entry (Hladik & McElrath, 2008), particularly when breaches occur in the epithelium, such as during sexual intercourse (Guimaraes, Vlahov, & Castilho, 1997; Norvell, Benrubí, & Thompson, 1984). Although post-coital microabrasions in the LGT may play a significant role in viral transmission (Haase, 2011), other studies, particularly those that have examined acute SIV infection in non-human primates indicate that HIV may preferentially invade through the endocervix of the UGT (Q. Li et al., 2009). The
large number of activated CD4+ T-cells that populate the cervical transformation zone may also provide a rich source of target cells for HIV-1 (Pudney et al., 2005). Furthermore, a recent study found that HIV-1 penetrated both intact human cervical columnar and squamous epithelial barriers to depths where the virus encountered potential target cells (Carias et al., 2013).

Together these results suggest that HIV transmission may be comprehensive and multifactorial, in that genital tract acquisition may take place at a variety of different tissues, perhaps depending on the inflammatory, hormonal or infectious milieu. Thus, elucidating the mechanisms associated with the early events of HIV infection in both the LGT and UGT will provide valuable information that can be used in the future to design targeted, effective therapeutics against HIV.

1.3.2 – Interactions Between HIV and Genital Epithelial Cells

The interactions that take place between HIV and GECs remain highly contentious and poorly understood. Early in vitro studies demonstrated that GECs and organ culture models could be infected with HIV (Asin et al., 2003; X. Tan, Pearce-Pratt, & Phillips, 1993; Wu, Chen, & Phillips, 2003), however the mechanism of HIV entry into GECs is likely distinct from canonical HIV entry pathways. Studies demonstrate conflicting information regarding the expression of CD4 on GECs. CD4 expression has been measured on primary ectocervical GECs during the early- and mid-proliferative stages of the menstrual cycle (Yeaman et al., 2004) and on endometrial GECs (Asin et al., 2004; Asin et al., 2003; Yeaman et al., 2003), primarily during the proliferative stage. In contrast, it has been shown that primary vaginal
and ectocervical GECs as well as three different human cervical cell lines and the endometrial cell line HEC-1A failed to express CD4 (Berlier et al., 2005; Bobardt et al., 2007; Dezzutti et al., 2001; Saidi, Magri, Nasreddine, Requena, & Belec, 2007). Detection of CCR5 and CXCR4 has also been inconsistent, with studies detecting both co-receptors (Asin et al., 2004; Bobardt et al., 2007; McClure et al., 2005; Yeaman et al., 2003), the exclusive expression of CXCR4 (Berlier et al., 2005; Saidi et al., 2007) or CCR5 (Yeaman et al., 2004) or neither co-receptor on GECs (Dezzutti et al., 2001).

In lieu of CD4, several other molecules have been suggested to mediate HIV attachment and entry into GECs. Cell surface galactosylceramide (GalCer) (Dezzutti et al., 2001; Yeaman et al., 2004) is a glycosphingolipid that has been found to bind HIV-1 gp120 on cervical GECs and foster transcytosis of the virus (Bomsel, 1997). Interactions of HIV gp120 with GEC transmembrane heparan sulphate proteoglycans (HSPGs), specifically syndecans, have also been shown to contribute to attachment and infection (Bobardt et al., 2007; Connell & Lortat-Jacob, 2013; Wu et al., 2003). Many viruses, such as herpes simplex virus type 2 (HSV-2) use heparan sulphate (HS) moieties on HSPGs in order to facilitate attachment and entry into cells (Shukla, Singh, & Shukla, 2009) and HS binding sites have been identified on HIV gp120 (Connell & Lortat-Jacob, 2013; Vives, Imberty, Sattentau, & Lortat-Jacob, 2005). GalCer and HSPGs are commonly expressed on epithelial cells and may promote HIV-1 binding and transport across the vaginal epithelium (Bobardt et al., 2007; Wu et al., 2003; Yeaman et al., 2004). Furthermore, gp340, a splice variant of salivary agglutinin, has been shown to specifically bind to HIV gp120 and facilitate
transcytosis of HIV through the genital epithelium giving it access to susceptible leukocytes (Stoddard et al., 2007; Stoddard et al., 2009).

It remains controversial whether HIV-1 can sustain a productive infection in GECs. For the purpose of this discussion, the term “productive infection” will refer to a completed HIV life cycle, including reverse transcription, integration and progeny virus production.

Both primary and immortalized uterine epithelial cells have been shown to support reverse transcription and integration of proviral DNA and to release infectious virus (Asin et al., 2004; Asin et al., 2003). In contrast, other studies have been unable to show evidence of productive HIV-1 infection in uterine GEC populations (Dezzutti et al., 2001; Greenhead et al., 2000; Wu et al., 2003). Whilst some studies support the view that HIV-1 can integrate into the genome of vaginal epithelial cells and produce progeny virus (Berlier et al., 2005; Furuta et al., 1994; Iversen et al., 1998; Phillips, Zacharopoulos, Tan, & Pearce-Pratt, 1994; X. Tan et al., 1993), others discount this view (Dezzutti et al., 2001; Greenhead et al., 2000; Spira et al., 1996; Wu et al., 2003).

In addition to productive infection, HIV virions have been theorized to traverse the epithelium through several putative pathways, including via microabrasions (Berlier et al., 2005; Dezzutti et al., 2001; Howell et al., 1997; Saidi et al., 2007; Wu et al., 2003) and transcytosis (Bobardt et al., 2007; Bomsel, 1997; Saidi et al., 2007). One mechanism of HIV-1 transmission across the mucosa is thought to occur through sequestration of the virus by epithelial cells, followed by transfer to permissive cells to establish a primary infection (Ballweber et al., 2011; Dezzutti et
al., 2001; J. Hu et al., 2000; C. J. Miller & Shattock, 2003; Shen, Richter, & Smith, 2011; Yeaman et al., 2004). In agreement with some of the earlier work done on epithelial cells lines, a recent study reported that ectocervical and endocervical epithelial cell lines became productively infected with cell-free HIV-1 in a CD4-independent manner and that this infection increased when inoculation occurred in the presence of semen-derived enhancer of virus infection (SEVI) fibrils (Micsenyi et al., 2013), which are positively charged amyloid fibrils derived from semen (Munch et al., 2007). Consequently, the de-novo virus was transmitted to target CD4 T cells in co-culture in a contact-dependent manner.

Similarly, work from our lab and others shows that HIV-1 binding to epithelial cells may directly impair mucosal barrier integrity, thus facilitating microbial translocation (Fanibunda, Modi, Gokral, & Bandivdekar, 2011; Nazli et al., 2010; Nazli et al., 2013). Previously we showed that gp120 interacts with TLR2, TLR4 and HS moieties on primary GECs resulting in downstream induction of proinflammatory cytokines such as TNF-α and IL-6 and concomitant disruption of epithelial barrier TJ proteins including ZO-1 and occludin (Nazli et al., 2013). Such mucosal barrier disruption resulted in HIV translocation across the epithelium, presenting a mechanism by which HIV acquisition may take place. Furthermore, we showed that gp120 also disrupted the gut epithelial barrier allowing for bacterial translocation, which may lead to immune activation (Nazli et al., 2010).

It is difficult to fully appreciate and compare past studies such as these because of the use of primary cells vs. cell lines, differing concentrations of virus, the use of LGT vs. UGT cells, different standards with respect to defining “infection” (i.e.
measuring p24 antigen vs. measuring integrated virus), etc. In addition, few studies have taken into consideration that factors present in the genital tract microenvironment, such as sex hormones, sexually transmitted infections (STIs) or inflammation, may play a significant role in determining the outcome of infection. Therefore, there remains a need for studies that comprehensively examine HIV-1 infection of primary GECs using highly sensitive and specific assays that measure *bona fide* productive infection.

1.3.3. - *Barriers to HIV Infection in the Female Genital Tract*

Previously we discussed how genital tract epithelium could act as a barrier against HIV acquisition in the FGT. In addition to this, the FGT contains a number of other endogenous barriers that provide protection against HIV. GECs of the FGT produce several biological and chemical factors that create an inhospitable environment for HIV including a hydrophilic surface layer of glycoproteins and glycolipids called the glycocalyx, and a thick hydrophobic glycoprotein mucus (Carson et al., 1998). Both the glycocalyx and mucus act as mucosal barriers against HIV-1 and other pathogenic microbes. A recent study demonstrated that human cervicovaginal mucus obtained from donors with normal lactobacillus-dominated vaginal flora efficiently traps HIV, causing it to diffuse 1,000-times more slowly than it would in water (Lai et al., 2009).

GECs, as well as cells of the FGT, such as macrophage, neutrophils and DCs, also secrete innate antimicrobial peptides (AMPs) with anti-HIV activity. These include secretory leukocyte protease inhibitor (SLPI), α- and β-defensins as well as
trappin-2/elafin (Kaushic et al., 2010). More recently, anti-proteases, such as serpins and cystatins expressed by GECs, have also been shown to inhibit HIV binding and replication and reduce local inflammation (Aboud, Ball, Tjernlund, & Burgener, 2014). AMPs are small proteins or peptides with the ability to neutralize or directly kill pathogens. Both the columnar epithelium that lines the endometrium as well as the cervico-vaginal epithelium have been shown to secrete a number of the AMPs, measured in genital tract secretions as well as in epithelial cell cultures. Moreover, the secretion of the AMPs has been shown to be regulated by the menstrual cycle (C. R. Wira, Patel, Ghosh, Mukura, & Fahey, 2011).

Human alpha and beta defensins are among the most well-studied and abundant AMPs present in the FRT. Of the six human alpha defensins (HADs) and six beta defensins (HBDs), HBDs 1-4 and HAD5 are expressed by endometrial epithelium, while HADs 1-3 and HBD2 are found in the cervico-vaginal secretions. Furthermore, many of the defensins are differentially regulated in the upper and lower reproductive tract during the menstrual cycle. Some reach their peak concentration in the proliferative phase (high estrogen), others in the secretory phase (high progesterone) (C. R. Wira, Fahey, et al., 2005). Human defensins have been shown to have antimicrobial properties against fungi, yeast, bacteria (Gram-negative and -positive) as well as HIV-1 (C. R. Wira et al., 2011).

In addition to AMPs, cells of the FGT can produce interferons (IFNs) that have a wide variety of immunomodulatory and antiviral effects. Type I IFNs (IFN-α, IFN-β) impede HIV replication by several mechanisms, including inducing the upregulation of restriction factors such as apolipoprotein B mRNA-editing enzyme-
catalytic polypeptide-like 3G (APOBEC3G) (K. Chen et al., 2006; Cremer, Vieillard, & De Maeyer, 2000), tripartite motif 5α (TRIM5α) (Stremlau et al., 2004), bone marrow stromal antigen 2 (BST2; also known as tetherin) (Neil, Zang, & Bieniasz, 2008) SAM domain and HD domain 1 (SAMHD1) (Hrecka et al., 2011; Laguette et al., 2011) and myxovirus resistance 2 (MX2 also known as MxB) (Kane et al., 2013). Interestingly, type I IFN has also been implicated as a contributor to HIV pathogenesis (J. J. Chang et al., 2013); elevated type I IFN is a component of the signature associated with chronic immune activation (G. A. Hardy et al., 2013). The benefit/harm of IFN responses may likely depend on the net outcome of a number of factors, including the stage of infection.

Two new mucosal IFN species have recently been described to possess anti-HIV activity. Unlike other type I IFNs, IFN-ε is expressed constitutively in mucosal tissues including the reproductive tract (M. P. Hardy, Owczarek, Jermiin, Ejdeback, & Hertzog, 2004). IFN-ε is the only type I IFN family member to be expressed by HeLa cells (Matsumiya, Prescott, & Stafforini, 2007). Moreover, seminal plasma was also found to upregulate expression of IFN-ε in cervicovaginal tissues (Sharkey, Macpherson, Tremellen, & Robertson, 2007), suggesting that IFN-ε may play a protective role in reproductive tissue. Interestingly, when IFN-ε was used in an intranasal/intramuscular heterologous HIV prime-boost immunization, elevated HIV-specific CD8 T-cell responses were observed in the spleen, genito-rectal draining lymph nodes and Peyer’s patches (Xi, Day, Jackson, & Ranasinghe, 2012).

Furthermore, the recently described Type III IFN-λ (IL-28/29), which has similar antiviral properties to Type I IFN, has been shown to block HIV-1 infection in
macrophages in vitro (Hou et al., 2009; M. Q. Liu et al., 2012) by inhibiting HIV-1 integration and post-transcriptional events (Tian et al., 2012). IFN-λ receptors are largely restricted to cells of epithelial origin. Together these results suggest that IFN-ε and IFN-λ may play a unique role in protecting the genital mucosa and future explorations of their potential role in protecting the FGT against HIV may prove valuable in the context of vaccine or microbicide development.

In addition to the innate factors described above, resident immune cells as well as non-immune cells of the FGT, such as GECs, express various pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which allows them to sense foreign microbes in their environment and rapidly relay messages to other innate and adaptive immune cells. Primary endocervical GECs express TLRs 1-3 and 6 (C. R. Wira, Fahey, et al., 2005). Primary human uterine GECs also express TLRs 1-9, indicating the potential to respond to a wide range of pathogens. Expression of NOD1 and NOD2 has also been detected in the human endometrium (King, Horne, Hombach-Klonisch, Mason, & Critchley, 2009). PRR recognition of pathogens typically initiates an intracellular signaling cascade resulting in the induction of inflammation (Schaefer, Desouza, Fahey, Beagley, & Wira, 2004).

1.4 - Cellular Trafficking of HIV

For many viruses, endocytosis – the process by which cells absorb molecules by engulfing them - is the primary way by which viral entry takes place (Marsh & Helenius, 2006). In contrast to this, HIV-1 is widely believed to undergo fusion at the
plasma membrane with target cells, such as CD4+ helper T-cells. In the case of HIV-1, endocytosis has classically been regarded as a pathway leading to virus degradation or non-productive infection (Agosto et al., 2009; Bedinger et al., 1988; Maddon et al., 1988; Stein et al., 1987). However, several new lines of evidence support the existence of an alternative endocytic pathway for HIV-1 entry. Firstly, HIV entry into endosomes has been observed by electron microscopy (EM) in monocytes, macrophages, T-cells as well as genital and mammary epithelial cells (Dorosko & Connor, 2010; Kinlock, Wang, Turner, Wang, & Liu, 2014; Marechal et al., 2001; Pauza & Price, 1988). Second, blocking the acidification of endosomal compartments can augment HIV infection, apparently by sparing the virus from degradation in lysosomes (Fredericksen, Wei, Yao, Luo, & Garcia, 2002). Interestingly, inhibition of clathrin-mediated endocytosis reduces the efficacy of HIV-cell fusion and infection in T-cells (Daecke, Fackler, Dittmar, & Krausslich, 2005).

Transcytosis describes a related selective vesicular transcellular pathway that is characteristic of polarized epithelia, such as columnar epithelial of the UGT. In transcytosis, cargo is endocytosed and subsequently transported from the apical pole of the cell to the basolateral pole, or vice versa. The cargo remains enclosed in transcytotic vesicles, which precludes access to the cytosol (Bomsel & Alfsen, 2003).

Conflicting reports have led to questions about whether transcytosis of HIV-1 is a mere artifact of some cell culture systems. While some reports suggest that transcytosis of HIV-1 through female GECs does not occur (Bouschbacher et al., 2008; K. B. Collins et al., 2000; Greenhead et al., 2000), data from other studies have demonstrated transcytosis of HIV-1 across cervical, intestinal, adult penile urethra
and other epithelial layers (Bobardt et al., 2007; Bomsel, 1997; Devito et al., 2000; Ganor et al., 2013; S. Gupta et al., 2013; Saidi et al., 2007), with cell-associated HIV-1 being transcytosed more efficiently compared to cell-free virus (Bomsel, 1997). The mechanism as to why cell-associated virus may transcytose at a higher rate compared to cell free virus is not completely understood but it has been suggested that HIV-1 viral synapse may play an important role in cell-associated virus entering into epithelial cells; the rate of transcytosis of cell-free HIV-1 through primary GECs has been estimated to be less than 0.02% of the initial inoculum (Bobardt et al., 2007). Despite the low rate of transcytosis of cell-free virus, both cell-associated and cell-free viruses found in semen have been documented to initiate HIV-1 infection (Anderson, 2010; K. B. Collins et al., 2000; C. J. Miller et al., 2005; Salle et al., 2010). However, an animal model study has shown that cell-associated virus is harder to cause infection in vivo, further implicating the importance of cell-free virus in HIV-1 transmission (Sodora, Gettie, Miller, & Marx, 1998). Theoretically, it only takes one infectious viral particle to successfully cross the epithelium to initiate systemic infection (Fischer et al., 2010; Keele et al., 2008); therefore, the passage of cell-free virus via transcytosis as a possible mode of facilitating HIV-1 transmission is quite feasible. Furthermore, the role of endogenous and exogenous factors in the FGT, such as female sex hormones or hormonal contraceptives, on HIV infection and viral trafficking inside the cell also remains unknown and needs to be elucidated.

1.5 - Co-infections in the Female Genital Tract
In the context of HIV-1 infection, a “co-infection” is defined as an infection that is acquired subsequently to, or in conjunction with, HIV-1. Many people living with HIV-1 are also co-infected with other pathogens, typically sexually transmitted microbes such as those that cause hepatitis, gonorrhea, syphilis, bacterial vaginosis, candidiasis and genital herpes. Co-infections have been associated with increased HIV genital shedding, transmission and susceptibility to HIV-1 as well as facilitating HIV-related pathology and the progression to AIDS (Corbett et al., 2002; Iqbal & Kaul, 2008).

1.5.1 – Herpes Simplex Viruses

The human herpes simplex viruses (HSV-1, HSV-2) were initially identified by Lowenstein in 1919 (Roizman, 2001). Later, these viruses were classified into two closely related members of the Alphaherpesviridae subfamily, showing a homology at the DNA level of 83% in protein coding regions (Dolan, Jamieson, Cunningham, Barnett, & McGeoch, 1998). Both HSV-1 and HSV-2 cause lifelong incurable infections. Primary infection of the epithelium is followed rapidly by entry of the virus into the central nervous system, thus establishing a latent infection (Corey, Adams, Brown, & Holmes, 1983). The course of disease is characterized by periodic reactivations that can be either symptomatic, causing ulcerative lesions in mucosal tissues, or asymptomatic, where there is evidence of ongoing viral replication in the absence of herpetic lesions (Augenbraun et al., 1995; Schacker, Zeh, Hu, Hill, & Corey, 1998; Wald, Huang, Carrell, Selke, & Corey, 2003).
HSV-1 and -2 are enveloped, icosahedral double-stranded DNA viruses with a capsid 100nm in diameter and an electron opaque core (Roizman, 2001). The bilayer envelope holds multiple copies of 12 different glycoproteins that protrude from the membrane, which serve several immunoregulatory functions besides their primary mechanical functions in viral adsorption and entry (Roizman, 2001). In the space between the envelope and the capsid lies an amorphous structure called the tegument, which consists of several viral proteins involved in the initial phase of viral infection and replication, such as the virus host shutoff (vhs) protein (Roizman, 2001; Zhou, Chen, Jakana, Rixon, & Chiu, 1999).

The HSV genome is expressed in a temporally regulated cascade characterized by immediate early (α), early (β1), delayed early (β2), early late (γ1) and late (γ2) genes, each with a certain characteristic group of promoters regulating their expression (Roizman, 2001). Generally, the α-gene products are transcription inducers, and they include infected cell protein-0 (ICP0). The β-gene products are viral enzymes, such as the thymidine kinase (TK), an enzyme that plays an important role in the replication of the viral genome (Roizman, 2001). Lastly, the products of γ-genes are the structural and accessory proteins of the viral particle, including tegument layer proteins, such as the vhs protein (Nishiyama, 2004; Rajcani, Andrea, & Ingeborg, 2004; Roizman, 2001). The vhs protein – which immediately degrades host mRNA following its release into the cytoplasm of the target cell - has major effects on the cytokine production of infected cells and reduces the quantity of type I IFN. This provides evidence that HSV-2 is armed with
machinery that is also able to overcome essential host antiviral responses (Nishiyama, 2004; Roizman, 2001; Zhou et al., 1999).

HSV-2 is one of the most prevalent viral STIs, infecting 20–30% of sexually active adults in North America and up to 80% in sub-Saharan Africa (Corey, Wald, Celum, & Quinn, 2004). Furthermore, up to 85% of people in sub-Saharan Africa living with HIV are also infected with HSV-2 (Weiss, 2004) and many studies indicate that a synergy exists between HIV-1 and HSV-2 infection. A recent meta-analysis demonstrated HSV-2 infection to be associated with a threefold increase in susceptibility to HIV in both men and women from the general population (Freeman et al., 2006). Part of this increased susceptibility may be attributed to HSV-2-induced ulcerations, which create breaches in the physical barrier of the genital epithelium (C. L. Celum, 2004). Genital HIV-1 shedding is also markedly increased during clinical HSV-2 reactivations, accompanied by an increase in HIV-1 plasma viral load (Mole, Ripich, Margolis, & Holodniy, 1997). Herpetic lesions and possibly asymptomatic HSV-2 mucosal shedding also generate an influx of activated CD4+ T cells into the FGT that persist for months after healing, which may facilitate the transmission of HIV (J. Zhu et al., 2009). Thus, there is a breadth of compelling research which shows a synergistic relationship between HSV-2 and HIV-1, but little is known about how these two viruses interact at the level of the genital epithelium, the main target site for HSV-2 and the predominant site of HIV-1 acquisition globally.

Recently it has also been suggested that HSV-1 may also play a role in mediating HIV-replication in the genital tract (D. H. Tan, Kaul, & Walsmley, 2009). HSV-1 co-infection has largely been ignored, yet there are several reasons that this
co-infection remains an important issue for study. First, the seroprevalence of HSV-1 is consistently higher than that of HSV-2 among both HIV-infected and HIV-uninfected populations (Ameli et al., 2006; J. S. Smith & Robinson, 2002; van Benthem et al., 2001). Furthermore, HSV-1 and HSV-2 are closely related viruses that share 83% genetic homology (Dolan et al., 1998). Their virological and pathobiological similarities suggest that their implications on HIV pathogenesis may be similar as well. Finally, HSV-1 is becoming increasingly relevant because the incidence of genital HSV-1 has risen (Coyle et al., 2003; Manavi, McMillan, & Ogilvie, 2004; C. M. Roberts, Pfister, & Spear, 2003; Schacker et al., 1998; Xu et al., 2006). In fact, recent studies have shown that in certain parts of the world, the majority of serologically confirmed primary genital herpes is attributable to HSV-1, particularly among younger cohorts, such as college-aged adults (Coyle et al., 2003).

1.5.2 – Neisseria gonorrhoeae

Bacterial STIs, such as Neisseria gonorrhoeae, have been epidemiologically associated with increasing susceptibility to HIV-1 (Johnson & Lewis, 2008; Rotchford, Strum, & Wilkinson, 2000). N. gonorrhoeae is a species of Gram-negative bacteria responsible for the sexually transmitted infection gonorrhea, of which there are an estimated 106 million cases each year worldwide (Unemo & Shafer, 2014). In the United States, it is the second-most common bacterial STI after Chlamydia trachomatis infection (CDC, 2012).

N. gonorrhoeae are facultative intracellular bacteria and typically appear in pairs (diplococci), in the shape of coffee beans (CDC, 2012). N. gonorrhoeae are
motile and possess type IV pili to adhere to surfaces. The type IV pili operate mechanistically similar to a grappling hook. Pili extend and attach to a substrate that signals the pilus to retract, dragging the bacterial cell forward (Cahoon & Seifert, 2011; Hansen & Forest, 2006). *N. gonorrhoeae* also have surface proteins called Opa proteins, which bind to receptors on immune cells causing suppression of immune responses (Stern, Brown, Nickel, & Meyer, 1986). Variation in surface proteins also makes it more difficult for immune cells to recognize *N. gonorrhoeae* and mount a response (Sadarangani, Pollard, & Gray-Owen, 2011). *N. gonorrhoeae* is naturally competent for DNA transformation as well as being capable of conjugation (Biswas, Thompson, & Sparling, 1989). These processes allow for the DNA of *N. gonorrhoeae* to acquire or spread new genes. Especially dangerous from the aspect of healthcare is the ability to conjugate, since this can lead to antibiotic resistance (Kruger & Stingl, 2011; Unemo & Shafer, 2014).

The epithelial cell layer of the UGT is one of the first tissues targeted by *N. gonorrhoeae*. Initial attachment to GECs is mediated by pili, and pilus retraction then allows tight secondary binding via an interaction between Opa proteins and CEACAMs expressed on apical columnar epithelium. CEACAM binding triggers bacterial engulfment and transcellular transcytosis through polarized epithelia, allowing efficient entry into the subepithelial spaces (Chin & Parkos, 2007). Bacterial products such as lipo-oligosaccharide, peptidoglycan and lipoproteins, stimulate NLRs and TLRs on GECs and basal dwelling immune cells such as DCs and macrophages, releasing gradients of proinflammatory cytokines, including IL-6, IL-8, IL-1β, IFN-γ and IL-17. These cytokines recruit neutrophils who bind and
phagocytose the bacteria. Recently, Russell and colleagues demonstrated that the Th17 pathway was responsible for the inflammatory response induced by *N gonorrhoeae* in the mouse model (Feinen, Jerse, Gaffen, & Russell, 2010). Th17 responses have also been detected in humans with gonorrhoea, and are induced by *N gonorrhoeae* in human monocyte-derived dendritic cells along with IL-10. Induction of the Th17 pathway by *N gonorrhoeae* is consistent with other mostly extracellular pathogens that elicit a strong neutrophil response (Jerse, Bash, & Russell, 2014).

Unfortunately, innate and TH17 responses are usually insufficient to clear the infection and the inflammation induced, as well as the massive recruitment of neutrophils following infection, contribute to developing immunopathology (Criss & Seifert, 2012). Furthermore, in humans the adaptive response to *N. gonorrhoeae* infection is poor. Repeated infections are common and there is no definitive evidence of protective responses in humans (W. Zhu et al., 2011).

Symptomatic gonococcal infections are characterized by purulent exudates that contain neutrophils with intracellular diplococci. Asymptomatic infections are common including 50–80% of infections in the FGT. If untreated, chronic infection in women may result in pelvic inflammatory disease (PID), an inflammatory condition of the uterus, Fallopian tubes, and/or ovaries that leads to scar formation and possible adhesions to nearby tissues and organs. PID causes significant fertility issues.

*N. gonorrhoeae* has been suggested to play an important role in potentiating HIV transmission (Johnson & Lewis, 2008; Rotchford et al., 2000). *N. gonorrhoeae*
infection can enhance HIV transcription in infected T cells (A. Chen, Boulton, Pongoski, Cochrane, & Gray-Owen, 2003). *N. gonorrhoeae*-infected women also have more endocervical CD4+ T cells providing more targets for HIV (Levine et al., 1998). In addition, *N. gonorrhoeae* has been shown to enhance HIV infection/replication in monocyte-derived dendritic cells (MDDCs) (J. Zhang et al., 2005).

### 1.6 - HIV and Inflammation

#### 1.6.1. – Inflammation

Inflammation is part of the complex biological response that the body produces against harmful stimuli, such as pathogens or damaged cells. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Acute inflammation, in the context of infection, is derived from immune cells and resident tissue cells, such as GECs, whose PRRs recognize conserved pathogenic motifs called pathogen-associated molecular patterns (PAMPs). These cells are then activated and release inflammatory mediators including cytokines and chemokines.

Cytokines, such as the interleukin (IL) family members or tumor necrosis factor-α (TNF-α), are a broad category of small proteins (~5–20 kDa) that are important in cell signaling and communication among cells of the immune system. In contrast, chemokines are specific types of cytokines that participate in cellular chemotaxis (K. Murphy, 2011).

#### 1.6.2 - Inflammation and HIV-1
Inflammation plays a critical role in HIV pathogenesis. HIV infection is associated with a rapid and intense release of a variety of proinflammatory cytokines, including IFN-γ, TNF-α, IL-6 and IL-15 (Stacey et al., 2009). Proinflammatory cytokines have been implicated in enhancing HIV infection at the cellular level. Studies of latently infected target cells have shown that the addition of cytokines such as TNF-α, IL-6 or IL-1β increase HIV replication, mediated through the HIV promoter, the HIV-LTR (Chun, Engel, Mizell, Ehler, & Fauci, 1998; Folks et al., 1989; Poli, Bressler, et al., 1990). In addition, HIV genital tract shedding was significantly associated with higher cervico-vaginal lavage (CVL) concentrations of IL-6, IL-1β, macrophage inflammatory protein (MIP-1α), and regulated on activation, normal T cell expressed and secreted (RANTES) (Herold et al., 2013). These results suggest that an inflammatory milieu in the FGT may be important for driving HIV replication. Interestingly, it has been observed that lower levels of IL-1β, IL-6, and TNF-α were measured in the unstimulated PBMCs of highly exposed persistently seronegative (HESN) women, suggesting an immunoquiescent phenotype among this resistant cohort (McLaren et al., 2010).

Together, these studies support a future exploration of the role of inflammation in the regulation of HIV-1 infection and replication in the FGT as it may provide valuable information regarding the early pathogenesis of HIV and how the infection may disseminate in the FGT under inflammatory conditions.

1.6.3 - Chronic Immune Activation
Chronic immune activation is a stage of HIV infection characterized by polyclonal B-cell activation, high T-cell turnover of both CD4+ and CD8 T+ cells, and high levels of circulating proinflammatory cytokines and chemokines; these signs persist even among HAART-treated individuals (Marchetti, Tincati, & Silvestri, 2013). Immune activation is believed to be one of the main driving forces for CD4+ T-cell depletion and promoters of HIV replication (Paiardini & Muller-Trutwin, 2013). In addition to this, chronic immune activation is strongly associated with the sequelea of disease associated with chronic HIV infection, such as cardiovascular disease, advanced aging, diabetes and neurodegenerative diseases, among others (Deeks, Lewin, & Havlir, 2013).

Chronic immune activation is said to take place due to microbial translocation of gut bacteria. Gut “translocation” of bacteria or other microbes is defined as the passage of the gastrointestinal microflora through the intestinal epithelial barrier and the lamina propria and eventually into the internal milieu of the body (Brenchley & Douek, 2008; Brenchley et al., 2006; Wolochow, Hildebrand, & Lamanna, 1966). Translocating bacteria and microbial components, such as lipopolysaccharide (LPS) may then stimulate innate immune cells through the TLR pathways as well as other innate immune pathways, thus contributing to the proinflammatory cytokine milieu and systemic immune activation associated with chronic HIV infection (Brenchley et al., 2006).

1.6.4 –Mechanism of Immune Activation: HIV gp120-mediated mucosal barrier disruption
The etiology of immune activation is incompletely understood, but work from our lab has shed significant light on one of the potential mechanisms by which mucosal barrier disruption may originate. Our past studies show that primary human columnar epithelial cells of the gut and genital tract directly interact with HIV-1 surface glycoprotein gp120 leading to the production of an array of proinflammatory cytokines (Nazli et al., 2010). Among these cytokines was TNF-α, which facilitates a rapid decrease in trans-epithelial resistance (TER), a measure of epithelial barrier integrity. The disruption in the barrier was accompanied with increased mucosal permeability as well as bacterial and viral translocation across the epithelium. Thus, increased mucosal permeability and microbial translocation resulted directly from early interactions between HIV-1 and epithelial cells. Further studies have since revealed that gp120 mediated activation of proinflammatory cytokine pathways in epithelial cells utilizes TLR2 and TLR4, in addition to cell surface HS moieties, to mediate the induction of inflammatory responses and the concomitant barrier breakdown (Nazli et al., 2013).

Because immune activation is typically observed during the chronic stages of infection, the window of opportunity to intervene and limit these processes likely occurs during the early stages of HIV infection. Blocking HIV-mediated barrier disruption by targeting inflammatory pathways may likely prevent microbial translocation and the genesis of immune activation, thus models evaluating such pathways would be valuable.
GECs from the UGT are capable of secreting a range of chemokines such as IL-8, monocyte chemotactic protein-1 (MCP-1), MIP-1β, and the cytokines IL-6, TNF-α, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), among others, during steady-state and infectious conditions (C. R. Wira, Fahey, et al., 2005).

As previously mentioned, GECs throughout the entire genital tract express PRRs, such as TLRs, and are capable of recognizing and responding to invading pathogens. Addition of TLR agonists to epithelial cells elicits the secretion of chemokines and cytokines, usually through intracellular signaling pathways that utilize the transcription factor, nuclear factor-κB (NF-κB). Addition of the TLR2 and TLR5 agonists zymogen and flagellin, respectively, stimulated the production of the chemokines IL-8 and MCP-1, respectively, as well as the cytokine IL-6 (Schaefer et al., 2004). Furthermore, cervical and endometrial GECs have been shown to upregulate the expression of IL-6, IL-8, TNF-α and IL-1β in response to the TLR3 agonist polyinosinic-polycytidylic acid [poly (I:C)], an analogue of double-stranded RNA as well as unmethylated CpG analogues (Nazli et al., 2009; Schaefer, Fahey, Wright, & Wira, 2005).

GEC responses to intact pathogens have also been partially explored in the literature. Previous studies showed that endocervical GECs respond to Chlamydia trachomatis (S. J. Rasmussen et al., 1997) and N. gonorrhoeae (Fichorova, Desai, Gibson, & Genco, 2001) with an array of cytokines and chemokines, including IL-6 and IL-8. In response to Trichomonas vaginalis, a parasitic STI with links to increased incidence of HIV-1, human endocervical, ectocervical and vaginal
epithelial cells selectively upregulated IL-8 and macrophage inflammatory protein 3-alpha (MIP-3α) (Fichorova et al., 2006).

GECs from both the LGT and UGT have also been shown to produce a variety of cytokines following exposure to HSV-2 infection including IL-6 and TNF-α (Drannik, Nag, Sallenave, & Rosenthal, 2013; H. Li et al., 2009; H. Liu, Chen, Feng, Wu, & Li, 2013; Triantafilou, Eryilmazlar, & Triantafilou, 2014). Furthermore, a study using primary cervical GECs found that in response to HSV-2 infection, the chemokine CXCL9 was upregulated and subsequently helped mobilize CD4+ T-cell responses against the virus (Huang et al., 2012). Clearly HSV-2 infection of GECs results in the induction of significant inflammatory responses however little is known about the requirements for these antiviral innate responses. For instance, is replication necessary or can GECs sense and respond to virus in the absence of a productive infection? These are critical questions that need to be answered since HSV-2 pathogenesis in the genital tract is poorly understood, which is surprising since it is the primary target site of the virus. Furthermore, in light of the fact that HSV-2 synergizes with HIV-1, understanding HSV-2 pathogenesis could provide new clues as to how HIV infection is modulated by herpes infection, and new therapeutic targets against HSV-2 and HIV-1 may be acquired in the process of these investigation.

1.6.6 – Direct Vs. Indirect Induction of HIV Replication by Co-Infections

Significant evidence links sexually transmitted co-infecting microbes with inducing HIV infection/replication. For example, both herpes simplex viruses and N.
gonorrhoeae have been shown to directly infect HIV target cells and induce HIV replication via trans-activation of the HIV-LTR promoter region (A. Chen et al., 2003; Ding et al., 2010; Golden et al., 1992; Schafer, Hiscott, & Pitha, 1996). Furthermore, evidence also suggests that in addition to intact microbes, pathogenic motifs, such as TLR ligands, may be sufficient to induce HIV replication in target cells. For instance, the TLR1/TLR2 ligand Pam3CSK4 was found to increase Langerhans cell capture of HIV-1 and subsequent trans-infection of T cells (de Jong et al., 2008).

Although co-infection has been linked to inducing HIV replication, most studies have focused on the direct interaction between HIV-harbouring target cells and co-infecting microbes. Less attention has been given to potential indirect mechanisms. For example, although inflammation has been linked to increasing HIV replication and co-infections have been shown to induce GEC inflammatory responses, it remains to be seen whether GEC responses to co-infecting microbes are capable of inducing sufficient inflammation to affect the outcomes of HIV infection. This is particularly important to elucidate since GECs are one of the first cells to encounter pathogens following sexual transmission and thus may act as desirable prophylactic targets to prevent HIV infection.

1.6.7 – Curcumin, A Potent Anti-inflammatory Compound

Curcumin (diferuloylmethane) is a polyphenol compound that gives the spice turmeric its distinctive yellow colour. Curcumin has been shown to exert potent anti-inflammatory activity such as suppressing proinflammatory cytokine (TNF-α, IL-1β, IL-6) production (Aggarwal & Sung, 2009). In vitro curcumin modulates the
inflammatory response by directly binding to and downregulating the activity of cyclooxygenase-2, lipoxygenase, inducible nitric oxide synthase as well as the transcription factors NFκB and AP-1 (S. C. Gupta et al., 2011). Furthermore, curcumin has been shown to block the expression of cell adhesion molecules (e.g., ICAM-1), which are necessary for the interaction of leukocytes with endothelial cells (Kumar, Dhawan, Hardegen, & Aggarwal, 1998). In addition to this, curcumin has also been shown to possess anti-cancer, anti-diabetic, antioxidant, cardio-protective and microbicidal activity (Prasad, Gupta, Tyagi, & Aggarwal, 2014), suggesting that curcumin likely exerts pleiotropic modes of action.

The extensive studies from cell-based and animal models have formed a solid basis for evaluating the safety and efficacy of curcumin against a plethora of human diseases. The first published clinical study investigating curcumin's efficacy was published back in 1937 where curcumin was found to provide relief from human biliary diseases (Oppenheimer, 1937). Since this initial discovery, observations from more than 65 human clinical trials of curcumin, which included more than 1000 patients, have been published, and more than 40 other clinical trials are under way (Prasad et al., 2014). In a human study where they administered 1,800 mg of curcumin orally, the half-life of the compound was measured at 6-7 hours (Jager et al., 2014). Extensive work in rats also demonstrated that the elimination half-life values for intravenous (10 mg/kg) and oral (500 mg/kg) curcumin were reported to be 28.1 and 7.5 hours, respectively (Yang, Lin, Tseng, Wang, & Tsai, 2007).
Curcumin has been used against human cancers including colorectal cancer, pancreatic cancer, breast cancer, prostate cancer, multiple myeloma, lung cancer, oral cancer, and head and neck squamous cell carcinoma. In these studies, curcumin was used for both prevention and treatment of cancer (Prasad et al., 2014). In terms of recent successful trials, curcumin was recently found to reduce the formation of aberrant crypt foci, the precursor of colorectal polyps (Carroll et al., 2011). In another study, curcumin was administered to patients with colorectal cancer after diagnosis and before surgery. Curcumin administration increased body weight, decreased serum TNF-α levels, increased the number of apoptotic cells and enhanced expression of p53 in tumor tissue (He et al., 2011). The efficacy of curcumin as maintenance therapy in several gut disorders including ulcerative colitis has also been evaluated clinically (Prasad et al., 2014). For instance, colitis relapse rates were 4.65% in curcumin-treated patients compared to 20.51% in the placebo group (Hanai et al., 2006). Ingestion of oral curcumin at 500 mg/day along with prednisone was also associated with clinical and endoscopic remission of disease in a patient with ulcerative colitis (Lahiff & Moss, 2011).

From the observations of some clinical trials, the efficacy of curcumin against human diseases seems promising. A search on www.clinicaltrials.gov revealed that curcumin is being evaluated for numerous human diseases including cancer, irritable bowel syndrome, inflammatory conditions, arthritis, neurological conditions, and diabetes. It is expected that these ongoing clinical trials will provide a deeper understanding of curcumin’s efficacy and mechanism of action against human diseases. Furthermore, direct antimicrobial effects of curcumin have also
been observed, suggesting that curcumin can also act in an extracellular way to neutralize microbes such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio cholera* (Sasidharan, Sreekala, Jacob, & Nambisan, 2014), underscoring the versatility and potential benefit of the compound.

Curcumin has also been evaluated as an HIV/AIDS treatment in several *in vitro* experiments and in one clinical trial. *In vitro* curcumin has been shown to directly inhibit HIV replication in susceptible cells (Gandapu, Chaitanya, Kishore, Reddy, & Kondapi, 2011; C. J. Li, Zhang, Dezube, Crumpacker, & Pardee, 1993), inhibit HIV-LTR activation (Barthelemy et al., 1998; Ferreira et al., 2011; Gandapu et al., 2011; C. J. Li et al., 1993) and enhance HAART activity (Riva et al., 2008). In contrast, no significant evidence of curcumin-associated reduction in viral load was observed in a clinical trial examining the effectiveness of curcumin as an antiviral agent in patients with AIDS (James, 1996). This clinical study was performed on AIDS patients and no evidence of the prophylactic benefit of curcumin was examined, as well as whether the compound reduced chronic immune activation. Furthermore, curcumin was administered orally, just like it is in the vast majority of clinical trails where the compound is used. It remains to be seen whether direct application to a mucosal surface may provide improved efficacy with respect to interfering with HIV infection or replication, particularly since studies suggest that oral administration of curcumin results in poor bioavailability due to its rapid metabolism *in vivo* (Prasad et al., 2014).

1.7 - Female Sex Hormones and Hormonal Contraceptives
1.7.1 - Estrogen

(17)β-estradiol (E2) is the dominant estrogen found in women. E2 is primarily produced by the granulosa cells of the ovaries and plays a prominent role in mediating sexual development, behaviour, reproductive functions and differentiation of various tissues. E2 exerts its effect by binding to intracellular estrogen receptors (ERs), of which two subtypes exist: ERα and ERβ. Both ERs are widely expressed in different tissue types; however, there are some notable differences in their expression patterns. ERα is found in endometrium, breast cancer cells, ovarian stromal cells and hypothalamus. In contrast, ERβ has been documented on ovarian granulosa cells, kidney, brain, lung, bone, heart, intestinal mucosa, prostate and endothelial cells (Bouman, Heineman, & Faas, 2005; Fish, 2008). These receptors are expressed by many types of immunocompetent cells, including T-cells, B-cells, DCs, macrophages, neutrophils and natural killer (NK) cells, as well as non-immune cells, such as GECs (Fish, 2008). In the uterus, elevated E2 levels are associated with increased proliferation of epithelial cells (Groothuis, Dassen, Romano, & Punyadeera, 2007). Physiological serum E2 levels fluctuate between $10^{-9}$ M to $10^{-12}$ M throughout the normal menstrual cycle and can reach as high as $10^{-8}$ M during pregnancy (Abbassi-Ghanavati, Greer, & Cunningham, 2009; Stricker, Eberhart, Chevailler, Quinn, & Bischof, 2006).

Hydrophobic sex hormones, such as E2 and progesterone, exert their effect after binding to their cognate intracellular steroid receptors. After binding, a nuclear localization signal is revealed, leading hormone bound receptors to dimerize and
translocate to the nucleus, where they associate with a variety of co-factors (or co-repressors) resulting in transcriptional trans-activation or trans-repression, respectively, of target genes that contain hormone response elements (HREs) in their promoters (Rosenfeld, Lunyak, & Glass, 2006).

In contrast to this, E2 also signals through a member of the seven-transmembrane (7TM) G-protein coupled receptor (GPCR) family, named G-protein receptor-30 (GPR-30). When E2 binds to membrane-bound GPR-30, it results in the intracellular release of calcium and synthesis of phosphatidylinositol (3, 4, 5)-triphosphate in the nucleus. This protein therefore plays a role in the rapid non-genomic signaling events widely observed following stimulation of cells and tissues with E2 (Prossnitz et al., 2008; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005).

1.7.2 – Progesterone

Progesterone (P4) plays a role in the differentiation of the endometrium, control of implantation and the maturation of mammary epithelium. The granulosa cells of the ovary and the corpus luteum, are the major sources of P4 during the menstrual cycle. The adrenal glands and central nervous system are additional sources of P4 production (Graham & Clarke, 1997; Schumacher et al., 2007). The major biological effects of progesterone are mediated by intracellular progesterone receptors (PRs), which are also expressed as two isoforms, PR-A and PR-B (Kastner et al., 1990; X. Li & O’Malley, 2003). Membrane-bound PRs have also been observed, however, their overall contributions to progesterone signalling remain unknown.
(Gellersen, Fernandes, & Brosens, 2009). In contrast to E2, P4 inhibits proliferation of the uterine epithelium. In contrast, P4 plays a role in making the endometrium receptive to implantation of the blastocyst, supports early pregnancy and increases uterine secretion (i.e. glycogen) by endometrial epithelial cells (Moyer & Felix, 1998). Physiological serum P4 levels normally fluctuate between $10^{-7}$ M and $10^{-10}$ M during a standard menstrual cycle and peak at $10^{-6}$ M during the third trimester of pregnancy (Abbassi-Ghanavati et al., 2009; Kratz, Ferraro, Sluss, & Lewandrowski, 2004; Stricker et al., 2006).

1.7.3 - Medroxyprogesterone Acetate

Medroxyprogesterone 17-acetate (MPA), or its injectable homologue depot medroxyprogesterone acetate (DMPA; commercial name Depo-Provera), is a first generation synthetic progestin. In females, the most common use of MPA is as an oral or subcutaneous injectable contraceptive. As a contraceptive, MPA works by inhibiting gonadotropin secretion from the pituitary, which in turn prevents ovarian follicle maturation and ovulation. It also thins the endometrium, making it less hospitable for implantation of the conceptus (Guilbert et al., 2009). MPA has a 100 times greater relative binding affinity for PR than P4 (Philibert et al., 1999) and it has been shown to bind and signal through ER, glucocorticoid receptor (GR), androgen receptor (AR) and mineralocorticoid receptor (MR) (Africander, Verhoog, & Hapgood, 2011; Kemppainen et al., 1999; Koubovec, Ronacher, Stubsrud, Louw, & Hapgood, 2005; Philibert et al., 1999). Globally, more than 100 million women use MPA, and it is one of the most popular contraceptive methods in the developing
world (Population Division, 2003). Physiologically relevant serum levels of MPA range from $10^{-9}$ M immediately following administration to $10^{-12}$ M, which is usually measured approximately 3 months following use (Goldman, 2000).

1.7.4 – The Menstrual Cycle

The menstrual cycle describes the natural changes that occur in the uterus and ovary in preparation of the uterus for pregnancy. A woman’s first menstruation is termed *menarche*, and occurs typically around age 12-13. The end of a woman’s reproductive phase of life is called the menopause, and this commonly occurs somewhere between the ages of 45 and 55. In humans, the length of a menstrual cycle varies greatly among women, with 28 days designated as the average length. Each cycle can be divided into three phases based on events in the ovary (ovarian cycle) or in the uterus (uterine cycle). The ovarian cycle consists of the follicular phase, ovulation, and luteal phase whereas the uterine cycle is divided into menstruation, proliferative phase, and secretory phase (Marieb, 2012).

By convention, the menstrual cycle begins on the first day of menstrual bleeding (See Appendix B). Stimulated by gradually increasing amounts of E2 in the follicular phase, menses slows then stops, and the lining of the uterus thickens. Follicles in the ovary begin developing under the influence of a complex interplay of hormones. Approximately mid-cycle, 24–36 hours after the luteinizing hormone (LH) surges, a follicle releases an ovum, or egg, in an event called ovulation. After ovulation, the remains of the dominant follicle in the ovary become the corpus
luteum, which primarily functions in producing large amounts of P4. Under the influence of P4, the endometrium (uterine lining) changes to prepare for potential implantation of an embryo to establish a pregnancy. If implantation does not occur within approximately two weeks, the corpus luteum will involute, causing sharp drops in levels of both P4 and E2, leading to the shedding of the uterine lining and menstruation (Marieb, 2012).

1.7.5 – Do Female Sex Hormones Regulate Susceptibility to HIV-1/SIV?

A number of studies in the past two decades indicate that endogenous female sex hormones and exogenous hormonal contraceptives may effect HIV-1 or SIV infection or disease progression. Non-human primate studies have consistently found that the administration of DMPA to rhesus macaques enhances the risk of acquiring SIV (P. A. Marx et al., 1996; Trunova et al., 2006; Veazey, Shattock, et al., 2003), whereas E2 has been shown to be protective against SIV infection (S. M. Smith, Baskin, & Marx, 2000). Human epidemiological studies have shown that P4-based contraceptives may lead to increased risk of HIV-1 acquisition and disease progression (Baeten et al., 2005; P. A. Marx et al., 1996; E. Stringer & Antonsen, 2008; E. M. Stringer et al., 2007). Furthermore, in a 10 year prospective cohort study, female sex workers from Kenya who either used DMPA or oral contraceptive pills (OCPs) were found to be at increased risk of acquiring HIV-1 compared to women who did not use hormonal contraceptives (Lavreys, Chohan, et al., 2004). These results have been supported by others (Lavreys, Baeten, et al., 2004; Leclerc, Dubois-Colas, & Garenne, 2008), but also refuted, including by one study which
found that among the 4,439 women recruited from family planning clinics in Uganda and Zimbabwe, neither OCPs nor DMPA were associated with HIV acquisition (C. S. Morrison et al., 2007). More recently, a prospective cohort study of nearly 3,800 serodiscordant couples from seven African nations found that the risk of acquiring HIV from an infected male partner was twice as high among women who used injectable hormonal contraceptives and that HIV-infected women who used injectable hormonal contraceptive were twice as likely to transmit HIV to an uninfected male partner (Heffron et al., 2012). In another recent study cervical tissue explants from 22 HIV-1 seronegative women were exposed to R5 HIV-1 \textit{ex vivo} and among the eight tissues that were productively infected, all were obtained from women in their secretory phase (high P4) of their menstrual cycle (Saba et al., 2013).

A number of reasons have been proposed for the increased HIV risk associated with contraceptive hormone usage (Blish & Baeten, 2011). Hormonal contraceptive use has been associated with cervical ectopy, which is the extension of the endocervical columnar epithelium onto the exocervical face (Critchlow et al., 1995). Cervical ectopy, in turn, has been associated with increased susceptibility to HIV-1 (Moss et al., 1991), perhaps due to enhanced exposure of endocervix, which has been implicated as the site of transmission for SIV in other studies (Q. Li et al., 2009).

Hormonal contraception is also associated with increased inflammation in the genital tract, including an increase in the number of CCR5-expressing T-cells (Baeten et al., 2001; Ghanem et al., 2005). This inflammation and recruitment of
potential HIV-1 target cells could increase the risk of HIV-1 acquisition in uninfected hormonal contraceptive users. Hormonal contraceptive use has also been associated with increasing the risk of cervical STIs and inflammation, particularly Chlamydia and non-specific cervicitis (Baeten et al., 2001; Ghanem et al., 2005; Lavreys, Chohan, et al., 2004; Louv, Austin, Perlman, & Alexander, 1989; C. S. Morrison et al., 2004; Prakash, Kapembwa, Gotch, & Patterson, 2002). In HIV-1-uninfected women, this increased risk of genital tract infection may increase the risk of HIV-1 acquisition and in HIV-1-infected women, increased genital HIV-1 shedding as a result of genital infections may increase the risk of transmitting HIV-1 to an uninfected partner (McClelland et al., 2001; Pedraza et al., 1999). In addition, hormonal contraceptives do appear to increase the risk of yeast vaginitis and decrease the number of protective H$_2$O$_2$-producing lactobacilli, both of which could enhance HIV transmission (H. L. Martin, Jr. et al., 1998; H. L. Martin et al., 1999; L. Miller et al., 2000).

Overall, the data supporting many of these mechanisms are limited, making it difficult to determine which, if any, of these mechanisms are actually relevant to HIV-1 transmission. The link between certain female sex hormones or contraceptive formulations and increased HIV susceptibility remains a highly contentious one. Recently, the WHO convened a technical consultation regarding hormonal contraception and HIV acquisition, progression and transmission. Due to a lack of sufficient evidence due to an absence of randomized controlled trails measuring HIV acquisition among contraceptive users, and an abundance of observational studies with opposing conclusions, the panel released a statement advising that women
should continue using injectable hormonal contraceptives, however, “...women using progestin-only injectable contraception should be strongly advised to also always use condoms, male or female, and other HIV preventive measures” (WHO, 2013).

All together these results suggest that P4 and P4-based contraceptives, in particular DMPA, may play a significant role in regulating susceptibility to genital tract infections, such as HIV-1, and underscore a need to elucidate the underlying mechanisms involved in this regulation as well as whether sex hormones may influence early events in the genital tract such as the interactions between HIV and GECs as well as the inflammatory milieu of the FGT.

1.7.6 - Modulation of GEC Immune Responses by Female Sex Hormones

Sex steroid hormones regulate the activity of immune cells, including lymphocytes, macrophages, granulocytes, as well as non-immune cells like fibroblasts and epithelial cells. The modulation of the immune system by sex steroids has both physiological and pathological implications (Fish, 2008; Kaushic et al., 2010; Nguyen et al., 2014).

In terms of its effect on GEC inflammatory profiles, E2 has been found to suppress the secretion and/or expression of proinflammatory cytokines. For example, in human keratinocytes, E2 exposure resulted in the suppression of MCP-1, RANTES, and inflammatory protein-10 (IP-10) (Kanda & Watanabe, 2003a, 2003b, 2003c). Previous data from a rodent model also shows E2 inhibits the constitutive and TLR-agonist-induced secretion of TNF-α and MIP3-α by uterine epithelial cells
(Grant-Tschudy & Wira, 2005). In similar experiments with polarized primary uterine epithelial cells, E2 enhanced the secretion and mRNA expression of the antimicrobials SLPI and human HBD2 (J. V. Fahey et al., 2008), while concomitantly reducing the LPS- and poly (I:C)- induced secretion of the proinflammatory cytokines macrophage migration inhibitory factor (MIF), IL-6, and IL-8, as well as a 70% inhibition of mRNA expression of NF-κB in primary uterine epithelial cells. Furthermore, in the endometrial epithelial cell line RL95-2, E2 also suppressed poly (I:C)-mediated induction of IL-6, IL-8 and IP-10, whereas P4 had no effect (Lesmeister, Jorgenson, Young, & Misfeldt, 2005). In vaginal epithelial cells incubated with P4 alone, no effect was seen on AMP production, but a combination of E2 and P4 reduced secretion of HBD2 significantly, perhaps due to the fact that PR expression depends on E2 (Ing & Tornesi, 1997). It was also found that primary vaginal cell secretion of the cytokine MIP-3α was elevated in P4-exposed cells, while undetectable in secretions from control and E2-treated cells (Patel, Fahey, Rossoll, & Wira, 2013; C. R. Wira, Fahey, Rodriguez-Garcia, Shen, & Patel, 2014).

Although less data is available with respect to the effects of hormonal contraceptives like MPA on primary GEC inflammatory responses, work by Govender et al, showed that MPA, unlike norethisterone acetate (NET-A) – another type of progestin-based contraceptive - decreased mRNA expression of the proinflammatory IL-6, IL-8 and RANTES genes, and IL-6 and IL-8 protein levels in End1/E6E7 endocervical epithelial cell line (Govender et al., 2014). Furthermore, in both the ectocervical and vaginal cell lines, Ect1/E6E7 and Vk2/E6E7, results show that unlike P4 which up-regulates IL-6 gene expression, both MPA and NET-A have
no effect on IL-6 production. In contrast, MPA, significantly up-regulated IL-8, while NET-A had no significant effect. Furthermore, unlike P4, which significantly up-regulated RANTES gene expression in the Ect1/E6E7 cell line, MPA significantly down-regulates RANTES gene expression, while NET-A had no effect (Africander, Louw, & Hapgood, 2013).

Together, these results suggest that in addition to potentially regulating susceptibility to HIV-1, sex hormones and hormonal contraceptives may also be modulating GEC immune responses. However, due to the use of non-physiologically relevant cell lines as well as a general lack of studies using primary human GECs, a need exists for a better exploration of the effect of sex hormones on immune responses, particularly those that could affect the outcome of HIV-1 infection, such as TNF-α, IL-6 and IL-1.

Although the effects of hormones on GEC inflammatory responses remains poorly explored in the literature, some studies have examined how hormones affect genital tissue inflammatory responses or how cytokine and chemokine profiles change during the menstrual cycle. This data can be mined to gauge the potential effect of female sex hormones on GEC inflammatory responses. Analysis of cervical-vaginal secretions demonstrate that chemokines and cytokines (IL-6 and IL-8) as well as endogenously produced AMPs (SLPI, HBD2, and lactoferrin, among others) dropped significantly at mid-cycle (day 13) and remained depressed for 7–10 days, returning to proliferative stage levels just before menstruation (Keller et al., 2007). In contrast, total protein and transforming growth factor-beta (TGF-β) levels remained unchanged throughout the menstrual cycle. IL-8 has been measured at
significantly higher levels during the early to mid-proliferative phase of the menstrual cycle (Arici, Seli, Senturk, et al., 1998; Arici, Seli, Zeyneloglu, et al., 1998), whereas MCP-1 levels are highest throughout the entire proliferative phase (Jones, Kelly, & Critchley, 1997). Furthermore, using tissue organ cultures, Sentman et al. demonstrated that E2 and P4 induce IP-10 mRNA transcription as well as protein expression in human endometrium, but MIP-1α and RANTES, among others, were not affected (Sentman, Meadows, Wira, & Eriksson, 2004). Accordingly, IP-10 mRNA production from primary endometrial GECs peaked in the late proliferative phase (Dominguez et al., 2008).

While several studies have examined the role of endogenous hormones on the induction of chemokines, little is known about the role of exogenous hormones, such as MPA, on the induction of chemokines from GECs from the URT, or, how the combination of hormones and secondary infections (such as HSV-2) can contribute to the production of chemokines, and therefore, HIV target cell recruitment. HIV target cells, which include CD4+ T-cells, macrophage and DCs, are recruited to tissues via chemokine gradients produced by resident cells in the tissue such as epithelial and stromal cells. Some of these produced chemokines are able to potently recruit macrophage and DCs (MCP-1, MIP-1α), CD4+ T-cells (IP-10, Eotaxin) or all three (IL-8, RANTES) (Rancez, Couedel-Courteille, & Cheynier, 2012). Thus, understanding how female sex hormones contribute to GEC chemokine production may ultimately provide clues as to how hormones may regulate HIV susceptibility in the FGT.
1.8 - Rationale, Hypothesis and Thesis Objectives

Although women constitute half of the estimated 34 million people living with HIV/AIDS worldwide, little is known about the early events of HIV-1 infection in the FRT, where 40% of all new HIV infections are established each year. The early pathogenesis of HIV infection in the FGT remain particularly elusive in the context of endogenous or exogenous factors found in the genital microenvironment that may influence susceptibility to HIV-1, such as inflammation. Two principal genital tract factors associated with initiating or regulating inflammation include female sex hormones and STIs. P4 and P4-based contraceptives as well as STIs such as HSV-2 and *N. gonorrhoeae* have been linked to increasing susceptibility to HIV-1 infection as well as inducing HIV replication, but little is known about the mechanism by which this takes place, particularly the role GEC inflammation plays in the process.

Given the importance of the genital tract microenvironment in determining the outcome of infection, we hypothesized that the sexually transmitted co-infecting microbes HSV-2 and *N. gonorrhoeae*, and the female sex hormones P4 and MPA, increased susceptibility to HIV in the FGT by inducing GEC inflammation that facilitated HIV infection and replication. Specifically, we predicted that GECs grown in the presence of P4 and in particular MPA, would upregulate inflammatory molecules such as TNF-α, IL-6 and IL-1α, that would facilitate HIV uptake and result in productive infection of primary human GECs, whereas E2 would decrease the levels of inflammatory molecules, thus protecting primary human GECs from HIV infection/replication. Furthermore, we predicted that in response to co-infecting microbes, specifically HSV-2 and *N. gonorrhoeae*,
primary human GECs would induce inflammatory responses that would increase HIV replication within infected T-cells. Lastly, based on the fact that inflammation leads to mucosal barrier disruption and has been associated with increasing HIV replication, we predicted that by blocking inflammatory pathways in primary human GECs, using the broad anti-inflammatory compound curcumin, we could decrease inflammation, protect the mucosal barrier against HIV and decrease HIV replication in T-cells.

The overall objective of this work was to elucidate the ways in which two major factors in the genital tract microenvironment, specifically female sex hormones and STIs contributed to the early events of HIV pathogenesis in the FGT, particularly in the context of inflammation. These objectives were addressed through the following aims:

1) To characterize the GEC innate immune responses to HSV-2, a common HIV-1 co-infecting pathogen, and to determine the viral factors that influence these responses

2) To determine whether GEC innate immune responses to bacterial and viral co-infecting microbes enhances HIV-1 replication in T-cells, and examine the underlying mechanism

3) To assess whether blocking GEC inflammatory pathways with curcumin decreases HIV-1 infection and replication.

4) To explore how female sex hormones regulate HIV-1 infection and replication in primary human GECs, in the context of inflammation.
Briefly, the experimental results presented in this thesis demonstrate that in response to HSV-2, primary GECs upregulated innate inflammatory responses, which were enhanced in the absence of the HSV-2 vhs protein and which required robust HSV-2 replication. Furthermore, we found that common co-infecting microbes, specifically HSV-1, HSV-2 or *N. gonorrhoeae*, directly induced HIV replication in T-cells and upregulated inflammatory processes in primary GECs that indirectly increased HIV replication. Based on these studies, we blocked inflammatory pathways using the broad anti-inflammatory compound curcumin and found that curcumin pre-treatment protected the genital epithelial barrier against HIV-1-mediated disruption, prevented the gp120-mediated upregulation of proinflammatory cytokines and chemokines by GECs, blocked GEC innate inflammatory responses and indirect induction of the HIV promoter in T-cells and blocked both HIV-1 and HSV-2 replication in T-cell sand GECs, respectively. Lastly, we found that P4 and in particular the synthetic P4-derived contraceptive hormone, MPA, increased uptake of HIV-1 and transcytosis, but not replication, across genital epithelial cells (GECs) – independent of an inflammatory milieu - and that this enhanced transcytosis resulted in increased infection of HIV target cells.

Furthermore, our work showed that

These results suggest that sex hormones and co-infection both play a role in regulating HIV-1 infection and replication in the FGT, with the former doing so in the absence of upregulated inflammation. Furthermore, our results suggest that anti-inflammatory compounds such as curcumin may offer paradigm shifting
prophylactic or therapeutic strategies against HIV-1 infection and future steps to investigate its potential benefit in *in vivo* should be taken.
Chapter 2

Proinflammatory cytokines and chemokines - but not interferon-β - produced in response to HSV-2 in primary human genital epithelial cells are associated with viral replication and the presence of the virion host shutoff protein.


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This study examined whether viral replication or viral components that subvert innate responses in immune-competent cells, specifically the vhs protein, play a role in determining primary GEC innate antiviral responses to HSV-2. We found that proinflammatory cytokines and chemokines were upregulated in primary GECs in response to replication-competent HSV-2, but suppressed in the presence of the vhs protein. In contrast, upregulation of biologically active IFN-β depended on viral replication but was not affected by VHS.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments. I was responsible for the generation and analysis of the data. Dr. Aisha Nazli provided technical assistance. Dr. Karen L. Mossman provided technical expertise. Dr. Charu Kaushic and I wrote and edited the manuscript.
Proinflammatory cytokines and chemokines - but not interferon-β - produced in response to HSV-2 in primary human genital epithelial cells are associated with viral replication and the presence of the virion host shutoff protein.

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Running Head: Female genital epithelial cell innate antiviral responses to HSV-2
ABSTRACT

**Problem:** It is unknown whether viral replication or viral components that subvert innate responses in other cells, specifically the virion host shutoff (VHS) protein, play a role in determining primary genital epithelial cell (GEC) innate antiviral responses.

**Method of Study:** Cultures of primary female GECs were exposed to wildtype (WT), VHS-deleted (vhsB) or UV-inactivated HSV-2. Antiviral pathway induction was evaluated by measuring nuclear factor-κB (NFκB) translocation by immunofluorescent microscopy. Proinflammatory cytokines, chemokines and interferon (IFN) were measured by Luminex or ELISA. Biological activity of IFN-β was evaluated via VSV-GFP bioassay, by blocking secreted IFN-β with neutralizing antibodies and by measuring interferon-stimulated genes by RT-PCR.

**Results:** Proinflammatory cytokines and chemokines were upregulated in primary GECs in response to replication-competent HSV-2, but suppressed in the presence of the VHS protein. In contrast, upregulation of IFN-β depended on viral replication but was not affected by VHS. However, the IFN-β produced was biologically active and reduced the viral burden.
**Conclusions:** Viral factors such as replication and the presence of the VHS protein play important roles in regulating innate antiviral responses against HSV-2 from primary GECs.

**Keywords:** genital epithelial cells, HSV-2, mucosal immunity, interferon, cytokines, viral immunology.
INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is a highly successful human pathogen with a worldwide prevalence exceeding 500 million (Looker, Garnett, & Schmid, 2008) and seroprevalence rates exceeding 20% among sexually active North American adults and 70% of sub-Saharan Africans (Nahmias, Lee, & Beckman-Nahmias, 1990; J. S. Smith & Robinson, 2002). HSV-2 is responsible for approximately two thirds of the sexually transmitted mucocutaneous lesions commonly referred to as genital herpes in North America, and is associated with an increased risk of HIV acquisition (C. L. Celum, 2004; Corey et al., 2004; Freeman et al., 2006; Wald & Corey, 2003). Clinical evidence suggests that women are more susceptible to herpes infections than men (Langenberg, Corey, Ashley, Leong, & Straus, 1999). The lack of understanding of HSV-2 pathogenesis in the female reproductive tract (FRT) precludes the development of specific and more effective antiviral therapies.

Primary HSV-2 infection preferentially targets the genital epithelium and subsequently establishes latency by rapidly escaping into the central nervous system (Corey et al., 1983). Herpes infection is characterized by periodic reactivations that can be either symptomatic, causing ulcerative lesions in mucosal tissues, or asymptomatic, where there is evidence of ongoing viral shedding and replication in the absence of lesions (Augenbraun et al., 1995; Schacker et al., 1998; Wald et al., 2003).

Epithelial cells, which line mucosal tracts, are considered to be the primary targets of HSV-2. These cells play an important barrier role by occluding harmful
pathogens from entering the internal milieu and participate in mounting innate and adaptive immune responses by secreting a number of immunological factors including proinflammatory cytokines and chemokines (C. R. Wira, Fahey, et al., 2005; C. R. Wira, Grant-Tschudy, & Crane-Godreau, 2005). Immortalized or transformed cell lines have been used to explore epithelial cell responses against HSV-2 (Huber et al., 2001; H. Li et al., 2006; J. Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003; Sato, Linehan, & Iwasaki, 2006; Schelhaas, Jansen, Haase, & Knebel-Morsdorf, 2003; Spear, 2004), but few studies have utilized primary human cells. Recently however, a study using primary cervical genital epithelial cells (GECs) found that in response to HSV-2 infection, the chemokine CXCL9 is upregulated and this helps to mobilize CD4+ T-cell responses against the virus (Huang et al., 2012).

Primary human GECs provide a more physiological alternative to immortalized or transformed cell lines. Our lab has previously developed a model to examine primary GEC susceptibility to HSV-2 infection (MacDonald et al., 2007; Nazli et al., 2009). Primary endometrial and cervical GECs were obtained from hysterectomy tissues and compared. Both types of epithelial cell cultures consisted of columnar epithelium that formed tight junctions and displayed very similar growth rates and morphology as compared under the microscope following haematoxylin staining. They were also remarkably similar with respect to cytokine production, sensitivity to Toll-like receptor (TLR) ligands and susceptibility to HSV-2 infection (Nazli et al., 2009). Because of the abundant availability of endometrial GECs, and the fact that they have a similar phenotype and functionality as cervical GECs, we can use endometrial cells to model cervical HSV-2 infection, which is more
common. Using these cells also allows us to explore endometrial HSV-2 infection, which although is less common, is associated with serious outcomes with respect to pregnancy including neonatal survival (Kropp et al., 2006).

Our previous studies showed that the kinetics of primary *ex vivo* HSV-2 GEC infection mirrored *in vivo* replication, with the earliest viral shedding measured at 18 hours post-exposure. Primary GEC monolayers could be infected apically or basolaterally, but preferential viral shedding occurred from the apical side of the cells (MacDonald et al., 2007). Furthermore, we showed that primary GECs were completely protected against HSV-2 infection when they were pre-treated with polyinosinic:polycytidylic acid (poly I:C), a synthetic double stranded RNA (dsRNA) analogue that acts via TLR3. Biologically active IFN-β and nitric oxide, produced by GECs in response to poly I:C stimulation correlated with antiviral activity and infection resulted in upregulation of inflammatory cytokines and chemokines and activation of immunoregulatory transcription factors, including interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NFκB) (Nazli et al., 2009).

HSV-2, which establishes a chronic persistent infection, has evolved complex and efficient machinery, such as the virally encoded mRNase virion host shut-off (VHS) protein, to subvert host innate immune responses in various cell types. In dendritic cells (DCs), the VHS protein has been implicated in impairing DC (Samady et al., 2003) and NFκB activation (Cotter et al., 2011), viral recognition pathways (Cotter et al., 2010) and chemokine production (Prechtel et al., 2005). It has also been shown to directly down-regulate antiviral responses including type I interferon (IFN), cytokines and chemokines in various cell types (Duerst &
as well as antimicrobial peptides, such as the secretory leukocyte protease inhibitor (SLPI) (Fakioglu et al., 2008). However, little is known about whether VHS plays similar roles in subverting responses against HSV-2 in primary human upper GECs, or whether essential viral processes, specifically viral replication, are required for the induction of GEC antiviral responses.
MATERIALS AND METHODS

Cell Lines and Virus

Vero green monkey kidney epithelial cells (ATCC, Manassas, VA, USA) were maintained in minimum essential medium-α (α-MEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada), 100 U/mL penicillin/streptomycin (pen/strep) (Sigma–Aldrich, Oakville, ON, Canada), 2 μM L-glutamine (L-glu) (Invitrogen) and 10 μM of HEPES. Wildtype (WT) HSV-2 and vhsB, both strain 333, were kindly provided by Dr. Jim Smiley (University of Alberta). WT and vhsB viruses were propagated on confluent monolayers of Vero cells at a multiplicity of infection (MOI) of 0.01. All viruses were purified over a 36% sucrose cushion gradient (Beckman Coulter Optima L-90K Ultracentrifuge, SW41 rotor, 4°C for 2h, 22,500 x g) to exclude defective particles (Pardoe & Dargan, 2002). UV inactivated HSV-2 was prepared by exposing WT HSV-2 to UV light using a Stratalinker2400 (Agilent, Mississauga, ON, Canada). UV-inactivation was confirmed by Vero plaque assay. VSV-GFP was kindly provided by Dr. Brian Lichty (McMaster University).

Source of Tissues and Epithelial Cell Preparation

This study was approved by the Hamilton Health Sciences Research Ethics Board. All participants provided voluntary informed written consent. Endometrial tissues were obtained from women aged 36-62 years (mean age 44.7 ± 4.5 years) undergoing hysterectomies for benign gynaecological reasons at Hamilton Health
Sciences Hospital. The most common reasons for surgery were uterine fibroids and menorrhagia (heavy bleeding). The detailed protocol for isolation and culture of GECs has been described elsewhere (Kaushic, Nazli, Ferreira, & Kafka, 2011; MacDonald et al., 2007). Briefly, endometrial tissues were minced into small pieces and digested in an enzyme mixture at 37°C with shaking for 30 minutes to an hour. GECs were isolated by a series of separations through nylon mesh filters (Small Parts Inc., Logansport, ID, USA), re-suspended in primary cell media, consisting of phenol red free DMEM/F12 (Gibco, Burlington, ON, Canada) supplemented with 10µM HEPES, 2µM L-glu, 100 U/mL pen/strep, 2.5% Nu Serum culture supplement (VWR, Mississauga, ON, Canada), 2.5% Hyclone defined FBS (Hyclone, Logan, UT, USA) and 250µM Fungizone (Invitrogen) and seeded onto 0.4-µm pore-sized polycarbonate tissue culture inserts (BD Falcon, Mississauga, ON, Canada). Polarized monolayers were formed within 5–7 days of cell culture. Confluence of GEC cultures was monitored by trans-epithelial resistance (TER) across polarized monolayers measured by a Volt ohm meter (World Precision Instruments, Sarasota, FL, USA). Monolayers showing TER values greater than 1 kΩ/cm were considered confluent and used for further experiments. Endometrial epithelial cell cultures were >95% pure. No traces of CD45⁺ hematopoietic cells were found in these cultures by flow cytometric analysis. The <5% remaining cells that could have been present in epithelial cell cultures were typically stromal cells of fibroblastic origin (MacDonald et al., 2007).

*Measuring GEC HSV-2 viral shedding and cell associated virus.*
Confluent monolayers of primary, female GECs with TERs above 1 kΩ/cm were infected apically with a standard inoculation dose of $10^4$ plaque-forming units (PFUs) (MOI 0.1) of WT HSV-2, vhsB or UV-inactivated HSV-2 in a volume of 100µL of serum free primary media. The cells were exposed to the virus for 2 hours at 37°C in 5% CO₂. After this time, the inoculum was removed and the cells were washed five times with 1x PBS and subsequently replenished with 300µL of fresh primary media on the apical side and 500µL on the basolateral side. To measure viral shedding from GECs, 24-hours post-infection, apical and basolateral supernatants were collected and titred for infectious virus using a Vero-plaque assay, described previously (A. E. Gillgrass, Fernandez, Rosenthal, & Kaushic, 2005). Alternatively, at 24-hours post-infection, HSV-2 exposed GECs were detached from the transwell inserts using 0.05% trypsin-EDTA (Gibco), pelleted (Eppendorf Centrifuge 5415 R, 4°C for 2 min, 12,000 x g) and subjected to 3 freeze-thaw cycles. Cell lysates were resuspended in 200µL of serum free, primary media and titred using a Vero plaque assay to measure cell-associated HSV-2.

**NFκB Staining**

GECs exposed to WT, UV-inactivated or vhsB HSV-2 at $10^4$ PFU, or Poly (I:C) at a concentration of 25µg/mL, were fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, USA) at specific time points post-exposure and permeabilized with blocking solution (2% goat serum) (Sigma) containing 0.1% Triton X-100 (Covidien Pharmaceuticals, Hazelwood, MO, USA). The primary antibody was a rabbit anti-p65/RELA NFκB (Santa Cruz Biotechnology
Inc., Santa Cruz, CA, USA) followed by AlexaFluor goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate detection antibody (Molecular Probes, Eugene, OR, USA). Propidium iodide (Molecular Probes) was used for nuclear counter-stain. Imaging was done on an inverted confocal laser-scanning microscope (LSM 510, Carl Zeiss Canada Ltd., Toronto, ON, Canada). Standard operating conditions were 63× objective, optical laser thickness 1 µm, image dimension of 512 × 512, lasers: argon (450 nm) for NFκB and helium-neon (543 nm) for propidium iodide. For each experiment, confocal microscope settings for image acquisition and processing were identical between control and treated monolayers and 3 separate, random images were acquired for each experimental condition.

**Cytokine/Chemokine Measurement**

Apical and basolateral supernatants were collected at pre-determined time-points following GEC exposure and analyzed for multiple cytokines and chemokines using the Luminex multi-analyte technology (Millipore, Billerica, MA, USA) as previously described [25]. Multiplex bead-based sandwich immunoassay kits were used to measure levels of interleukin 1 alpha (IL-1α), IL-1β, IL-6, IL-8, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 3 (CCL3), CCL4, CCL5, granulocyte-macrophage colony stimulating factor 2 (CSF2) and tumour necrosis factor alpha (TNF-α) as per the manufacturer’s instructions. ELISAs were also performed to measure human IFN-β (Antigenix America, Huntington Station, NY, USA), IFN-α, IFN-λ1 and IFN-λ2 (Biolegend, San Diego, CA, USA) in cell culture supernatants, as per the manufacturer’s instructions.
**VSV-GFP Bioassay**

To assess the presence of biologically active IFN in GEC supernatants, a vesicular stomatitis virus (VSV) plaque reduction assay was used. This method was based on measuring the ability of VSV-GFP, a lytic but IFN-sensitive virus that expresses green fluorescent protein (GFP), to replicate within Vero cell cultures (Nazli et al., 2009; Paladino, Cummings, Noyce, & Mossman, 2006). Briefly, supernatants were collected from primary GECs exposed to WT, vhsB or UV-inactivated HSV-2 for 24 hours. These supernatants were exposed to UV light to inactivate any live virus in the supernatant that could interfere with the assay. Supernatants were added to Vero cell cultures and incubated at 37°C for 24 hours, after which the supernatants were removed and $10^4$ PFU of VSV-GFP was added to the cells for one hour. The virus was removed and Vero cells were overlaid with 2% methylcellulose/ 2xF11/MEM medium (1:1 ratio) and incubated for 48 hours. Levels of GFP fluorescence (indicative of VSV replication) were quantified using a Typhoon scanner (Amersham Bioscience, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Fluorescence was inversely proportional to biological interferon activity and relative to Vero cells incubated with cell culture media and exposed to VSV-GFP (positive control).

**HSV DNA Extraction and Genome Copies to PFU Ratio.**

The extraction of purified HSV nucleic acid from viral stocks was performed using a QIAamp MiniElute Virus Spin Kit (QIAGEN, Mississauga, ON, Canada),
according to instructions. Quantitative real time PCR was performed by adding 1µg of the extracted DNA, or serially diluted quantified HSV-2 DNA (Advanced Biotechnologies, Inc., Columbia, MD, USA) to 12.5 µL of RT² Real Time™ SYBR Green/ROX PCR master mix (SABioscineceses), 1µM DNA polymerase forward (5’-TCCCGGTACGAAGACCAG-3’) and reverse (5’-AGCAGGCGCTGTCCCT-3’) primers (Mobix, McMaster University, Hamilton, ON, Canada) and RNase/DNase free water (Invitrogen) for a total reaction volume of 25µL. Real-time PCR was performed using an ABI Prism 7900 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). PCR templates were initially denatured for 10 minutes at 95°C, followed by 40 cycles of amplification (15 seconds at 95°C, then 60 seconds at 60°C), with the addition of a dissociation step. Taking the number of DNA polymerase copies measured in the viral preps and dividing by the number of previously measured PFUs for that viral prep gave the genome copies to PFU ratio.

**Real Time RT-PCR Measurement of ISGs**

Primary GECs were exposed to WT, vhsB or UV-inactivated HSV-2 as described above. Culture media was used as a negative control for infection. At 2- and 24-hours post-exposure, total RNA was extracted from primary GECs following treatment with TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The following oligonucleotide primer sequences (Mobix) for interferon stimulated genes (ISGs) were used: MX1 F: 5’-GCTACACACCGTGACGGATATGG-3’, MX1 R: 5’-CGAGCTGGATTGAAAGCCC-3’
(Antonelli et al., 1999); *OAS1* F: 5'-TCAGAAGAGAAGCCAACGTGA-3', *OAS1* R: 5'-CGGAGACAGCGA GGTAAAT-3' (Farrugia & Cann, 1999); *ISG15* F: 5'-CAGAAGCAGACTCCTTAATTC-3', *ISG15* R: 5'-AGACCTCATATATATGTTGCTGTG-3' (Nacionales et al., 2006); *CXCL10* F: 5'-ATCATCCCTGCGAGCCTAT-3', *CXCL10* R: 5'-ATTCTTGCTTCGGCAGTTAC-3' (Nacionales et al., 2006). Real time PCR was performed using 5µL of 1:20 diluted cDNA, RT2 Real Time™ SYBR Green/ROX PCR master mix (SABiosceinesces), 1 µM of forward and reverse primers (Mobix) as well as RNase/DNase free water for a total reaction volume of 25µL. Amplification was performed using an ABI Prism 7900 sequence detection system with the previously described conditions. A comparative ΔΔC_{T} value was calculated using the C_{T} values of the genes of interest relative to uninfected controls and normalized to the C_{T} values of *RPL13A*, an endogenous housekeeping gene (*RPL13A* F: 5'-CATAGGAAGCTGGGAGCAAG-3', *RPL13A* R: 5'-GCCCTCCAATCAGTCTTCTG-3').

**IFN-β Neutralization**

IFN-β polyclonal neutralizing antibody (Millipore) (25µg/mL), or isotype control (Southern Biotech, Birmingham, AL, USA) was added to primary GECs during the initial 2-hour exposure to HSV-2. Following this exposure, the inoculum and antibodies were removed and the cells were washed thoroughly with 1x PBS. Next, serum free primary media containing the IFN-β neutralizing antibody or isotype control at 25µg/mL was added to the GECs. At 24-hours post-infection, viral shedding and cell-associated virus were measured as previously described to
determine whether blocking IFN-β had any effect on GEC HSV-2 viral shedding and cell-associated virus.

**Statistical Analysis**

GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used to compare three or more means by one-way analysis of variance (ANOVA). When an overall statistically significant difference was measured (p<0.05), a Bonferroni post-test was performed to adjusted the p-value for multiple comparisons.
RESULTS

Deletion of VHS impairs viral replication in primary, female GECs.

Prior to determining GEC responses to WT and VHS deleted virus, we first examined whether there were any differences in replication of the two viruses in GECs. Confluent monolayers of primary, human GECs were exposed to WT or vhsB HSV-2 and at 24-hours post-exposure, apical and basolateral cell culture supernatants were collected to measure shed virus, or cells were disrupted in order to assess cell-associated virus.

GECs exposed to vhsB shed significantly less virus into the apical supernatant (Figure 1A) and contained less cell-associated virus (Figure 1B) compared to cells exposed to WT virus. The shed and cell-associated virus from cells exposed to vhsB were reduced by 2.6-fold, and 2.2-fold, respectively. Shed virus was not detected on the basolateral side at 24-hours post-infection, regardless of the virus used (results not shown), which is in agreement with previous studies from our group (Ferreira et al., 2011; MacDonald et al., 2007). As expected, no shed virus or cell-associated virus was detected from primary GECs exposed to UV-HSV-2. Together these results suggest that deletion of VHS results in a small, but statistically significant decrease in HSV-2 replication in primary GECs, relative to WT virus.

Since our main goal was to compare the GEC antiviral responses against WT and VHS-deleted HSV-2, we next compared the particle composition of our viral stocks, since mutant virus preparations often contain a higher number of non-infectious DNA filled viral particles that may be replication-deficient, but can
contribute to the activation of antiviral pathways (Dohner, Radtke, Schmidt, & Sodeik, 2006). To determine differences in particle composition, we measured the ratio of HSV-2 gene copies (using DNA polymerase as a reference gene) to particle forming units (PFUs) in viral stocks for WT and vhsB. No statistical differences were measured in gene copy to PFU ratio between the viruses used in this study (Figure 1C), suggesting that any observed differences measured in innate antiviral pathways are due to the intrinsic nature of the viruses and not to differences in viral particle counts in our viral preparations.

*Exposure of primary GECs to replication competent or VHS-deleted HSV-2 induces robust NFκB nuclear translocation.*

Next we measured the ability of HSV-2 exposure to induce nuclear translocation of the inflammatory transcription factor NFκB. Primary GECs were exposed to virus for 2, 4 or 6 hours and subsequently fixed and examined for NFκB nuclear translocation by immunofluorescent microscopy. Exposure of cells to WT or vhsB HSV-2 resulted in peak translocation of NFκB at 2 hours post-exposure (Figure 2), with decreased translocation observed by 4 hours post-exposure and a return to baseline expression by 6 hours (*results not shown*). GECs treated with Poly (I:C), a TLR3 synthetic ligand known to activate the NFκB pathway in primary GECs (Nazli et al., 2009), demonstrated similar induction characteristics as WT and vhsB with respect to both time course and strength of activation. In contrast, NFκB translocation was not visibly detected in GECs exposed to UV-HSV-2 or to media alone control, at any of the time points examined. These results suggest that acute
NFκB activation in GECs is produced in response to replication competent virus and that in absence of the VHS protein, NFκB activation is maintained.

*Induction of proinflammatory cytokines and chemokines is dependent on viral replication and IL-8 and CCL2 are suppressed in the presence of the VHS protein.*

Given that WT and vhsB HSV-2 upregulated robust NFκB translocation in primary, human GECs, we next determined whether various proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6) and chemokines (IL-8, CCL2, CCL3, CCL4, CCL5, CSF2) downstream of NFκB were upregulated as well. GEC apical and basolateral supernatants were collected at 6- or 24-hours post-infection and the aforementioned immune factors were measured using the multi-analyte Luminex bead-based system.

Significant upregulation of TNF-α (Figure 3A), IL-8 (Figure 3B), CCL2 (Figure 3C) and IL-6 (Figure 3D) required viral replication, as levels of these factors were significantly decreased in supernatants from cells exposed to UV-inactivated virus in comparison to supernatants from cells exposed to replication competent WT virus. Only apical IL-8 levels were significantly upregulated in GECs exposed to UV-inactivated virus, relative to mock-infected controls (Figure 3B). Interestingly, the greatest production of these factors occurred in cells exposed to vhsB, where in fact levels of CCL2 and IL-8 were significantly greater compared to levels in the supernatants of cells exposed to WT virus. With respect to basolateral supernatants, only cells exposed to vhsB produced significantly higher levels of proinflammatory cytokines compared to mock controls, and of the cytokines measured only TNF-α
levels at 6-hours and IL-6 levels at 24-hours post-exposure were significantly elevated (results not shown). Together, these results suggest that HSV-2 replication is required to upregulate GEC cytokine and chemokine responses, and that in the presence of the VHS protein, these responses are suppressed, particularly IL-8 and CCL2 production.

_The VHS protein does not suppress biologically active IFN-β responses from primary GECs._

Among innate factors produced or recognized by GECs, type I and III IFNs play an important role in inducing an antiviral state to limit the spread of infection (Ank & Paludan, 2009; Decker, Muller, & Stockinger, 2005). Since we found differential induction of proinflammatory cytokines depending on viral replication and the presence of VHS, and since previous studies in other cell types showed that the VHS protein was capable of repressing IFN responses (Duerst & Morrison, 2004; J. A. Murphy et al., 2003; Suzutani et al., 2000), we next examined whether primary GECs exposed to WT, vhsB or UV-HSV-2 produced differential amounts of type I IFN (IFN-α, IFN-β) or type III IFN (IFN-λ1, IFN-λ2).

The results show that among all the IFNs examined, only IFN-β was upregulated in significant amounts following exposure of GECs to HSV-2 (Figure 4). At early time points (6-hours post-infection), GECs exposed to vhsB, but not WT or UV-inactivated HSV-2 secreted significantly elevated levels of IFN-β apically compared to uninfected controls (Figure 4A). At 24-hours post-exposure, WT and vhsB virus, but not UV-inactivated HSV-2, induced significant IFN-β production.
apically from primary GECs, compared to mock infection. Closer examination showed that apical IFN-β levels were significantly higher following vhsB exposure relative to UV-HSV-2 exposure at all time points measured. Unlike the significantly higher induction of proinflammatory cytokines seen in the absence of VHS, IFN-β induction was not significantly different between WT and vhsB exposure (Figure 4A, B). Furthermore, although primary GECs secreted IFN-β basolaterally, there was no difference in IFN-β production following viral exposure into basolateral supernatants at either of the time points measured (Figure 4B). There were no changes in IFN-α or Type III IFN secretion in response to HSV-2 (Figure 4D, E).

Since no differences were found in IFN-β levels between cells exposed to WT or vhsB, and previous studies had demonstrated a role for VHS in suppressing IFN pathways in other cells, we examined whether the VHS protein alternatively affected the biological activity of the IFN-β produced in GECs. In order to assess the bioactivity of the IFN produced, an established and previously described vesicular stomatitis virus (VSV)-green fluorescent protein (GFP) bioassay was used (Nazli et al., 2009). VSV replication was reduced significantly at 6 and 24-hours post-infection in cells exposed to apical supernatants from GECs exposed to WT or vhsB HSV-2 (Figure 5A), whereas supernatants from GECs exposed to UV- HSV-2 did not show any decrease in VSV replication, relative to uninfected positive controls, indicating that IFN-β produced by GECs could suppress VSV replication. Although supernatants collected from GECs exposed to VHS-deleted virus showed greater VSV replication reduction compared to supernatants collected from GECs exposed to WT, these differences were not statistically significant.
Our results showed that deletion of VHS did not significantly affect induction of biologically active IFN-β beyond that seen with WT HSV-2. Therefore, we further evaluated whether VHS-deletion had an effect on the IFN pathway by measuring IFN-stimulated genes (ISGs). IFNs generate their effects on target cells by binding to specific cell surface receptors and inducing the activation of ISGs, which ultimately give rise to proteins that mediate the biological effects of IFNs (Platanias, 2005). We measured four hallmark ISGs: chemokine (C-X-C motif) ligand 10 (CXCL10), myxovirus resistance gene 1 (MX1), 2′,5′-oligoadenylate synthetase 1 (OAS1) and ISG15 ubiquitin-like modifier (ISG15), at 24-hours post-exposure by RT-PCR. CXCL10, OAS1 and ISG15 were significantly upregulated (>3-fold increase) following exposure to all viral conditions; however the greatest increase in ISG induction followed exposure to WT or vhsB HSV-2 (Figure 5B) and there was also significant upregulation of ISG15 by vhsB, and OAS1 by WT and vhsB, relative to UV-HSV-2. However, no significant differences were measured between cells exposed to WT or vhsB HSV-2. This confirmed that in GECs, HSV-2 VHS protein does not potently regulate the IFN pathway.

**IFN-β produced by primary GECs in response to HSV-2 reduces the viral burden.**

IFN-β plays an important role in inducing an antiviral state, which limits the spread of infection. Therefore, we decided to directly assess if IFN-β produced by primary GECs could curb viral replication. In order to measure the functional anti-HSV capability of the IFN-β secreted by primary, human GECs in response to WT or vhsB HSV-2, we added an IFN-β polyclonal neutralizing antibody to primary GEC
cultures during the course of viral exposure. Blocking IFN-β significantly increased both shed virus (approximately 4-fold) and cell-associated virus (approximately 3-fold) following vhsB or WT infection (Figure 6), suggesting that GEC IFN-β responses may play an important role in limiting the initial replication of virus and subsequent spread of infection.
DISCUSSION

To the best of our knowledge this is the first study examining the influence of viral factors such as viral replication and the presence of the VHS protein on innate responses in primary, human GECs. Our results suggest that HSV-2 viral replication influences the induction of proinflammatory cytokines and chemokines from primary endometrial GECs, specifically TNF-α, IL-6, IL-8 and CCL2. In the presence of the VHS protein, these responses are downregulated, in particular, the chemokines IL-8 and CCL2. HSV-2 exposure also induced IFN-β responses and this was negatively correlated with shed and cell-associated virus, suggesting these GEC responses may play a role in controlling viral replication at the site of infection. However, unlike proinflammatory cytokine responses, IFN-β production did not correlate with the presence or absence of VHS, although it was increased in response to replication competent virus (vhsB and WT).

GECs are capable of producing a plethora of cytokines and chemokines, which act as a first wave of antiviral responses against sexually transmitted pathogens such as HSV-2. Proinflammatory cytokines and chemokines play direct and indirect roles in curbing the spread of HSV-2 infection. Others have shown that TNF-α inhibits HSV-2 infection in cervical carcinoma cells (HeLa), but only in the presence of IFN-γ (Adams et al., 2004). In addition, a study performed by Leblanc and colleagues (LeBlanc, Pesnicak, Cabral, Godleski, & Straus, 1999), compared WT mice to IL-6 knockout mice and found that animals lacking IL-6 were less able to survive an ocular challenge with HSV-1. Furthermore, IL-8 was found to dramatically enhance antigen-specific Th1 cellular immunity in the context of lethal
HSV-2 challenge (Sin, Kim, Pachuk, Satishchandran, & Weiner, 2000) and CCL2 was found to directly neutralize HSV-1 (Lehrer, Daher, Ganz, & Selsted, 1985). Here we show that GECs respond to replication competent HSV-2 by upregulating TNF-α, IL-6, IL-8 and CCL2, and that in the presence of the VHS protein, some of these factors (CCL2 and IL-8) are suppressed. All together, these results suggest that the proinflammatory cytokines and chemokines upregulated by GECs in response to HSV-2 likely play an important role in neutralizing the virus, but that the potency of these responses may likely depend on the presence of the VHS protein.

IFN-β antiviral GEC responses likely play an active and essential role in decreasing the initial viral burden associated with HSV-2 infection. In our study we found that blocking GEC IFN-β responses resulted in increased shed virus and cell-associated virus, indicating that primary GEC innate antiviral responses may be important in lowering the viral burden as opposed to completely clearing the infection. The importance of a small but significant decrease in the initial viral replication has been demonstrated clearly within in vivo mouse models where half a log differences in the intravaginal viral inoculation dose can determine whether an infection will have a lethal or non-lethal outcome (A. E. Gillgrass, Fernandez, et al., 2005; Kaushic, Ashkar, Reid, & Rosenthal, 2003). Furthermore, higher HSV-2 viral loads are associated with increased pathology and inflammation (A. E. Gillgrass, Fernandez, et al., 2005). Interestingly, clinical and stochastic mathematical HSV-2 studies suggest that the rate of containment of infected cells by the mucosal immune system is the major driver of duration and severity of HSV-2 reactivation in the immunocompetent host (Schiffer et al., 2010). Thus the biologically active IFN-β
produced by GECs as seen in this study could help decrease the initial viral burden, thus improving the outcome of the infection.

When comparing our results to studies using cultures of submerged epithelial cells, we see some interesting similarities. Submerged corneal epithelial cells and transformed cervical epithelial cells both upregulated NFκB that was concomitant with the production of IL-6 and IFN-β in response to HSV-1 and HSV-2, respectively (H. Li et al., 2009; H. Li et al., 2006). Furthermore, in a mouse model of corneal epithelial cell HSV-1 infection, the induction of the chemokines CXCL1, CCL4, CXCL2 and CCL2 as well as the cytokines IL-1, IL-6, IL-12 and TNF-α, depended on viral replication (Thomas, Kanangat, & Rouse, 1998). In our study we found that primary columnar epithelial cells also induced potent NFκB activation in responses to replication competent HSV-2 or VHS-deleted HSV-2 which peaked at 2-hours post-exposure, which was associated with downstream upregulation of IL-6, IL-8, CCL2, TNF-α and IFN-β, peaking at 24-hours post-exposure. In our study, we also found that the presence of the VHS protein resulted in significant downregulation of proinflammatory cytokines and chemokines. Very few studies have explored the role of the VHS protein in regulating epithelial cell responses, but recently one study found that HSV-2 VHS was associated with shutting down IFN-β production and IFN pathways in submerged cultures of the human vaginal epithelial cell line Vk2 following HSV-2 infection (Yao & Rosenthal, 2011). Interestingly though, we did not find that IFN-β pathways were affected by the presence of the VHS protein in primary GECs.
When we compare our results to studies looking at innate antiviral responses against HSV in immune-competent cells, we see pronounced differences. IFN-β production or IFN sensing pathways are disrupted in human DCs and murine embryonic fibroblasts by the VHS protein (Duerst & Morrison, 2004), but are not in primary endometrial GECs. In contrast to our findings, IFN-β induction is fully independent of HSV replication in DC models (Cotter et al., 2011; S. B. Rasmussen et al., 2007). Generally, leukocyte infection with HSV triggers a biphasic production of chemokines and cytokines: an early response not requiring replication-competent virus, but dependent on the presence of viral surface and tegument proteins and a later cytokine response dependent on virus replication (Malmgaard, Melchjorsen, Bowie, Mogensen, & Paludan, 2004; Melchjorsen, Pedersen, Mogensen, & Paludan, 2002; Melchjorsen, Siren, Julkunen, Paludan, & Matikainen, 2006; Paludan, 2001; Paludan & Mogensen, 2001). In our studies, we find that in the absence of viral replication, primary endometrial GECs are poorly able to induce proinflammatory cytokine responses, regardless of the time point, further emphasizing the difference in antiviral immune responses between immune competent cells and GECs.

The correlation between ability of HSV-2 to replicate with the induction of robust innate responses in our study may provide some insight into prophylactic strategies against HSV-2. In the last two decades, HSV-2 vaccine trials (K. Roth, Ferreira, & Kaushic, 2012) have typically utilized formulations consisting primarily of recombinant HSV glycoproteins, such as gB and gD. Our results suggest that potent induction of proinflammatory cytokines and chemokines – which contribute to the recruitment and activation of antigen presenting cells and the generation of
effective adaptive memory responses – requires replication and the absence of viral regulatory proteins such as VHS, which suppress these responses. Therefore our results suggest that future formulations may want to consider a replication competent vaccine virus that is still attenuated enough to avoid causing morbidity. Interestingly, mouse models using attenuated strains of HSV-2, such as thymidine kinase deleted (Bhavanam, Snider, & Kaushic, 2008; K. L. Roth et al., 2013), VHS-deleted (Keadle et al., 2002; T. J. Smith, Morrison, & Leib, 2002) or highly attenuated replication-competent strains of HSV-1 (Luo et al., 2012), have been shown to provide complete protection against genital HSV-2 challenge. All together, these studies underscore the need for more comprehensive vaccine strategies that explore alternate options (K. Roth et al., 2012).

In conclusion, this study shows that viral factors such as viral replication and the presence of the VHS protein both play an important role on regulating innate antiviral responses against HSV-2 from primary GECs. Further the regulation of these innate pathways is distinct in GECs compared to other cell types, emphasizing the importance of examining cell-specific innate responses. These studies will provide valuable information regarding early HSV pathogenesis in the FRT and insights into developing better therapeutics against HSV-2, such as vaccines and microbicides.
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FIGURE LEGENDS

FIGURE 1 – Primary GEC shed and cell associated virus, and assessment of gene copy to PFU ratio. To measure shed virus in GEC supernatants (A), *ex vivo* monolayers of primary, female GECs were exposed to $10^4$ PFU (approximate MOI of 0.1) of WT, vhsB, or UV-HSV-2 for 2 hours. Next, the viral inoculum was removed and the cells were washed five times with PBS and subsequently replenished with fresh media. 24-hours post-exposure, apical and basolateral supernatants were collected and measured for shed virus using a vero plaque assay. To measure cell-associated virus (B), at 24-hours post-exposure, GECs were detached from transwell inserts using 1x trypsin, pelleted down and subjected to disruption by liquid nitrogen freeze-thaw. Cell lysates were resuspended in 0% α-MEM and titred using a vero plaque assay. Results are presented in PFU/mL. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001. Gene copies to PFU ratios were measured for WT and vhsB (C). HSV-2 DNA was extracted from previously titred viral preparations and the number of copies of DNA polymerase was measured against a reference standard of quantified HSV-2 DNA using quantitative real time PCR. To obtain the genome copies to PFU ratio, the calculated number of DNA polymerase copies was divided by the previously measured PFUs for that viral prep. Data shown represents mean ± SEM of 4 separate experiments.

FIGURE 2 – NFκB translocation following exposure of primary, female GECs to HSV-2. Primary, female GECs were exposed to $10^4$ PFU of WT, vhsB or UV-HSV-2,
cell culture media (negative control), or Poly (I:C) at 25µg/mL (positive control) for several time points post-infection and stained for the NFκB (green stain). Nuclear counter-staining, as seen in red, was achieved using propidium iodide. Overlap of NFκB and propidium iodide staining can be observed in the merge panel. All samples were imaged on an inverted confocal laser-scanning LSM 510 microscope. Images are presented en face and are representative of one of three separate experiments.

**FIGURE 3** - Cytokine and chemokine profiles of apical and basolateral supernatants from primary, female GECs exposed to HSV-2. Confluent primary, female GECs were exposed to WT, vhsB or UV-HSV-2, and at 6 and 24-hours post-exposure, apical supernatants were collected and measured for IL-1α, IL-1β, IL-6, IL-8, CCL2, CCL3, CCL4, CCL5, CSF2 and TNF-α using Multiplex bead-based sandwich immunoassay. Cells exposed to media alone (mock) were used as a negative control. Results are shown for TNF-α (A), IL-8 (B), CCL2 (C) and IL-6 (D). Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01 and ***p<0.001.

**FIGURE 4** – Type I and III IFN production from primary, female GECs exposed to HSV-2. Confluent primary, female GECs were exposed to WT, vhsB or UV-HSV-2, and at 6 and 24-hours post-infection, apical (A) and basolateral (B) supernatants were collected and measured for IFN-β by ELISA. Alternatively, apical and basolateral supernatants were collected and measured at 24-hours post-exposure, for IFN-α (C),
IFN-λ1 (D) and IFN-λ2 (E) by ELISA. In all cases, cells were exposed to media alone or Poly (I:C) for negative and positive controls, respectively. Data shown represents mean ± SEM of 3 separate experiments. N.S. indicates a comparison that is not statistically significant. ** p<0.01 and *** p<0.001.

**Figure 5 - IFN-mediated reduction in VSV replication and induction of interferon stimulated genes.** Biologically active interferon was measured in apical supernatants collected from primary, female GECs 6 and 24 hours post-exposure to WT, vhsB or UV-HSV-2 by VSV-GFP assay (A). Supernatants collected from GECs exposed to media alone (mock), or Poly (I:C) were used as negative and positive controls, respectively. Fluorescence values of GEC supernatant-treated and VSV-GFP infected vero cells were compared with positive controls of VSV-GFP infected vero cells exposed to media alone. Confluent primary, female GECs were exposed to WT, vhsB or UV-HSV-2 and total RNA was extracted from the cells at 24-hours post-exposure to measure ISG induction of MX1, CXCL10, ISG15 and OAS1 (B). Real time RT-PCR was performed and a comparative CT value (2^ΔΔCT) was calculated by comparing the CT values of the samples of interest with a control (uninfected media control) and these values were normalized to the RPL13A endogenous housekeeping gene. Data shown represents mean ± SEM of 4 separate experiments. N.S. indicates a comparison that is not statistically significant. * p<0.05, ** p<0.01 and *** p<0.001.

**Figure 6 - Neutralization of primary GEC IFN-β responses increases viral shedding and cell-associated virus.** Primary, female GECs were treated with an
IFN-β polyclonal neutralizing antibody and exposed to $10^4$ PFUs of WT or vhsB HSV-2. After the inoculum was removed and the cells were washed, fresh media containing the same neutralizing antibody was added to the cells. At 24-hours post-exposure, apical supernatants were collected to measure shed virus (A) or the cells were disrupted to measure cell-associated virus (B), both using vero plaque assay as previously described. An isotype control antibody was also used to confirm the specificity of the polyclonal anti-IFN-β antibody. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01 and ***p<0.001.
Figure 1

A. 

B. 

C.
Figure 2

![Image of cellular staining experiment results](image-url)
Figure 3

A.

B.

C.

D.
Figure 4
Figure 5

A.

B.
Figure 6
Chapter 3

Endometrial epithelial cell responses to coinfecting viral and bacterial pathogens in the genital tract can activate the HIV-1 LTR in an NFκB-and AP-1-dependent manner.


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In this study we attempted to describe the mechanism by which STIs increase HIV replication in the FGT. We found that HSV-1, HSV-2, N. gonorrhoeae and TLR ligands FimH (TLR-4), flagellin (TLR-5), and poly (I:C) (TLR-3), representative of various bacterial and viral STIs, directly induced HIV-LTR activation in 1G5 (Jurkat) T cells. Furthermore, supernatants collected from GECs exposed to these agents indirectly induced HIV-LTR promoter activation in T-cells. Production of TNF-α, IL-6, IL-8 and MCP-1 was elevated in GECs exposed to co-infecting microbes. Interestingly, when NFκB and AP-1 signaling pathways were impaired in 1G5 T cells, both direct and indirect HIV-LTR activation was abrogated.

Drs. Charu Kaushic, Aisha Nazli and I were responsible for the design and interpretation of the experiments. Ghaznia Khan and I were responsible for the generation and analysis of the data. Dr. Aisha Nazli provided technical assistance. Drs. M. Firoz Mian, Ali A. Ashkar, Scott Gray-Owen and Rupert Kaul provided technical expertise and reagents. Dr. Charu Kaushic and I wrote and edited the manuscript.
Endometrial epithelial cell responses to co-infecting viral and bacterial pathogens in the genital tract can activate the HIV-1 LTR in an NFκB and AP-1 dependent manner.

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Running Title: HIV-LTR activation by co-infections
FOOTNOTE

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ABSTRACT

Background: Sexually transmitted infections (STIs) are associated with increased HIV-1 susceptibility and viral shedding in the genital tract, but the mechanisms underlying this association are poorly understood.

Methods: Direct activation of HIV-LTR, a proxy measure for HIV-1 replication, was measured following treatment of 1G5 T cells with TLR ligands, HSV-1/2 or N. gonorrhoeae. For indirect activation, 1G5 T cells were incubated with supernatants from female primary genital epithelial cells (GECs) previously exposed to these agents. Proinflammatory cytokines and chemokines were measured in GEC supernatants. Proinflammatory pathways were blocked to determine the mechanisms of direct and indirect HIV-LTR activation.

Results: HSV-1/2, N. gonorrhoeae and TLR ligands FimH (TLR 4), flagellin (TLR5), and Poly (I:C) (TLR3) directly induced HIV-LTR activation in 1G5 T cells. Supernatants collected from GECs incubated with these agents indirectly induced HIV-LTR activation. Production of TNF-α, IL-6, IL-8 and MCP-1 was elevated in GECs exposed to co-pathogens. Inhibition of NFκB and AP-1 signalling pathways in 1G5 T cells abrogated both direct and indirect HIV-LTR activation.
Conclusions: STIs may increase HIV-1 replication in the female genital tract via pro-inflammatory signalling pathways directly and indirectly via their effects on GECs. This increased HIV-1 replication may enhance sexual and vertical HIV transmission.

Key words: Sexually transmitted infections, Co-infection, Female Genital Tract, Epithelial Cells, HIV-1, HSV, Neisseria gonorrhoeae, TLR
INTRODUCTION:

Heterologous infection with viruses, bacteria and other organisms such as protozoan parasites may significantly influence the clinical course of HIV infection, as well as the acquisition, replication and transmission of the virus (Blanchard, Montagnier, & Gougeon, 1997; M. S. Cohen, 1998; Kaul et al., 2008). UNAIDS statistics indicate that women constitute over 50% of HIV infected individuals globally (UNAIDS, 2008). Women also carry a disproportionately higher burden of sexually transmitted infections (STIs) compared to men (Fish, 2008). STIs such as Neisseria gonorrhoeae and herpes simplex virus (HSV) may play an important role in increasing HIV replication in the female genital tract. N. gonorrhoeae is among the most common bacterial STIs in people living with HIV/AIDS (Page, Moore, Wilgus, Gindi, & Erbelding, 2008). Symptomatic gonococcal infection is associated with increased detection rates of HIV DNA in the urogenital tract (CDC, 2006; Ding et al., 2010; Ghys et al., 1997; J. Zhang et al., 2005). With prevalence rates ranging from 10 to 60%, genital herpes is a viral infection that also poses a substantial burden on people living with HIV/AIDS (R. Gupta, Warren, & Wald, 2007). Genital herpes is primarily caused by HSV-2, although HSV-1 infection is increasingly being confirmed in cases of primary genital herpes (Coyle et al., 2003; R. Gupta et al., 2007; C. M. Roberts et al., 2003). Clinical studies show that HSV-2 reactivation is frequently accompanied by an increase in HIV plasma viral load and conversely, treatment of HSV-2 is seen to slow down progression of HIV (Lingappa et al., 2010; McClelland et al., 2002; Schacker et al., 1998).
Heterosexual transmission across genital tissue is the primary route by which women acquire HIV (Royce, Sena, Cates, & Cohen, 1997). Sexually transmitted co-pathogens may be present in the female genital tract prior to, during, or following HIV exposure. However, little is known about the mechanism by which these co-infections may enhance HIV replication in the female genital tract. In HIV infected women, T cells in the female genital tract may come into direct contact with the co-infecting pathogens resulting in activation of the 5'HIV-long terminal repeats (LTR) region resulting in enhanced HIV replication. Alternatively, proinflammatory cytokines present in the genital tract may indirectly enhance HIV replication in infected T cells. Genital epithelial cells (GEC) are the first cells in the female genital tract to encounter sexually transmitted pathogens. In response to interactions with microbes, GECs have been shown to produce proinflammatory cytokines, including TNF-α and IL-6, which can increase HIV replication (Poli, Bressler, et al., 1990; Poli, Kinter, et al., 1990). Therefore, HIV replication may be upregulated in infected cells exposed to proinflammatory cytokines produced by GECs, both during initial exposure or after established infection. This possibility is supported by our recent study showing that exposure of GECs to HIV upregulated production of TNF-α, which caused impairment of the mucosal epithelial barrier in the genital and intestinal tract, facilitating viral and bacterial translocation (Nazli et al., 2010).

This current study was designed to examine if sexually transmitted co-infections were capable of enhancing HIV replication in the female genital microenvironment. We found that viral and bacterial Toll-like receptor (TLR) ligands as well HSV-1, HSV-2 and N. gonorrhoeae were capable of directly driving
the 5’-LTR promoter in transfected T-cells in a mechanism dependent on both NFκB and AP-1. Furthermore, in response to bacterial TLR ligands, as well as infection by HSV-1, HSV-2 or \textit{N. gonorrhoeae}, GECs secreted immune factors that could indirectly activate the HIV-LTR, in a mechanism also dependent on NFκB and AP-1.
MATERIALS AND METHODS

Pathogens and Cell Lines

Vero cells (ATCC, Manassas, VA, USA), were maintained in minimum essential medium-α (α-MEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada), 100 U/mL penicillin/streptomycin (pen/strep) (Sigma-Aldrich, Oakville, ON, Canada), 2 μM L-glutamine (L-glu) (Invitrogen) and 10 μM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). HSV-1 KOS and HSV-2 333 were propagated using vero cells at an MOI of 0.01. Supernatants were collected and infected cells were disrupted by sonication in order to release the virus remaining inside the vero cells. Preparations were spun down and virus was resuspended in supplemented FBS-free α-MEM and titred using a standard vero plaque assay. Neisseria gonorrhoeae clinical strain 2071 was grown at 37°C in a 5% CO₂ humidified incubator from frozen stocks on GC agar base supplemented with 1% (vol/vol) IsoVitaleX enrichment (Becton, Dickinson-DIFCO, Franklin Lakes, NJ, USA). Gonococcal colonies were resuspended in 1x PBS and assessed for colony forming units (CFUs) based on optical density measurements. The bacteria were then diluted to desired concentrations using antibiotic and phenol red free Dulbecco’s modified Eagle’s medium (DMEM)/F12 (GIBCO, Burlington, ON, Canada). 1G5 Jurkat T-cells were maintained in Roswell Park Memorial Institute (RPMI) media supplemented with 10% FBS, 100 U/mL pen/strep, 2 μM L-glu and 10 μM of HEPES.
**Source of Genital Tissue and Epithelial Cell Preparation**

Female genital tract tissues were obtained from women aged 34-56 years (mean age 44.8 ± 6.7) undergoing hysterectomies for non-malignant gynecological purposes at McMaster University Medical Centre in Hamilton, Ontario. Written informed consent was received in accordance with the approval of the Hamilton Health Sciences-McMaster University Research Ethics Board. The most common reasons for surgery were uterine fibroids or heavy bleeding. Uterine tissues were only used if free of malignancy or any other clinically observed disease. Protocol for isolation, culture and details regarding purity of primary GECs has been described previously (MacDonald et al., 2007; Nazli et al., 2010; Nazli et al., 2009). Briefly, endometrial tissues were minced into small pieces and digested in an enzyme mixture for 1 h at 37°C. GECs were isolated by a series of separations through nylon mesh filters of different pore sizes (Small Parts, Inc. Logansport, IN, USA) in order to retain sheets of endometrial GECs, while removing stromal cells, unwanted cellular debris and mucus. GECs were seeded onto and grown on Matrigel (Becton, Dickinson, Franklin Lakes, NJ, USA) coated, 0.4-μm pore-size polycarbonate membrane tissue culture inserts (BD Falcon, Mississauga, ON, Canada) with phenol red free DMEM/F12 supplemented with 10μM HEPES, 2μM L-glu, 100 U/mL pen/strep, 2.5% Nu Serum culture supplement (VWR, Mississauga, ON, Canada), and 2.5% Hyclone defined fetal bovine serum (Hyclone, Logan, UT, USA). Polarized monolayers were formed within 5–7 days. The confluency of GEC cultures was monitored by trans-epithelial resistance (TER) across polarized monolayers measured by a Volt ohm meter (World Precision Instruments, Sarasota, FL, USA).
Monolayers showing TER values higher than $1000\Omega/cm$ were considered confluent and used for further experiments.

**Toll-like Receptor (TLR) Ligands**

The TLR2 ligands lipoteichoic acid ([LTA](#)) from *Staphylococcus aureus* and peptidoglycan ([PG](#)) from *S. aureus*, the TLR3 ligand polyinosinic:polycytidylic acid ([Poly [I:C]](#)) and TLR4 ligand lipopolysaccharide ([LPS](#)) from *Escherichia coli* 026:B6 were purchased from Sigma-Aldrich (Oakville, ON, Canada). FimH, a novel TLR4 ligand (Ashkar, Mossman, Coombes, Gyles, & Mackenzie, 2008; Mossman et al., 2008), and flagellin, a TLR5 ligand were prepared at McMaster University. The TLR7 ligand loxoribine was purchased from Invivogen (San Diego, CA, USA). CpG-oligonucleotides (TLR9 ligands) were prepared by Mobix Facility at McMaster University and sequences for CpG-A-2216, CpG-B-2006 and CpG-C-2395 have been previously published (Nazli et al., 2009). All ligands were diluted to a final concentration of $10\mu g/mL$, with the exception of Poly (I:C) and loxoribine, which were prepared to $25\mu g/mL$ and $339\mu g/mL$ concentrations, respectively.

**Cytokine/Chemokine Measurement**

Apical and basolateral supernatants were analyzed for multiple cytokines using the Luminex multi-analyte technology (Millipore, Billerica, MA, USA) as previously described (Fernandez, Gillgrass, & Kaushic, 2007). Multiplex bead-based sandwich immunoassay kits were used to measure levels of IL-1$\alpha$, IL-6, IL-8, MCP-1, MIP-1$\alpha$, GM-CSF, RANTES and TNF-$\alpha$ as per the manufacturer’s instructions.
Direct Activation of the HIV-LTR

To measure direct activation of the HIV-LTR in 1G5 cells by TLR ligands or intact pathogens, 1x10^6 1G5 cells were treated with TLR ligands for 24 hours at 37°C, or incubated with 10^4 PFU of HSV-1 KOS, HSV-2 333 or 10^6 CFU of N. gonorrhoeae strain 2071 for 16 hours at 37°C, after which the cells were lysed and luciferase activity was determined using a luciferase assay (Agilent, Mississauga, ON, Canada) as per the kit’s instructions. Mock treated controls consisting of a suspension of disrupted uninfected vero cells (for HSV-1/2) or a washing from GC agar plates (for N. gonorrhoeae) were also included, in addition to media controls.

Indirect Activation of the HIV-LTR

To measure indirect activation of the HIV-LTR in 1G5 cells by TLR ligands, confluent (TER > 1000Ω/cm) primary endometrial GECs were treated with TLR ligands for 2 hours at 37°C. The cells were washed 5 times with 1xPBS and replenished with fresh media and incubated at 37°C. 24-hours post-treatment apical and basolateral supernatants were collected. 100µL of apical and basolateral supernatants were added to 1x10^6 1G5 cells for 24 hours at 37°C after which the cells were lysed and luciferase activity was determined. To measure indirect HIV-LTR activation by HSV-1, HSV-2 or N. gonorrhoeae, confluent primary GECs were infected with 10^4 PFU of HSV-1 KOS, HSV-2 333 or 10^6 CFU of N. gonorrhoeae strain 2071 for 2 hours, washed 5 times and supernatants were collected from the apical and basolateral chambers. Supernatants collected from HSV-infected cells were
exposed to one cycle of 10 kJ of UV-energy using a Stratalinker (Agilent) to ensure there was no residual live virus. Supernatants collected from \textit{N. gonorrhoeae}-infected GECs were filter sterilized using a 0.2\textmu m filter (BD Falcon) to remove any residual bacteria. The supernatants were then incubated with $1 \times 10^6$ 1G5 cells for 24 hours and luciferase activity was measured. Mock infected controls consisting of supernatants collected from primary GECs incubated with a suspension of disrupted uninfected vero cells (for HSV-1/2) or a washing from GC agar plates (for \textit{N. gonorrhoeae}) were also included, in addition to media controls.

\textbf{Inhibition of Intracellular Pathways.}

In order to block anti-inflammatory pathways, 1G5 cells were pre-treated with 5\textmu M curcumin (Sigma-Aldrich), 10\textmu M NF\kappa B inhibitor pyrrolidine dithiocarbamate (\textbf{PDTC}; Sigma-Aldrich), p38 MAP kinase inhibitor SB203580 (Invivogen) or c-Junk N-terminal kinase (\textbf{JNK}) inhibitor SP600125 (Invivogen) for 1 hour, after which the cells were washed with 1xPBS before being exposed to direct or indirect treatments.

\textbf{Statistical Analysis}

GraphPad Prism version 4 (GraphPad Software, San Diego, CA) was used to compare three or more means by two-way analysis of variance (ANOVA). When an overall statistically significant difference was seen ($p<0.05$), post-tests were performed to compare pairs of treatments, using the Bonferroni post-test method to adjust the p-value for multiple comparisons.
RESULTS

**TLR ligands can directly and indirectly drive HIV-LTR activation in 1G5 cells.**

To examine how HIV co-infecting pathogens in the genital tract may play a role in increasing HIV replication, we tested whether TLR ligands representative of viral and bacterial pathogens, were capable of directly or indirectly trans-activating the HIV-LTR in 1G5 Jurkat T-cells that expressed the luciferase gene under the control of the HIV-LTR promoter (A. Chen et al., 2003). For direct HIV-LTR activation to occur, a ligand would directly interact with its cognate receptor, causing an intracellular cascade resulting in HIV-LTR activation. 1G5 cells treated with Poly (I:C) (TLR3 ligand) (p<0.05), the Gram-negative bacteria fimbriae protein FimH (TLR4 ligand) (p<0.001) and the bacterial flagella protein flagellin (TLR5 ligand) (p<0.001) induced potent HIV-LTR activation in comparison to mock treatment (Fig. 1a). Peptidoglycan (TLR2 ligand), lipoteichoic acid (TLR2 ligand), LPS (TLR4 ligand), loxoribine (TLR7 ligand) and CpG oligonucleotides (TLR9) did not directly induce HIV-LTR activation in 1G5 cells.

In order to examine indirect activation of HIV-LTR in 1G5 T cells, we tested whether TLR ligands could induce GECs to secrete factors that could induce indirect activation of the HIV-LTR. 1G5 cells were incubated with apical or basolateral cell culture supernatants from TLR ligand treated primary endometrial GECs. Both apical (Fig. 1b) and basolateral (Fig. 1c) supernatants collected from FimH- and flagellin-treated GECs increased HIV-LTR activation in comparison to supernatants collected from primary cells that had been mock treated. To rule out that the
indirect activation could be due to TLR ligand contamination in primary GEC supernatants, PBS washes collected 2 hours after incubation of TLR ligand with primary GECs were incubated with 1G5 cells. LTR activation similar to mock treated controls was observed in the last 4 of 5 total washes, suggesting that no residual TLR ligands remained in the cell culture supernatants after the last PBS wash (results not shown). These results suggest that specific TLR ligands can directly activate the HIV-LTR in 1G5 T cells, or induce primary GECs to secrete factors that can indirectly activate the HIV-LTR in 1G5 cells.

Co-pathogens can directly and indirectly drive the HIV-LTR in 1G5 cells.

Since we observed that TLR ligands could directly drive HIV-LTR activation, we next examined whether herpes simplex viruses or N. gonorrhoeae could similarly increase HIV-LTR activation. 1G5 cells exposed directly to HSV-1 KOS (p<0.01), HSV-2 333 (p<0.01) or N. gonorrhoeae (p<0.001) all induced potent HIV-LTR activation in comparison to mock-infected controls (Fig. 2a).

In order to determine whether these common sexually transmitted pathogens could also indirectly induce HIV-LTR activation in the genital tract, 1G5 T cells were incubated with apical and basolateral supernatants from primary GEC cultures grown in transwell inserts and infected with either HSV-1, HSV-2 or N. gonorrhoeae. Apical supernatants collected from HSV-1, HSV-2 or N. gonorrhoeae-infected primary GEC cultures activated HIV-LTR in 1G5 T cells (Fig. 2b). Of the basolateral supernatants, only those collected from primary GECs treated with N. gonorrhoeae induced HIV-LTR activation (Fig. 2b). Basolateral supernatants
collected from HSV-1 or HSV-2 infected GECs did not induce HIV-LTR activation. These results suggest that common STIs that co-infect HIV-infected individuals may directly induce HIV-LTR activation in infected T-cells, and also cause GECs to secrete immune factors that indirectly drive HIV replication.

**Primary GECs infected with co-pathogens produce elevated levels of proinflammatory cytokines and chemokines**

GECs can respond to incoming pathogens by producing proinflammatory cytokines and chemokines (H. Li et al., 2009). These can activate the HIV LTR and promote HIV replication, as well as drive chronic systemic HIV immune activation (Aziz, Nishanian, & Fahey, 1998; J. L. Fahey, 1998; Mellors, Griffith, Ortiz, Landry, & Ryan, 1991; Poli, Bressler, et al., 1990; Poli, Kinter, et al., 1990; L. Roberts et al., 2010). We therefore measured co-pathogen exposed GEC supernatants for 8 different cytokines and chemokines known to be produced by GECs. Apical supernatants from primary GECs infected with HSV-1, HSV-2 or *N. gonorrhoeae* contained elevated levels of TNF-α, IL-6, IL-8 and MCP-1 compared to uninfected controls (Fig.3a). Basolateral supernatants from *N. gonorrhoeae* infected primary GECs contained elevated levels of these cytokines as well (Fig. 3b). However, HSV-1 and HSV-2-infected GECs consistently secreted very low levels of most cytokines into the basolateral supernatant, likely explaining why these basolateral supernatants demonstrated a diminished capacity to indirectly drive the HIV-LTR (Fig 2b). No supernatant contained elevated levels of IL-1α, MIP-1α, GM-CSF or RANTES following infection (results not shown). These results suggest that in
response to common co-infecting pathogens, GECs secrete a number of proinflammatory cytokines and chemokines that have previously been implicated in increasing HIV replication.

**The direct and indirect activation of HIV-LTR can be abrogated by curcumin**

Since we observed that pro-inflammatory factors were produced by primary GECs in response to HSV-1, HSV-2 or *N. gonorrhoeae* infection, we next blocked intracellular inflammatory pathways by treating 1G5 cells with curcumin, a potent broad-spectrum inhibitor of anti-inflammatory pathways (Kunnumakkara, Anand, & Aggarwal, 2008), prior to incubation with pathogens or GEC supernatants. Pretreatment of 1G5 cells with curcumin completely blocked the ability of HSV-1, HSV-2 and *N. gonorrhoeae* to directly drive the HIV-LTR (**Fig. 4a**). Furthermore, pre-treatment of 1G5 cells with curcumin also completely inhibited indirect HIV-LTR activation by apical cell culture supernatants from primary GECs infected with HSV-1, HSV-2 or *N. gonorrhoeae* (**Fig. 4b**). Curcumin pre-treatment did not affect 1G5 cell viability, as measured by trypan blue cellular staining (not shown) indicating that inhibition of HIV-LTR activation was due specifically to the anti-inflammatory effects of curcumin. This suggests that viral and bacterial co-infections of the genital tract directly activate inflammatory pathways in 1G5 T cells, resulting in direct HIV-LTR activation. Furthermore, these pathogens induce primary GECs to secrete immune factors which can trigger inflammatory pathways in HIV infected T cells, resulting in indirect HIV-LTR activation.
Direct and indirect HIV-LTR activation by co-infecting pathogens requires NFκB and AP-1.

Curcumin is a broad-spectrum compound known to inhibit growth factors, anti-apoptotic mechanisms, protein kinases and transcription factors such as nuclear factor (NF)κB and activator protein (AP)-1, both of which modulate cytokine production and have been shown to play important roles in the induction of HIV replication (Duh, Maury, Folks, Fauci, & Rabson, 1989; Kunnumakkara et al., 2008; Pereira, Bentley, Peeters, Churchill, & Deacon, 2000). We next determined if blocking these intracellular inflammatory pathways could abrogate HIV-LTR activation in 1G5 cells. 1G5 cells were pre-treated with PDTC, an inhibitor of NFκB activation (Schreck, Meier, Mannel, Droge, & Baeuerle, 1992), and inhibitors of p38 mitogen-activated protein (MAP) kinase (SB203580) and c-Jun N-terminal kinase (JNK) (SP600125), both of which activate AP-1 (Bennett et al., 2001; Jarnicki et al., 2008; Schreck et al., 1992). 1G5 cells pre-treated with pathway-specific inhibitors were exposed to HSV-1, HSV-2, N. gonorrhoeae or cell culture supernatants from primary GECs infected with these pathogens. Direct activation of the HIV-LTR by HSV-1, HSV-2 and N. gonorrhoeae was significantly decreased in 1G5 T cells pre-treated with specific inhibitors of either NFκB or AP-1. The greatest decrease, comparable to uninfected controls in HIV-LTR activity, was observed when 1G5 T cells were pre-treated with a combination of both inhibitors. This indicates that direct activation of HIV-LTR in 1G5 T cells required both NFκB and AP-1 (Fig. 5).

The NFκB and AP-1 pathways were also found to be the main intracellular pathways involved in indirect HIV-LTR activation by GEC supernatants as the dual
blockade of NFκB and AP-1 led to greatest abrogation of indirect HIV-LTR activation in 1G5 cells (Fig. 6). The p38 MAP kinase did not seem to play a significant role in this process, as neither its inhibition individually or in concert with the other inhibitors had a significant effect on HIV-LTR activation in 1G5 cells (Fig. 7). These results suggest that both direct and indirect activation of HIV-LTR is mediated primarily by NFκB and JNK mediated AP-1 signalling pathways in infected T-cells.
DISCUSSION

In this study we addressed how heterologous co-infection with HSV-1, HSV-2 or *N. gonorrhoeae* can influence HIV replication in infected T cells in the upper female genital tract. We found that TLR ligands, representative of viral (Poly I:C) and bacterial (FimH, flagellin) co-infections, as well as the common sexually transmitted pathogens HSV-1, HSV-2 and *N. gonorrhoeae*, were capable of directly activating the HIV-LTR promoter in stably transfected T-cells. Furthermore, these TLR ligands and co-infecting pathogens induced GEC to secrete immune factors that indirectly activated the HIV-LTR promoter. Key factors secreted by GECs that could activate the HIV-LTR included the proinflammatory cytokines TNF-α and IL-6 and chemokines IL-8 and MCP-1. The direct and indirect activation of the HIV-LTR was completely abrogated when inflammatory pathways were blocked. The direct and indirect activation of the HIV-LTR was mediated by transcription factors NFκB and AP-1.

These results support previous studies which have suggested that common STIs, such as HSV-1, HSV-2 and *N. gonorrhoeae* are all capable of directly inducing HIV replication (A. Chen et al., 2003; Ding et al., 2010; Golden et al., 1992; Margolis, Rabson, Straus, & Ostrove, 1992; Schafer et al., 1996). Additionally, the present study shows that recognition of pathogens by TLRs on infected T cells could directly activate pro-inflammatory signalling pathways thereby activating HIV-LTR. More importantly, the present study provides new insight into the effect of a pro-inflammatory milieu in the upper female genital tract on HIV replication. Bacterial TLR ligands, FimH and flagellin, as well as HSV-1/2 and *N. gonorrhoeae* were all seen
to induce GECs to secrete pro-inflammatory cytokines and chemokines that indirectly contributed to increasing HIV replication in transfected T-cells. These results suggest that direct contact between a pathogen and a T-cell harbouring proviral HIV DNA may not be necessary to induce HIV replication in co-infected individuals. STIs could enhance HIV replication by inducing production of pro-inflammatory cytokines and chemokines from GECs in the genital tract and this may be sufficient to enhance HIV replication. These results are supported by clinical observations that among HSV-2/HIV co-infected individuals, increased HIV shedding was most dramatic among women with elevated chemokines and proinflammatory cytokine levels in cervico-vaginal lavage fluid (Rebbapragada et al., 2007).

Another novel finding in the present study was that bacterial and viral co-pathogens in the genital tract may induce distinct responses from GECs resulting in differential potentiation of HIV-LTR activation. In response to HSV-1 and HSV-2 infection, primary GECs secreted pro-inflammatory factors that could indirectly drive HIV-LTR activation only onto the apical or luminal side. On the other hand, bacterial TLR ligands, as well as N. gonorrhoeae, successfully activated the HIV-LTR with supernatants from both the apical and basolateral sides. Since HIV-infected T cells would predominantly be present in the subepithelial lamina propria of the genital mucosa, bacterial infection may be more likely to induce HIV replication indirectly. The apical pro-inflammatory factors secreted by GECs in response to HSV infection may act in an autocrine manner to upregulate HIV-LTR in GECs. Although controversial, GECs have been shown to be infected by HIV (Dezzutti et al., 2001;
Howell et al., 1997; Wu et al., 2003), and it may be possible that the apical factors induced by HSV-1/2 may facilitate HIV replication in these cells. Ongoing studies in our lab are examining this possibility.

In the current study we were able to identify the intracellular pathways involved in direct and indirect induction of HIV-LTR in transfected T cells. Curcumin, a broad spectrum inhibitor, could abrogate both direct and indirect LTR activation. Unlike “smart drugs,” which inhibit one factor alone, curcumin has been referred to as a “dirty drug,” because it has been shown to suppress multiple signalling pathways (Kunnumakkara et al., 2008). By using specific inhibitors of NFκB and AP-1, transcription factors that have been implicated in HIV transcription (Pereira et al., 2000), we were able to show completely abrogation of LTR activation. In contrast, inhibition of the AP-1 activating MAP kinase p38, did not affect either direct or indirect HIV-LTR activation, suggesting a JNK mediated AP-1 and NFκB pathways regulate HIV-LTR activation in T cells.

In conclusion, this study demonstrates, for the first time, the cellular mechanism that may drive HIV replication in the presence of viral or bacterial co-infections. These co-pathogens could directly activate HIV replication in infected T cells in the genital tract. More importantly, even in the absence of any direct contact between pathogens and infected cells, the pro-inflammatory factors secreted by GECs may be sufficient to activate HIV-LTR. These results indicate that inhibition of inflammatory pathways may also be effective in reducing HIV replication in co-infected individuals in addition to anti-microbial treatments.
ACKNOWLEDGEMENTS

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FIGURES

FIGURE 1 -- TLR ligands can directly and indirectly activate the HIV-LTR in 1G5 cells. To assess whether co-infections directly contribute to HIV-LTR activation (a), 1x10^6 1G5 Jurkat T-cells expressing the luciferase gene under the control of the HIV-LTR promoter were treated with PG, LTA, Poly (I:C), LPS, FimH, flagellin, loxoribine or CpG-oligonucleotides for 24 hours. Following this, the cells were lysed and luciferase activity was measured and reported in RLUs. Data shown represents mean± SEM of 4 separate experiments. To assess whether co-infections in the female genital tract could indirectly contribute to HIV-LTR activation, primay endometrial GEC cultures were grown to confluency on transwell inserts and treated with the same TLR ligands described above for 2 hours. Treatments were removed and the cells were washed 5 times with 1xPBS and subsequently replenished with fresh media. 24 hours following treatment, apical (b) and basolateral (c) supernatants were collected and incubated with 1x10^6 1G5 cells for 24 hours, after which the cells were lysed and luciferase activity was measured. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001.

FIGURE 2 – HSV-1, HSV-2 and N. gonorrhoeae can directly and indirect increase HIV-LTR activation in 1G5 cells. To assess whether common co-infections of the female genital tract could directly contribute to HIV-LTR activation (a), 1x10^6 1G5 cells were infected with 10^4 PFU of HSV-1 KOS, HSV-2 333, or 10^6 CFU of N.
gonorrhoeae clinical strain 2071 for 16 hours. Following this, the cells were lysed and luciferase activity was measured and reported in RLUs. Data shown represents mean ± SEM of 3 separate experiments. To assess whether common co-infections of the female genital tract could indirectly contribute to HIV-LTR activation (b), primary GEC cultures were grown to confluency on transwell inserts and infected with 10⁴ PFU of HSV-2 333, HSV-1 KOS, or 10⁶ CFU of N. gonorrhoeae clinical strain 2071 for 2 hours. Following this, the inocula were removed and the cells were washed 5 times with 1xPBS and subsequently replenished with fresh media. Twenty-four hours following infection, the apical and basolateral supernatants were collected and either briefly exposed to UV-light, or filter sterilized to kill/remove any live virus or bacteria, respectively, remaining in the supernatant. The supernatants were incubated with 1x10⁶ 1G5 cells for 24 hours, after which the cells were lysed and luciferase activity was determined. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001.

**FIGURE 3** - Cytokine and chemokine profiles of apical supernatants from primary endometrial GECs infected with HSV-1, HSV-2 or N. gonorrhoeae.

Confluent primary endometrial GECs were mock infected or infected with 10⁴ PFU of HSV-2 333, HSV-1 KOS, or 1x10⁶ CFU of N. gonorrhoeae for 2 hours. Following this, the inocula were removed and the cells were washed 5 times with 1xPBS and subsequently replenished with fresh media. Sixteen hours following infection, apical (a) and basolateral (b) supernatants were collected and assayed for IL-1α, IL-6, IL-8, MCP-1, MIP-1α, GM-CSF, RANTES and TNF-α using a multi-analyte Luminex assay.
The results for apical TNF-α, MCP-1, IL-8 and IL-6 are presented above. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01 and ***p<0.001.

**FIGURE 4 – The direct and indirect effect of HSV-1, HSV-2 and N. gonorrhoeae infection on HIV-LTR activation in 1G5 cells is inhibited by curcumin.** To measure whether proinflammatory pathways played a role in inducing direct (a) or indirect (b) HIV-LTR activation, 5 µM of curcumin was added to 1x10^6 1G5 cells for 1 hour. The cells were then washed and either infected with 10^4 PFU HSV-1 KOS, HSV-2 333 or 10^6 CFU N. gonorrhoeae for 16 hours (a); or they were exposed for 24-hours to apical supernatants collected from primary GECs infected with 10^4 PFU HSV-1 KOS, HSV-2 333 or 10^6 CFU N. gonorrhoeae, as previously described (b). Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001.

**FIGURE 5 – Direct HIV-LTR activation by HSV-1, HSV-2 and N. gonorrhoeae in 1G5 cells is dependent on NFκB and AP-1.** In order to determine whether direct HIV-LTR activation required NFκB or AP-1, 1G5 cells were pre-treated with either 10µM PDTC, or SP600125 for 1 hour. The cells were then washed thoroughly with 1x PBS before being infected with HSV-1, HSV-2 or N. gonorrhoeae as previously described. The cells were then lysed and luciferase activity was measured. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001.
**FIGURE 6 – Indirect HIV-LTR activation by HSV-1, HSV-2 and *N. gonorrhoeae* in 1G5 cells relies on NFκB and AP-1.** In order to determine whether indirect HIV-LTR activation required NFκB or AP-1, 1G5 cells were pre-treated with either 10μM PDTC or SP600125 for 1 hour. The cells were then washed thoroughly with 1x PBS before being incubated with supernatants from primary GECs that had been infected with HSV-1, HSV-2 or *N. gonorrhoeae* as previously described. Following incubation, the 1G5 cells were lysed and luciferase activity was measured. Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001.

**FIGURE 7 – The direct and indirect effect of HSV-1, HSV-2 and *N. gonorrhoeae* infection on HIV-LTR activation in 1G5 cells does not depend on p38 MAP kinase.** To determine whether the AP-1 activating enzyme p38 MAP kinase played an important in inducing direct (a) or indirect (b) HIV-LTR activation, 1x10^6 1G5 cells were pre-treated singularly with 10μM SB203580, or with SB203580 in combination with 10μM of PDTC and SP600125 for 1 hour. The cells were then washed and either infected with 10^4 PFU HSV-1 KOS, HSV-2 333 or 10^6 CFU *N. gonorrhoeae* for 16 hours (a); or they were exposed for 24-hours to apical supernatants collected from primary GECs infected with 10^4 PFU HSV-1 KOS, HSV-2 333 or 10^6 CFU *N. gonorrhoeae*, as previously described (b). Data shown represents mean ± SEM of 3 separate experiments *p<0.05, **p<0.01, ***p<0.001. N.S. (not significant).
Figure 1

a. 

b. 

c.
Figure 2

a. HIV-LTR Activation (in RLUs)

b. HIV-LTR Activation (in RLUs)
Figure 3

(a) Apical cytokine production (pg/mL)

(b) Basolateral cytokine production (pg/mL)

Treatments

Media, HSV-1 KOS, HSV-2 333, N. gonorrhoeae

Cytokines: TNF-α, IL-6, IL-8, MCP-1

Significance levels: * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 4

(a) 

HIV-LTR Activation (in RLUs)

**

***

Curcumin Pre-treated

(b) 

HIV-LTR Activation (in RLUs)

**

* 

***

Curcumin Pre-Treated

Treatments
Figure 5

HIV-LTR Activation (in RLUs)

**PDTC**
- + - - - + - - - + - - - + - - -

**SP600125**
- - + - - - + - - - + - - - + - -

**PDTC + Sp600125**
- - - + - - - + - - - + - - - + -
**Figure 6**

The figure illustrates the activation of HIV-LTR (in RLU's) for different treatments and pathogens:

- **PDTC**
- **SP600125**
- **PDTC + Sp600125**

The y-axis represents HIV-LTR Activation in RLU's, ranging from 0 to 3000.

The x-axis lists different treatments and pathogens:
- HSV-1
- HSV-2
- N. gonorrhoeae

The data points are indicated with asterisks (*) and double asterisks (**) to denote statistical significance.
Chapter 4

The anti-inflammatory activity of curcumin protects the genital mucosal epithelial barrier from disruption and blocks replication of HIV and HSV-2

Victor H. Ferreira, Aisha Nazli and Charu Kaushic

This manuscript has been submitted to AIDS (July 2014) and is currently in revision.

Given its potent anti-inflammatory properties, we decided to investigate whether curcumin could be used to abrogate inflammatory processes that facilitate HIV-1 acquisition in the FGT or contribute to HIV amplification. Our results suggest that curcumin can a) protect the genital epithelial barrier against HIV-1-mediated disruption by preventing the HIV gp120-mediated induction of inflammatory responses from primary GECs, b) prevent the gp120-mediated upregulation of chemokines that recruit HIV target cells to the FGT, c) block GEC innate inflammatory responses to co-infecting microbes thereby preventing indirect activation of the HIV promoter in T-cells, d) decrease HIV amplification in chronically infected T-cells and e) block HSV-2 viral replication in GECs by a mechanism that likely involves NFκB.

Drs. Charu Kaushic, Aisha Nazli and I were responsible for the design and interpretation of the experiments. Dr. Aisha Nazli and I were responsible for the generation and analysis of the data. Dr. Charu Kaushic and I wrote and edited the manuscript.
The anti-inflammatory activity of curcumin protects the genital mucosal epithelial barrier from disruption and blocks replication of HIV and HSV-2

Running Head: Curcumin protects the genital mucosa from HIV

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Total number of words used: 3,498
Abstract:

Objective: Inflammation is a known mechanism that facilitates HIV acquisition and the spread of infection. In this study, we evaluated whether curcumin, a potent and safe anti-inflammatory compound, could protect the genital mucosal barrier from HIV-mediated disruption, dampen inflammation or decrease HIV and HSV-2 replication.

Design & Methods: Primary, human genital epithelial cells (GECs) were pretreated with curcumin and exposed to HIV-1 or gp120, both of which have been shown to disrupt tight junctions. Mucosal barrier integrity was assessed by measuring tight junction protein expression, trans-epithelial electrical resistance across the monolayer and inflammatory cytokine production in GEC supernatants. HIV-LTR promoter activation was measured in T-cells exposed to supernatants from primary GECs pretreated with curcumin and exposed to sexually transmitted co-infecting pathogens. Lastly, amplification of HIV and HSV-2 were measured in chronically infected T-cells and primary GECs, respectively, treated with or without curcumin.

Results: Pre-treatment with curcumin prevented disruption of the mucosal barrier by HIV-1 gp120 and abrogated the concomitant upregulation of the proinflammatory cytokines TNF-α and IL-6. Furthermore, curcumin prevented gp120-mediated upregulation of chemokines (IL-8, RANTES and IP-10) that recruit HIV target cells to the FGT. GECs treated with curcumin and exposed to STIs were unable to elicit innate inflammatory responses that have been shown to indirectly
activate the HIV promoter. Finally, curcumin treatment resulted in significantly decreased HIV amplification in chronically infected T-cells and HSV-2 viral shedding in GECs.

Conclusions: Our results suggest that the use of anti-inflammatory compounds such as curcumin may offer paradigm-shifting alternatives for prevention and/or controlling HIV replication.
Introduction

According to the WHO and UNAIDS, women comprise more than half of all people living with HIV-1 (UNAIDS, 2011b). An estimated 40% of all annual global infections occur through HIV invasion of the female genital tract (FGT) via exposure to HIV-1 containing semen (Hladik & McElrath, 2008). The FGT is lined by genital epithelial cells (GECs), which are one of the first cells to encounter the virus during sexual transmission. It has been proposed that following exposure, a short phase of local viral amplification in the FGT is necessary for successful establishment of HIV-1 infection (Q. Li et al., 2009). We have previously demonstrated that HIV directly impairs the genital mucosal barrier, leading to viral translocation that could initiate infection of underlying target cells (Nazli et al., 2010; Nazli et al., 2013). Thus protecting the mucosal barrier could play a critical role in preventing HIV infection and provide an early window for prophylactic strategies.

The binding of HIV-1 gp120 to GECs results in the upregulation of numerous proinflammatory cytokines, most notably tumor necrosis factor-α (TNF-α) through activation of TLR-2 and -4 pathways; it is this inflammation that mediates disruption of the mucosal barrier (Nazli et al., 2010; Nazli et al., 2013). A number of other studies have demonstrated that inflammation facilitates the acquisition and transmission of HIV-1 infection, as well as contributing to the sequelea of disease associated with chronic infection, including cardiovascular disease, diabetes and neurodegenerative disorders (Deeks et al., 2013). Studies of latently infected monocyte and T cell lines have shown that the addition of TNF-α, interleukin-6 (IL-6) or IL-1β can activate HIV-1 replication, mediated through the HIV-long terminal
repeat (LTR) promoter region (Chun et al., 1998; Folks et al., 1989; Poli, Bressler, et al., 1990). Furthermore, lower levels of IL-1β, IL-6 and TNF-α were measured in unstimulated peripheral blood mononuclear cells (PBMCs) of highly exposed persistently seronegative (HESN) women, suggesting an immunooquiescent phenotype among this resistant cohort (McLaren et al., 2010). These studies strongly suggest that lower levels of inflammation may decrease susceptibility to HIV-1 and decrease viral replication, perhaps impairing transmission.

A number of studies have shown that sexually transmitted co-infections increase HIV genital shedding and transmission (C. C. Chang et al., 2013). Herpes simplex virus type 2 (HSV-2) is one of the most prevalent viral STIs, infecting 20–30% of sexually active adults in North America, and up to 85% of people in sub-Saharan Africa living with HIV are also infected with HSV-2 (Weiss, 2004). A recent meta-analysis demonstrated HSV-2 infection to be associated with a threefold increase in susceptibility to HIV by both men and women (Freeman et al., 2006). In addition to viral co-infections, sexually transmitted bacteria such as Neisseria gonorrhea have also been suggested to play an important role in enhancing HIV infection or replication (A. Chen et al., 2003; Johnson & Lewis, 2008; Klotman et al., 2008; Levine et al., 1998; Rotchford et al., 2000; J. Zhang et al., 2005). GECs are the first cells to come into contact with both HIV and other sexually transmitted pathogens. We previously showed that not only could co-infecting microbes, specifically HSV-1, HSV-2 and N. gonorrhea, directly induce HIV replication in T-cells, but that in response to these pathogens, GECs upregulated inflammatory mediators that indirectly induced HIV replication (Ferreira et al., 2011).
Curcumin (**diferuloylmethane**), the principal curcuminoid of the spice turmeric, is a highly pleiotropic compound that has been shown to possess anti-inflammatory activities (Aggarwal & Sung, 2009). Curcumin has been shown to modulate multiple cell signaling molecules such as proinflammatory cytokines (TNF-α, IL-1β, IL-6) and transcription factors such as NFκB and AP-1 (Aggarwal & Sung, 2009). Curcumin has also shown tremendous beneficial anti-inflammatory properties in clinical trials for Crohn's disease and rheumatoid arthritis, among others (S. C. Gupta, Patchva, & Aggarwal, 2013).

Given its potent anti-inflammatory properties, we decided to investigate whether curcumin could be used to abrogate inflammatory processes that facilitate HIV-1 acquisition in the FGT or contribute to HIV amplification. Our results suggest that curcumin can a) protect the genital epithelial barrier against HIV-1-mediated disruption by preventing the HIV gp120-mediated induction of inflammatory responses from primary GECs b) prevent the gp120-mediated upregulation of chemokines that recruit HIV target cells to the FGT c) block GEC innate inflammatory responses to co-infecting microbes thereby preventing indirect activation of the HIV promoter in T-cells d) decrease HIV amplification in chronically infected T-cells and e) block HSV-2 viral replication in GECs by a mechanism that likely involves NFκB.
Materials & Methods

Cell Lines, Viruses and Bacteria

HIV-IIIIB was prepared from the chronically infected H9 cell line, followed by virus concentration by Amicon Ultra-15 filtration system (Millipore, Billerica, MA, USA) (Nazli et al., 2010). All HIV-1 stocks were titred for infectious viral units (IVUs)/mL by TZM-bl (ATCC, Manassas, VA, USA) indicator cell assay, as previously described (Kimpton & Emerman, 1992). Vero cells (ATCC) were maintained in α-MEM media (McMaster University, Hamilton, ON, Canada) supplemented with 5% FBS (Life Technologies Inc., Burlington, ON, Canada), 100 U/mL penicillin/streptomycin (pen/strep) (Sigma-Aldrich, Oakville, ON, Canada), 2 μM L-glutamine (L-glu) (Life Technologies Inc.), and 10 μM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (McMaster University). HSV-1 KOS and HSV-2 333 viral stocks were prepared by infecting Vero cells at a multiplicity of infection of 0.01 for 24–48 hours. HSV-1 KOS was a kind gift from Dr. Karen Mossman (McMaster University). N. gonorrhoeae clinical strain 2071, a kind gift from Dr. Scott Gray-Owen (University of Toronto), was grown at 37°C in a 5% CO₂ humidified incubator from frozen stocks on GC agar base supplemented with 1% IsoVitaleX enrichment (BD, Mississauga, ON, Canada). 1G5 Jurkat T cells were maintained in RPMI (McMaster University) media supplemented with 10% FBS, 100 U/mL pen/strep, 2 μM L-glu and 10 μM of HEPES (Ferreira et al., 2011).

Source of Tissues and Epithelial Cell Preparation
FGT tissues were obtained from women undergoing hysterectomies for non-malignant gynecological purposes at McMaster University Medical Centre in Hamilton, ON, Canada. Written informed consent was received in accordance with the approval of the Hamilton Health Sciences-McMaster University Research Ethics Board. The protocol for isolation, culture and assessment of primary GEC culture purity are described elsewhere (Kaushic, Nazli, et al., 2011; MacDonald et al., 2007). Briefly, endometrial and endocervical tissues were minced into small pieces and digested in an enzyme mixture and GECs were isolated by a series of separations through nylon mesh filters (Small Parts, Inc. Logansport, IN, USA). Approximately 1x10^5 GECs were seeded onto Matrigel (BD) coated tissue culture transwell inserts (BD) and cells were grown until they formed confluent monolayers, as measured by a trans-epithelial resistance (TER) >1 kΩ/cm. The purity of GEC monolayers was between 95 and 98%, with no trace of any hematopoietic cells. TERs were measured before exposing the cells to HIV or gp120 (pre-treatment TER) and 24 hours after, and expressed as a percent of pre-treatment TER.

**Confocal Microscopy**

Confluent primary GECs were grown on transwell culture inserts and pretreated with 5μM curcumin (Sigma-Aldrich) or primary media as control for 1 hour prior to exposure to 10^5 IVUs of HIV, recombinant gp120 (Immunodiagnostics, Woburn, MA, USA) at 0.1μg/mL or primary media (mock infection). This standard dose of curcumin was selected because it is sufficient to block the induction of TNF-α from LPS-stimulated monocytes (M. M. Chan, 1995). The recombinant HIV-1
gp120 protein was endotoxin free and viral stock preparations were determined to be free of TNF-α, IL-6, IL-8, and other cytokines via the multiplex bead-based sandwich immunoassay (Luminex Corporation, Austin, TX), described elsewhere in detail (Nazli et al., 2010; Nazli et al., 2013). At 24 hours post exposure, GECs were fixed, permeabilized and stained as described before (Nazli et al., 2010). Imaging was done on an inverted confocal laser-scanning microscope (LSM 510, Carl Zeiss Canada Ltd., Toronto, ON, Canada). For each experiment, confocal microscope settings for image acquisition and processing were identical between controls and treated monolayers and 3 random images were acquired and analyzed for each experimental condition.

*Magpix for Cytokine and Chemokine Measurements.*

Supernatants were collected and TNF-α, IL-6, inflammatory protein-10 (IP-10), eotaxin, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), IL-8 and regulated on activation, normal T cell expressed and secreted (RANTES) were measured using the Magpix multi-analyte technology system (Millipore, Billerica, MA, USA), as per the manufacturer's instructions.

*Measuring HIV Replication in Chronically Infected T-cells*

5x10⁵ chronically HIV-infected H9 T-cells were exposed once or daily to 5 or 50 μM curcumin or serum-free RPMI as control. At several time points post-treatment, supernatants were collected and HIV-1 p24-antigen was measured using
a commercial p24 ELISA kit (Zeptometrix Corp., Buffalo, NY, USA), as per the manufacturer's instructions.

**Indirect Activation of the HIV-LTR in 1G5 Cells**

IG5 Jurkat T cells were used to measure trans-activating the HIV-LTR promoter by epithelial cell innate inflammatory responses (A. Chen et al., 2003; Ferreira et al., 2011). Briefly, confluent primary GECs pre-treated with or without curcumin for 1 hour were exposed to $10^4$ PFUs of HSV-1 KOS or HSV-2 333 or $10^6$ CFUs of *N. gonorrhea* strain 2071 for 2 hours, washed 5 times, and supernatants were collected. To ensure that there was no residual live HSV-2, supernatants were exposed to 1 cycle of 10 kJ of UV energy (Stratalinker, Agilent, Santa Clara, CA, USA). Supernatants collected from *N. gonorrhea*-infected GECs were filter sterilized using a 0.2-μm filter (BD) to remove any residual bacteria. The supernatants were then incubated with $10^6$ 1G5 cells for 24 hours, and luciferase activity in the T-cells was measured using a luciferase assay (Agilent) in accordance with the kit’s instructions.

**Measuring HSV-2 Shed Virus in Cell Culture Supernatants**

Primary GECs were grown to confluency and were pre-treated with media (control) or 5 μM or 50μM of curcumin, pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich), an NFκB inhibitor, SB203580 (Invivogen, San Diego, CA, USA), a p38 MAP kinsase inhibitor or SP600125 (Invivogen) an inhibitor of c-Jun N-terminal kinase (JNK) for 1 hour, prior to exposure to $10^4$ PFU of HSV-2 strain 333 for 2 hours. Virus-exposed cells were washed with 1x PBS and fresh media was added to the
cells. At 24-hours post-infection, apical cell culture supernatants were collected and the amount of shed HSV-2 was measured using a standard Vero plaque assay, as previously described (A. E. Gillgrass, Tang, Towarnicki, Rosenthal, & Kaushic, 2005).

**Statistical Analysis**

All experiments were repeated at least three times, using three or more replicates per experiment. GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) was used to compare 3 or more means by one-way analysis of variance (ANOVA). When an overall statistically significant difference was measured (p<0.05), a Bonferroni post-test was performed to adjust the p-value for multiple comparisons.
Results

Curcumin blocks the disruption of epithelial cell tight junction proteins and protects the epithelial barrier against HIV-1 gp120.

Previously we have shown that in the presence of HIV gp120, tight junction (TJ) proteins, including ZO-1 and occludin, expressed by primary GECs, are downregulated due to innate inflammation, leading to the disruption of barrier integrity and subsequent microbial translocation (Nazli et al., 2010; Nazli et al., 2013). Given the potent anti-inflammatory effects of curcumin, we decided to measure whether curcumin could block the barrier disrupting effects of HIV gp120. In the presence of HIV-1 or recombinant gp120, ZO-1 (Figure 1a) and occludin (Figure 1b) protein expression in GEC monolayers was disrupted, which was not seen in monolayers pre-treated with curcumin. Curcumin on its own had no visible effect on TJ protein expression. Pre-treatment with curcumin also prevented the HIV gp120- and TNF-α-mediated decrease in TER (Figure 1c), a measure of epithelial barrier integrity (Nazli et al., 2010). These results suggest that curcumin can prevent HIV-mediated mucosal barrier impairment by maintaining TJ protein expression and maintaining TER in GECs.

Curcumin prevents the induction of proinflammatory cytokines associated with barrier disruption.

Previously we have shown that epithelial cell exposure to gp120 results in the upregulation of inflammatory mediators, including TNF-α (Nazli et al., 2010;
Nazli et al., 2013), which mediate the disruption of TJ proteins. Therefore, we sought to determine whether the gp120-mediated upregulation of proinflammatory mediators could be blocked by curcumin. At 24-hours following exposure to recombinant gp120, TNF-α (Figure 2a) and IL-6 (Figure 2b) were significantly upregulated relative to untreated cell culture supernatants. Pre-treatment with curcumin abrogated gp120-mediated induction of these proinflammatory cytokines, suggesting that curcumin may block inflammatory mediators associated with HIV-mediated mucosal barrier disruption.

Curcumin blocks the gp120-mediated induction of chemokines associated with the recruitment of HIV-target cells.

HIV target cells, which include CD4+ T-cells, macrophage and dendritic cells (DCs), are recruited to the genital tract via chemokine gradients that include MCP-1, MIP-1α, IP-10, eotaxin, IL-8 or RANTES produced by resident tissue cells, such as GECs (Nazli et al., 2010; Nazli et al., 2013). Exposure of GECs to gp120 resulted in significant upregulation of IL-8, IP-10 and RANTES (Figure 2c-e) but not MCP-1, eotaxin or MIP-1α (results not shown). Interestingly, gp120-mediated chemokine induction was abrogated in GEC monolayers pretreated with curcumin. These results show that pre-treatment with curcumin blocks gp120 mediated upregulation of chemokines that can recruit HIV target cells to the FGT.

Curcumin pre-treatment blocks HIV amplification in chronically infected T-cells.
Next we determined if curcumin could directly block HIV replication in chronically infected T-cells. Chronically infected H-9 T-cells exposed to a single curcumin pre-treatment secreted significantly less virus into cell culture supernatants relative to untreated H-9 T-cells. After 24 hours of exposure, the effect of curcumin started to diminish but HIV replication remained significantly lower than untreated cells (Figure 3a). Interestingly, when H-9 T-cells were exposed to curcumin once daily, HIV levels remained significantly suppressed relative to controls (Figure 3b). Both low (5 μM) and high (50 μM) curcumin doses appeared to be equally effective in controlling HIV replication in H-9 cells, indicating that curcumin could have a potent effect on HIV replication within infected T cells.

Curcumin pre-treatment of primary GECs blocks co-infection mediated innate inflammatory responses that cause indirect induction of HIV replication.

Previous studies from our group have shown that in the presence of co-infecting STIs, specifically HSV-1, HSV-2 or N. gonorrhea, primary GECs secrete inflammatory factors that cause indirect activation of the HIV-LTR in T-cells, a process synonymous with HIV replication (Ferreira et al., 2011). Therefore, we sought to determine whether blocking inflammatory pathways in primary GECs using curcumin could abrogate indirect activation of HIV replication in T-cells. Cell culture supernatants collected from GECs pretreated with curcumin and exposed to co-infecting STIs were unable to indirectly activate the HIV-LTR (Figure 4). In contrast, supernatants collected from untreated GECs exposed to co-infecting microbes potently induced HIV-LTR activation in T-cells, suggesting that pre-
treatment with curcumin can block co-infection-mediated inflammation that could lead to increased HIV replication.

**Curcumin blocks HSV-2 replication.**

HSV-2 infection is associated with a threefold increase in susceptibility to HIV in both men and women (Barnabas & Celum, 2012; R. Gupta et al., 2007; McClelland et al., 2002) and controlling HSV-2 replication has been associated with decreased HIV replication (C. Celum et al., 2010). Thus, we decided to test the antiviral activity of curcumin in primary human GECs. In cells pretreated with the 5μM curcumin, HSV-2 shed approximately 1,000-fold less virus relative to untreated primary cell controls infected with HSV-2 (Figure 5a). At 50μM of curcumin no HSV-2 virus could be detected in cell culture supernatants. Since curcumin blocks various inflammatory pathways (Aggarwal & Sung, 2009), we pre-treated GECs with inhibitors of curcumin-regulated transcription factors including NFκB (PDTC), p38 MAP kinase (SB203580) or c-Jun N-terminal kinase (JNK) (SP600125), prior to infecting the cells with HSV-2. Our results show that the NFκB inhibitor PDTC completely blocked viral shedding in primary GECs exposed to HSV-2 (Figure 5b), whereas blocking p38 MAP kinase or JNK did not have any effect on HSV-2 viral replication (*results now shown*).
Discussion

We have described in this study the multiple pathways by which curcumin may have a beneficial role in protecting the FGT against HIV-1. Our results show pleiotropic effects of curcumin, mediated by its potent anti-inflammatory effects. Curcumin prevented mucosal barrier impairment mediated by HIV-1 gp120 and abrogated gp120-mediated upregulation of proinflammatory cytokines and chemokines. Furthermore, curcumin pre-treatment of primary GECs prevented co-infection mediated upregulation of inflammatory molecules that indirectly activate the HIV promoter. Lastly, we found that curcumin could decrease HIV-1 amplification in T-cells and block HSV-2 shedding from primary GECs.

Based on our results, we posit that protecting the mucosal barrier with curcumin may play a significant role in preventing mucosal transmission of HIV. Previously, we showed that gp120 interacts with TLR2, TLR4 and heparan sulphate moieties on primary GECs resulting in downstream induction of TNF-α, TJ disruption and barrier impairment (Nazli et al., 2013). Such mucosal barrier disruption resulted in HIV translocation across the epithelium, presenting a mechanism by which HIV transmission may take place (Nazli et al., 2010). In our current study, we found that curcumin pre-treatment prevented gp120-mediated disruption of the TJ proteins ZO-1 and occludin, and maintained TERs across the genital monolayer. Furthermore, pre-treating primary GECs with curcumin prevented the gp120-mediated induction of barrier-breaking proinflammatory cytokines (TNF-α, IL-6) and HIV-target cell recruiting chemokines (IL-8, IP-10, RANTES). Together these results suggest that curcumin and other compounds,
which reduce inflammation and maintain mucosal barrier integrity, may be useful candidates to test for future prophylactic formulations used to prevent HIV acquisition.

Curcumin may also play a significant role in preventing or reducing chronic immune activation. Microbial translocation due to mucosal barrier impairment is not only associated with HIV acquisition but is strongly believed to be one of the main contributors to chronic immune activation (Brenchley et al., 2006), characterized by increased inflammatory markers and immune cell activation that persists even in HAART-treated individuals (Hazenberg et al., 2003; Redd et al., 2009). Immune activation is believed to be one of the main driving forces of CD4+ T-cell depletion and promoter of HIV replication (Paiardini & Muller-Trutwin, 2013).

Repeated exposure to HIV gp120 in mucosal tissues from shed virus or from unbound gp120 may continuously provide a source for generating inflammation in mucosal tissues that facilitate barrier breakdown and microbial translocation. Because immune activation is typically observed during the chronic stages of infection, the window of opportunity to intervene and limit these processes likely occurs during the early stages of HIV infection. Blocking HIV-mediated barrier disruption by curcumin may likely prevent microbial translocation and the initiation of immune activation, suggesting that curcumin treatment, may also contribute to decreasing the chronic inflammatory state that contributes to HIV pathology.

Our results show that curcumin may block HIV replication by multiple mechanisms including i) decreasing tissue inflammation and ii) controlling STIs that enhance HIV replication, such as HSV-2. Elevated levels of proinflammatory
cytokines (TNF-α, IL-6 or IL-1β) have been shown to increase HIV replication (Chun et al., 1998; Folks et al., 1989; Poli, Bressler, et al., 1990). In this study, we observed that curcumin pre-treatment significantly decreased gp120-mediated induction of proinflammatory cytokines by GECs. Previously we found that innate inflammatory responses to *N. gonorrhoeae*, HSV-1 or HSV-2 increased HIV promoter activation in T-cells (Ferreira et al., 2011). In the current study, we found that pre-treating GECs with curcumin was sufficient to block this co-infection mediated induction of the HIV promoter. Together these results suggest that curcumin may play a role in regulating the early tissue inflammation that may be required for viral amplification in the FGT.

HSV-2 has been identified as a significant facilitator of HIV infection. HSV-2 infection contributes to HIV infection by several mechanisms including causing ulcerations in mucosal tissues that facilitate HIV acquisition (Van de Perre et al., 2008) and recruiting a long-lasting population of CD4+ T-cells to the genital tract (J. Zhu et al., 2009). Interestingly, our studies show that curcumin may block HSV-2 replication in GECs in a mechanism that likely requires NFκB, suggesting that curcumin may exert anti-HIV activity by also directly inhibiting co-infecting microbes.

By suppressing replication in chronically infected T-cells, curcumin may provide a means of controlling HIV acquisition and amplification during the early phases of infection. HIV crosses the mucosal epithelial barrier to establish a small founder population that then expands locally possibly due to an influx of newly recruited target cells caused by upregulation of chemokines (Haase, 2011). These
early events represent a window of maximum opportunity for interventions to prevent systemic infection. Our results show that in addition to protecting the mucosal barrier, curcumin may curb HIV by limiting local propagation and expansion in T-cells, thus potentially preventing systemic dissemination, and by suppressing the local chemokine environment thus curbing target cell chemotaxis. Our results also showed that repeated administration of curcumin worked best at suppressing HIV amplification in chronically infected T-cells, suggesting that therapies that continuously release curcumin may provide novel tools by which HIV viral replication can be reduced in the FGT.

*In vitro* curcumin has been shown to directly inhibit HIV replication in susceptible cells (Gandapu et al., 2011; C. J. Li et al., 1993), inhibit HIV-LTR activation (Barthelemy et al., 1998; Ferreira et al., 2011; Gandapu et al., 2011; C. J. Li et al., 1993) and enhance HAART activity (Riva et al., 2008). In contrast, no significant evidence of curcumin-associated reduction in viral load was observed in a clinical trial examining the effectiveness of curcumin as an antiviral agent in patients with AIDS (James, 1996). These conflicting results between *in vivo* and *in vitro* studies underscore the need to reconsider the method of curcumin application. *In vivo*, curcumin’s poor bioavailability is attributed to poor absorption, rapid metabolism, and rapid systemic elimination (Anand, Kunnumakkara, Newman, & Aggarwal, 2007). The use of compounds, such as piperine, that can block hepatic and intestinal glucuronidation of curcumin have been shown to increase the bioavailability of curcumin in healthy human volunteers by 2,000% (Shoba et al., 1998). Other promising approaches to increase the bioavailability of curcumin in
humans include the use of nanoparticles (Sasaki et al., 2011) or liposomes (Basnet, Hussain, Tho, & Skalko-Basnet, 2012) for delivery. Most clinical studies reporting poor bioavailability have administered curcumin via the enteral route. Our results suggest that direct mucosal application may be the best use for curcumin; therefore developing microbicidal or topical formulations of curcumin may provide a unique way of controlling HIV infection.

In conclusion, our results indicate the promising potential of anti-inflammatory compounds such as curcumin, which protect the mucosal barrier and/or limit inflammation, unlike current ARV therapies that target infection and replication. Inflammation may contribute to the acquisition or spread of HIV-1 infection, as well as contribute to the sequelea of chronic HIV infection. Thus a paradigm shift toward limiting inflammation in the genital tract and/or systemically, may have a significant impact on HIV infection and disease progression.


**FIGURE 1 – Curcumin prevents tight junction disruption and breakdown of epithelial barrier integrity caused by HIV-1 gp120.** Primary GECs were grown to confluency on transwell culture inserts and pre-treated with 5μM curcumin or media as control for 1 hour. Next, GECs were exposed to 10^5 IVU of HIV ADA, recombinant gp120 at 0.1μg/mL or media (mock infection). At 24 hours post exposure, GECs were fixed in 4% PFA and permeabilized with blocking solution. The primary antibodies were rabbit anti-ZO-1 (A) or anti-occludin (B), followed by FITC-labelled goat anti-rabbit detection antibody. Imaging was done on an inverted confocal laser-scanning microscope. For each experiment, confocal microscope settings for image acquisition and processing were identical between control and treated monolayers and 3 separate, random images were acquired and analyzed for each experimental condition. Images are presented en face and are representative of one of three separate experiments. (C) Primary GECs were grown to confluency and pre-treated with media (mock) or 5μM curcumin for 1 hour and TERs were measured (pre-treatment TER). Next, the cells were exposed to media control (untreated), gp120 at 0.1μg/mL or recombinant TNF-α at 100 ng/mL (positive control) for 24 hours, after which TERs were measured again and the percent of pre-treatment TER was calculated. Data shown represents the mean ± SEM of three separate experiments. **p<0.01, ***p<0.001.
FIGURE 2 – Curcumin prevents the gp120-mediated induction of proinflammatory cytokines or chemokines that recruit HIV-target cells. Confluent primary GECs were pre-treated with 5μM curcumin or media (untreated) for 1 hour after which the cells were exposed to gp120 at 0.1 μg/mL. Apical cell culture supernatants were collected at 24 hours post-exposure and measured for the proinflammatory cytokines TNF-α (A) or IL-6 (B), as well as the chemokines IL-8 (C), IP-10 (D) and RANTES (E) via the Magpix multi-analyte system. Data shown represents the mean ± SEM of three separate experiments *p<0.05, **0.01, ***p<0.001.

FIGURE 3 – Curcumin prevents the amplification of HIV-1 in chronically infected T-cells. 5x10^5 chronically HIV-infected H9 T-cells were exposed once to 5 or 50 μM curcumin or serum free RPMI as control in a U-bottom 96-well plate (A) and at 24, 48 or 72 hours post-treatment, supernatants were collected and HIV-1 p24-antigen was measured using a commercial p24 ELISA kit. Alternatively, 5x10^5 H9 T-cells were exposed to curcumin once every 24 hours at 5 or 50 μM curcumin after which supernatants were collected and HIV-1 p24-antigen was measured. Data shown represents the mean ± SEM of three separate experiments *p<0.05, ***p<0.001.

FIGURE 4 – Curcumin pre-treatment of primary GECs can prevent the indirect activation of the HIV-LTR mediated by sexually transmitted co-infecting
**microbes.** Confluent primary GECs were pre-treated with 5uM curcumin or media (untreated) for 1 hour after which the cells were exposed to $10^4$ PFU of HSV-1 KOS, HSV-2 333 or $10^5$ CFU of *N. gonorrhoea* (NGO) clinical strain 2071 for 2 hours. After this, the inocula were removed and the cells were washed 5 times with 1x phosphate buffered saline and subsequently replenished with fresh media. The supernatants were incubated with $10^6$ 1G5 cells for 24 hours, after which the cells were lysed and luciferase activity was measured as a readout for HIV-LTR activation. Data shown represents the mean ± SEM of three separate experiments. ***p<0.001.

**FIGURE 5 - Inhibition of inflammatory signalling pathways decreases the amount of shed HSV-2 virus found in cell culture supernatants.** Primary GECs were grown to confluency and were pre-treated with media (untreated) or 5 uM or 50uM of curcumin (A) or PDTC the NFκB inhibitor, SB203580 a p38 MAP kinase inhibitor or SP600125 an inhibitor of c-Jun N-terminal kinase (JNK) (B) for 1 hour. The cells were then exposed to $10^4$ PFU of HSV-2 strain 333 for 2 hours. At 24-hours post-infection, apical cell culture supernatants were collected and the amount of shed HSV-2 was measured using a standard vero plaque assay. Data shown represents the mean ± SEM of three separate experiments. ***p<0.001.
Figure 1

A. (Images of different treatments: Media, gp120, HIV-1, Curcumin, Curcumin + gp120, Curcumin + HIV)

B. (Images of different treatments: Unlabeled)

C. (Graph showing Percent Pre-Treatment TBR: Untreated, gp120, INF-a 100ng/mL)
Figure 2
Figure 3

A.

B.
Figure 4

HIV-LTR Activation (in RLU)

Treatments

- Media
- HSV-1
- HSV-2
- NGO

- Untreated
- Curcumin

Significance levels indicated by asterisks: ***
Figure 5
Chapter 5

Medroxyprogesterone acetate regulates HIV-1 uptake and transcytosis, but not replication, in primary genital epithelial cells, resulting in enhanced T-cell infection.

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This manuscript examines the role of female sex hormones and MPA on HIV infection and replication in primary human GECs. This study demonstrates that HIV uptake was significantly increased within GECs grown in the presence of P4 and in particular, MPA. HIV-1 uptake by GECs primarily took place via endocytosis and EM confirmed that HIV-1 was localized to intracellular vesicular compartments. Despite uptake into GECs, reverse transcription, HIV DNA integration or RNA splicing were not measured, regardless of hormone exposure, suggesting a non-productive infection. Nevertheless, transcytosis of infectious HIV-1 was significantly increased among GECs grown in the presence of MPA, which resulted in increased infection of T-cells. Furthermore, supernatant analysis did not find increased inflammatory cytokines in cell culture supernatants from P4 or MPA-treated cells suggesting that sex hormones regulate GEC susceptibility in the absence of induced inflammation.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments. I was responsible for the generation and analysis of the data. Dr. Aisha Nazli, Jessica K. Kafka, Kristen Mueller and Sara Dizzell provided technical assistance and processed tissues. Drs. Michel J. Tremblay and Alan Cochrane provided technical expertise and reagents. Dr. Charu Kaushic and I wrote and edited the manuscript.
Medroxyprogesterone acetate regulates HIV-1 uptake and transcytosis, but not replication, in primary genital epithelial cells, resulting in enhanced T-cell infection.

Short Title: Sex hormones regulate HIV infection

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ABSTRACT

Although women constitute half of all people living with HIV/AIDS worldwide, the early events of HIV-1 infection in the female genital tract (FGT) are poorly understood. Genital epithelial cells (GECs) are the first cells to encounter HIV-1 during sexual transmission. Significant, albeit controversial, clinical and experimental evidence indicates that sex hormones and hormonal contraceptives regulate susceptibility to HIV infection, however little is understood about how this occurs in the FGT. In this study, primary GEC cultures were grown in the presence or absence of physiological concentrations of estrogen, progesterone (P4) or medroxyprogesterone acetate (MPA) prior to HIV-1 exposure. HIV uptake by GECs was significantly increased in endometrial and cervical epithelial cells grown in the presence of P4 and MPA. GEC uptake of HIV-1 occurred primarily via endocytosis, however no productive infection was measured, regardless of hormone exposure. HIV captured in GECs was released into both apical and basolateral compartments, with significantly higher transcytosis observed in the presence of MPA. Consequently, in GEC:T-cell co-cultures, maximum infection and replication in T-cells was observed in the context of MPA. Finally, we found that MPA broadly downregulated GEC production of proinflammatory cytokines and upregulated chemokines. These results suggest that P4, and especially MPA, may play a significant role in HIV acquisition in the FGT.
INTRODUCTION

Women comprise approximately half of all people living with HIV/AIDS (UNAIDS, 2011b). Approximately 40% of all new HIV infections originate in the female genital tract (FGT) (Haase, 2005; Hladik & McElrath, 2008), which is lined by genital epithelial cells (GECs), and as such, are one of the first cells to encounter HIV during sexual transmission. The FGT can be divided into two major compartments: the lower genital tract (LGT), consisting of the vagina and ectocervix, which is lined by stratified squamous epithelium; and the upper genital tract (UGT) consisting of the endocervix, endometrium and Fallopian tubes, which is lined by simple columnar epithelium (C. R. Wira, Fahey, et al., 2005).

The primary site of HIV-1 transmission in the FGT remains unresolved. Post-coital microabrasions and specialized cells such as dendritic cells (DCs) expressing DC-SIGN have been implicated in HIV acquisition in the LGT (Haase, 2011; Hladik & McElrath, 2008). Conversely, studies of acute simian immunodeficiency virus (SIV) in non-human primates, suggest that HIV may preferentially invade through the UGT (Q. Li et al., 2009; C. J. Miller et al., 1992). In humans, there is evidence that large number of activated CD4+ T-cells populate the cervical transformation zone making it a prime target site for HIV (Pudney et al., 2005). A recent study also found that HIV-1 was able to penetrate both intact human cervical columnar and vaginal squamous epithelial barriers to depths where the virus encountered potential target cells further supporting the idea that HIV can target multiple FGT sites (Carias et al., 2013).
Significant controversy also exists regarding whether HIV can replicate in GECs. *In vitro* studies demonstrate that primary and immortalized GECs can support HIV replication (Asin et al., 2004; Asin et al., 2003; Furuta et al., 1994; Micsenyi et al., 2013; Phillips et al., 1994; X. Tan et al., 1993), while other studies have been unable to show evidence of productive infection (Dezzutti et al., 2001; Greenhead et al., 2000; Wu et al., 2003). HIV virions have also been proposed to traverse the epithelium via transcytosis (Belec et al., 2001; Bobardt et al., 2007; Bomsel, 1997; Carreno, Krieff, Irinopoulou, Kazatchkine, & Belec, 2002; S. Gupta et al., 2013; Hocini et al., 2001; Kinlock et al., 2014; Saidi et al., 2007; Stoddard et al., 2009), a selective vesicular transcellular pathway where cargo is transported from the apical side of a polarized epithelial cell to the basolateral side (Bomsel & Alfsen, 2003).

The interactions that take place between HIV and GECs remain incompletely understood, particularly in the context of female sex hormones or hormonal contraceptives. Throughout the menstrual cycle, fluctuating levels of 17β-estradiol (E2) and progesterone (P4) regulate ovulation and preparation of the uterus for implantation and pregnancy. Medroxyprogesterone 17-acetate (MPA), or its injectable homologue depot medroxyprogesterone acetate (DMPA; commercial name Depo-Provera™), is a first generation synthetic progestin which is a very popular method of injectable hormonal contraception used by more than 100 million women (Population Division, 2003). A number of studies in the past two decades indicate that female sex hormones and hormonal contraceptives may affect susceptibility to HIV-1 or SIV infection. Epidemiological studies have noted a significant increase in HIV susceptibility and transmission with contraceptive usage,
particularly DMPA (Heffron et al., 2012; Polis & Curtis, 2013). Furthermore, studies in non-human primates and mice have consistently found that the administration of DMPA to animals enhances the risk of acquiring sexually transmitted viruses such as HSV-2 and HIV (A. Gillgrass, Chege, Bhavanam, & Kaushic, 2010; A. E. Gillgrass, Ashkar, Rosenthal, & Kaushic, 2003; A. E. Gillgrass, Fernandez, et al., 2005; A. E. Gillgrass, Tang, et al., 2005; P. A. Marx et al., 1996; S. M. Smith et al., 2000; Trunova et al., 2006; Veazey, Shattock, et al., 2003).

Given the lack of understanding of the early events in the FGT following sexual transmission, we investigated the role of female sex hormones and MPA on regulating HIV infection in GECs. Our results show that P4 and in particular MPA increase uptake of HIV-1 and transcytosis, but not replication, across GECs, and that this enhanced transcytosis results in increased infection of HIV target T-cells. These results suggest that P4 and MPA regulate GEC susceptibility to HIV-1, which may have significant ramifications with respect to HIV acquisition in the FGT.
MATERIALS & METHODS

Cells and Viruses

HIV-ADA (R5, macrophage-tropic strain) and HIV-IIIB (X4, T-cell tropic strain) were prepared by infection of adherent monocytes from human PBMCs or from the chronically infected H9 cell line, respectively, as previous described (Nazli et al., 2010). Jurkat T-cells (ATCC, Manassas, VA, USA) were maintained in RPMI media (McMaster University, Hamilton, ON, Canada) supplemented with 10% FBS (Life Technologies Burlington, ON, Canada), 10μM HEPES (Life Technologies), 2μM L-glutamine (Life Technologies) and 100 U/mL penicillin/streptomycin (Life Technologies).

Source of Tissues and Epithelial Cell Preparation

FGT tissues were obtained from women undergoing hysterectomies for non-malignant gynecological purposes at McMaster University Medical Centre in Hamilton, ON, Canada. Written informed consent was received in accordance with the approval of the Hamilton Health Sciences-McMaster University Research Ethics Board. The detailed protocol for isolation, culture and assessment of primary GEC culture purity is described elsewhere (Kaushic, Nazli, et al., 2011; MacDonald et al., 2007). Briefly, pure epithelial sheets lining the endometrium and endocervix were isolated after enzymatic digestion and filtration steps. Approximately $10^5$ GECs were seeded onto Matrigel (BD, Mississauga, ON, Canada) coated tissue culture transwell inserts (BD) and cells were grown until they formed confluent monolayers, as
measured by a trans-epithelial resistance >1 kΩ/cm. The purity of GEC monolayers was between 95 and 98%, with no trace of any hematopoietic cells. E2, P4 or MPA (Sigma-Aldrich, Oakville, ON, Canada) were prepared to the appropriate concentrations in phenol-red free DMEM/F12 (Life Technologies) supplemented with 10 µM HEPES, 2 µM L-glutamine, 100 units/mL penicillin/streptomycin and 5% charcoal-stripped FBS (Life Technologies). GECs were grown in hormone-supplemented media until confluent (~7 days).

**HIV-1 Infection of Primary Genital Epithelial Cells**

Primary GECs, isolated from human endometrial or endocervical tissues were exposed apically to 10^5 IVUs or 2ng/mL p24 antigen of HIV-ADA or HIV-IIIB at 37°C for up to 4 hours. For longer time-points (24 hours or more), after 4 hours of exposure, the inoculum was removed and the cells were acid-treated with cold 1% acetic acid in 0.5M NaCl for 1 minute to remove virus attached to the cell surface, as described before (Vidricaire, Gauthier, & Tremblay, 2007). The cells were then washed with PBS and fresh hormone media was added to the cells. At designated time points, the cells were disrupted using a commercial disruption buffer (ZeptoMetrix Corporations, Buffalo, NY, USA) for protein collection or TRITol (Life Technologies) for nuclei acid extraction. In order to inhibit endocytosis, primary GECs were pre-treated with Dynasore (Sigma-Aldrich) at a concentration of 80 µM for 30 minutes at 37°C (Macia et al., 2006). To ensure the epithelial barrier was protected and that virus crossed GECs specifically due to transcytosis and not paracellular leakage between cells, GECs were pre-treated with anti-human TNF-α
neutralizing antibody (25 µg/ml) (R&D Systems, Minneapolis, MN, USA) (Nazli et al., 2010), or 10 µM colchicine (Sigma-Aldrich) (Parry, Zhang, Koi, Arechavaleta-Velasco, & Elovitz, 2006) for 1 hour prior to virus exposure. To measure HIV-1 within disrupted cells or in cell culture supernatants a commercial HIV-1 p24 antigen ELISA kit was used (ZeptoMetrix Corporations). Supernatants were titred for infectious virus using the TZM-bl cell (ATCC) infection assay, as previously described (Kimpton & Emerman, 1992).

Confocal Microscopy

At designated time points, GECs were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, USA), permeabilized and stained for ZO-1 and immunofluorescent microscopy was performed, as previously described (Nazli et al., 2010). As a positive control for disrupting the mucosal epithelial barrier, GECs were exposed to recombinant TNF-α for 24 hours at 100 ng/ml prior to fixing (R&D Systems).

Real Time RT-PCR for Measuring HIV-1 RNA Splicing

HIV exposed GECs were disrupted and RNA was extracted and purified using an RNeasy Mini Kit (Qiagen, Toronto, ON, Canada). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Real time RT-PCR was performed using primers specific for unspliced, single spliced or multi-spliced HIV-1 RNA, according to a previously published protocol (Table 1) (N. Lund et al., 2011). The housekeeping gene, RPL13a, was selected.
because it was not regulated by female sex hormones (data not shown). All real-time PCR reactions were performed using the 7900 HT Fast Real Time PCR system (Applied Biosystems) and analyzed with SDS version 2.2 software (Applied Biosystems). Jurkat cells that were chronically infected with HIV-IIIB were disrupted and used as a positive control for all PCR assays. All PCR products were run on a 2% agarose gel, stained with EZ-Vision DNA Dye (Amresco, Solon, OH, USA) as loading buffer, and visualized using a UV transilluminator (WEALTEC Corp., Sparks, NV, USA).

TaqMan Alu-Gag Real-Time PCR for Measuring HIV DNA Integration

A previously described real-time nested PCR approach was used to measure the amount of integrated proviral HIV DNA (Suzuki et al., 2003). Briefly, a PCR reaction was conducted using extracted DNA, Taq DNA polymerase (Promega, Madison, WI, USA) and Alu and Gag M661 primers (Table 1). PCR products were subsequently diluted 1:5 and subjected to a real time PCR reaction targeting the HIV-1 R/U5 promoter region using the M667 and AA55 primer pair (Table 1). A fluorogenic probe (Biosearch Technologies, Novato, CA, USA) was used to increase the specificity of the reaction.

TaqMan Real Time PCR for Measuring Reverse Transcription

To measure HIV reverse transcription products, TaqMan real time RT-PCR was performed using Strong-stop, Env, Gag and RPL13a DNA primers and probes, as described previously (Table 1) (Doitsh et al., 2010). 1 µg of DNA was mixed with
12.5µL TaqMan Universal PCR Master Mix (Applied Biosystems), 0.8 µM forward and reverse primers (Mobix McMaster University, Hamilton, ON, Canada), 0.3 µM fluorogenic probe (Biosearch Technologies) and RNase/DNase free H₂O (Life Technologies) for a final volume of 25 µL with the program: 94°C for 10 min, and 40 cycles of 94°C for 15s and 60°C for 1 min.

Electron Microscopy

GECs were exposed to 10⁵ IVUs of HIV for 4 hours, fixed in 4% PFA in PBS and post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at room temperature, dehydrated in ascending concentrations of ethanol, infiltrated and embedded with Spurr’s resin (Sigma-Aldrich). 100nm sections were mounted on 200 mesh copper palladium grids and counterstained with uranyl acetate and lead citrate. Samples were examined with a TEMSCAN 1200 (JEOL, Peabody, MD, USA) at 80 kv.

GEC:T-cell Co-Culture Assay

Primary GECs were grown to confluency with or without hormones and exposed to HIV-IIIB for 4 hours, at which point, the inoculum and basolateral supernatants were removed. Cells were washed with PBS and fresh media was added to the apical side. The transwell was transferred to a new well containing 5x10⁵ uninfected Jurkat T-cells and incubated for 7 days. Supernatants from the basolateral chamber (containing the T-cells) were collected and p24 antigen was measured by ELISA.
Multiplex Cytokine Assay.

Supernatants were collected from cultures of primary GECs grown in E2, P4, MPA or hormone naïve conditions and analyzed for cytokines and chemokines using the 64-Plex Discovery Luminex Assay from Eve Technologies (Calgary, AB, Canada). Analytes included in this panel can be found on [http://www.evetechnologies.com/discoveryAssayListHuman.php](http://www.evetechnologies.com/discoveryAssayListHuman.php).

Statistical Analysis

GraphPad Prism version 5 (GraphPad Software) was used to compare 3 or more means by one-way analysis of variance (ANOVA). When an overall statistically significant difference was measured (p<0.05), a Bonferroni post-test was performed to adjust the p-value for multiple comparisons.
RESULTS

Progestrone, and in particular medroxyprogesterone acetate, increase HIV-1 uptake into primary genital epithelial cells.

In order to determine whether female sex hormones regulate HIV-1 uptake by GECs, primary endometrial GECs were grown in the presence or absence of physiological concentrations of E2 (10^{-9} M), P4 (10^{-7} M) or MPA (10^{-9} M) and exposed to HIV-ADA (M-tropic) or HIV-IIIB (T-tropic). Sex hormone and MPA levels correspond to peak serum levels during the menstrual cycle or following administration of contraceptive, respectively (Abbassi-Ghanavati et al., 2009; Goldman, 2000; Halpern, Combes, Dorflinger, Weiner, & Archer, 2014; Kratz et al., 2004; Stricker et al., 2006). GECs were acid-treated following virus exposure in order to remove any externally attached HIV-1 (Vidricaire et al., 2007). Subsequently, cells were disrupted and HIV uptake was measured by HIV p24 antigen ELISA. At 2 hour post-exposure, uptake of HIV-1 was significantly increased in cells grown in the presence of MPA for HIV-ADA (Figure 1A) and MPA and P4 for HIV-IIIB (Figure 1B). At 24-hours post-exposure, HIV uptake increased, but a significant increase was only measured in cells grown in MPA (Figure 1C, D).

Studies implicate the endocervix as a preferential site for HIV transmission (Carias et al., 2013; Q. Li et al., 2009; Pudney et al., 2005). In order to confirm that HIV uptake was similar in all columnar GECs of the UGT, we measured HIV-1 uptake in endocervical epithelial cells. Our results showed that similar to the endometrial epithelial cells, HIV uptake at 2 hours post-exposure was significantly increased in
endocervical epithelial cells grown in the presence of P4 (ADA) and MPA (ADA & IIIB) (Figure 1E & F).

Next, we performed a dose-response experiment to measure how GEC HIV uptake varied over a range of physiological and supraphysiological concentrations of E2 ($10^{-7} - 10^{-11}$ M), P4 ($10^{-6} - 10^{-10}$ M) and MPA ($10^{-7} - 10^{-11}$ M). Overall uptake of HIV was consistently lower in GECs grown in E2 compared to P4 and MPA (Figure 2). A dose dependent decrease in HIV uptake was observed as E2 concentrations increased. HIV uptake into GECs did not vary much with escalating amounts of P4, but significant uptake of HIV-1 was measured for several MPA concentrations, including known circulating serum levels ($10^{-9}$ M) in women on Depo-Provera (Goldman, 2000; Halpern et al., 2014).

**HIV-1 entry into primary genital epithelial cells takes place primarily via endocytosis.**

Others and unpublished work from our lab have shown that canonical HIV attachment and entry receptors that lead to HIV fusion with the host cell membrane (CD4, CXCR4, CCR5) are poorly or not expressed on human GECs (Berlier et al., 2005; Bobardt et al., 2007; Dezzutti et al., 2001; Saidi et al., 2007; Yeaman et al., 2004). Others have shown that HIV enters/crosses epithelial cells through other means such as endocytosis and transcytosis (Belec et al., 2001; Bobardt et al., 2007; Bomsel, 1997; Carreno et al., 2002; S. Gupta et al., 2013; Hocini et al., 2001; Kinlock et al., 2014; Saidi et al., 2007; Stoddard et al., 2009). Thus, we decided to examine in more detail if the HIV uptake seen in the primary GECs in our studies was due to
endocytosis. When GECs were treated with Dynasore, an inhibitor of dynamin-1, -2 and Drp1-mediated endocytosis (Macia et al., 2006), uptake of HIV-1 was significantly abrogated, with the largest decrease (~80%) measured in cells exposed to MPA (Figure 3A). Electron microscopy data show that internalized HIV localized to endocytic vesicles within primary GECs exposed to HIV for 4 hours (Figure 3B). To determine whether the decrease in HIV uptake following Dynasore treatment could be due to off-target effects, such as effects on cell viability and/or disruption of tight-junction (TJ) networks, we pre-treated cells with Dynasore and measured expression of the TJ protein ZO-1 by confocal microscopy. We found no changes in ZO-1 expression relative to media controls (Figure 3C). These results indicate that endocytosis plays a significant role in HIV-1 entry into primary human GECs and MPA may regulate endocytic uptake of HIV into GECs.

**HIV-1 exposure to primary genital epithelial cells results in a non-productive infection.**

Since we observed that approximately 80% of HIV entry into primary GECs takes place via dynamin-1, -2 or Drp1-mediated endocytosis, we next decided to measure whether this uptake resulted in a productive infection and whether female sex hormones influenced HIV replication. At 24 hours post-exposure, integrated HIV proviral DNA was not measured in primary GECs, regardless of hormone exposure or viral strain, but was measured in chronically infected Jurkat T-cells (Figure 4A). Cells were also tested for HIV proviral DNA 48 and 120 hours post-exposure, but no HIV integration was measured in primary GECs (results not shown). Although we
measured genomic, or unspliced HIV RNA under all conditions in GECs, we were unable to measure single spliced or multi-spliced RNA transcripts in GECs (Figure 4B), which indicate productive infection. Lastly, at 24 (Figure 4C) or 48 hours (data not shown) post-exposure, we were unable to measure early (strong stop DNA), intermediate (Env) or late (Gag) reverse transcription products in GECs exposed to HIV. All together, these results indicate that despite successful entry, HIV does not replicate in primary GECs and female sex hormones or MPA do not influence permissivity to HIV-1 in GECs.

_Progesterone, and in particular medroxyprogesterone acetate, increase transcytosis of HIV-1 across genital epithelial cell._

Polarized epithelial cells are capable of producing unique apical and basolateral secretions (Fernandez et al., 2007; Johansen & Kaetzel, 2011), and certain viruses, such as HSV-2, are known to replicate in GECs and are preferentially shed through the apical compartment (Ferreira, Nazli, Mossman, & Kaushic, 2013; MacDonald et al., 2007; Nazli et al., 2009). In the case of HIV-1, it is not known whether the virus that gets taken up is preferentially shed from a particular pole and whether female sex hormones or MPA influence HIV trafficking. We collected apical and basolateral supernatants from HIV-exposed GECs grown in female sex hormones or MPA. No significant differences were measured in apically recycled HIV-1 in cells exposed to HIV-ADA (Figure 5A) or IIIB (Figure 5B). In contrast, the amount of HIV-ADA in basolateral supernatants of MPA-exposed GECs was significantly greater compared to those seen in basolateral supernatants of cells
grown in hormone naïve conditions (Figure 5C) and HIV-IIIB was significantly higher in basolateral supernatants of GECs grown in P4 or MPA (Figure 5D). Together these results indicate that viral particles taken up via endocytosis are recycled apically as well as transcytosed to the basolateral compartment and that P4 and MPA may enhance HIV transcytosis across the epithelium.

Transcytosed HIV is infectious, amplifies in T-cells and is enhanced in the presence of medroxyprogesterone acetate.

To confirm that the virus detected in apical and basolateral compartments was released via endocytic transport, GECs were pre-treated with Dynasore, which inhibited >99% of p24 released into both the apical (Figure 6A) and basolateral (Figure 6B) supernatants. Next, we decided to confirm HIV transcytosis through primary GECs in these studies was responsible for the presence of virus in basolateral compartments in rather than paracellular leakage via gp120-mediated disruption of the epithelial barrier (Nazli et al., 2010; Nazli et al., 2013). Previous studies have shown that following longer exposures to HIV-1, TNF-α produced by GECs, plays a major role in tight junction disruption and neutralization of TNF-α prevents paracellular leakage of HIV. In spite of TNF-α neutralization, we detected HIV in the basolateral supernatant (Figure 6C). In contrast, no HIV was measured in basolateral supernatants of GECs treated with colchicine, a previously validated inhibitor of HIV transcytosis in GECs (Bomsel, 1997; Hocini et al., 2001; Parry et al., 2006).
To determine whether the transcytosed virus was infectious, we utilized the TZM-b1 indicator cell assay that measures infectious HIV. GEC basolateral supernatants were collected 24 hours post-HIV exposure and incubated with TZM-b1 cells; infectious virus was found in basolateral supernatants under all conditions and we measured an increase in the amount of infectious virus transcytosed by GECs grown in the presence of MPA (Figure 6D). Further confirmation that transcytosed virus was infectious and could replicate and amplify in target cells was obtained by co-culturing GECs with uninfected T-cells. After 7 days of co-culture, we measured a greater than five-fold increase in HIV replication in T-cells co-cultured with GEC grown in MPA (Figure 6E). Together, these results suggest that HIV that is transcytosed across GECs is infectious, capable of replicating in target cells and maximum virus is transcytosed under the influence of MPA.

**GECs enhance production of chemokines but not proinflammatory cytokines under the influence of medroxyprogesterone acetate.**

Work by Carreno et al, showed that transcytosis of R5-tropic HIV particles was increased 2-fold in endometrial epithelial cell line HEC-1A in a proinflammatory microenvironment consisting of LPS, IL-1α and TNF-α (Carreno et al., 2002). Since we observed enhanced transcytosis in P4 and MPA treated GECs, we decided to characterize the inflammatory cytokine profile of our GEC cultures to see whether enhanced transcytosis in the context MPA was concomitant with enhanced proinflammatory factors. Although we measured a significant increase in IL-1α production by GECs exposed to HIV and MPA, relative to hormone naïve conditions,
MPA broadly suppressed proinflammatory cytokines, including TNF-α and IL-6 (Table 2). Thus, our results suggest that enhanced transcytosis by P4 and in particular MPA, takes place independently of a proinflammatory state. Interestingly, MPA induced significant upregulation of the chemokines MCP-1, RANTES and eotaxin-2. Furthermore, while not statistically significant, trends indicated that MPA, and to a certain extent P4, broadly induced chemokine responses. Together, our results suggest that enhanced transcytosis in the context of MPA may be independent of an inflammatory milieu, but that P4, and in particular MPA, may play a role in promoting chemokine induction by primary GECs.
DISCUSSION

We have described in this study the mechanism by which female sex hormones and the hormonal contraceptive MPA regulate early events of HIV infection in the FGT. Our results show that HIV uptake is increased in primary endocervical and endometrial epithelial cells grown in P4 and MPA. Entry into primary GECs occurred primarily via endocytosis and virus was recycled apically and transcytosed through the genital epithelium. In spite of increased uptake, there was no productive infection, and neither viral tropism nor the presence of hormones altered GEC permissivity to HIV. Nevertheless, HIV transcytosis was significantly increased by MPA, which resulted in enhanced infection and replication in T-cells. Lastly, we found that enhanced transcytosis occurred in the absence of a proinflammatory environment, although MPA did increase chemokine responses from GECs.

Our results support controversial animal and human epidemiological studies, which find that hormonal contraceptives such as MPA, increase the risk of acquiring HIV-1 (P. A. Marx et al., 1996; Polis & Curtis, 2013; Trunova et al., 2006; Veazey, Shattock, et al., 2003), and provide insight into the biological mechanism whereby this may occur. HIV uptake by primary GECs as well as transcytosis to basal dwelling T-cells was increased in the presence of MPA. This resulted in enhanced HIV infection and replication in T-cells. We also found that in the presence of MPA, GECs increased production of chemokines that recruit HIV target cells such as macrophages, DCs and CD4+ T-cells (Rancez et al., 2012). Thus our results suggest that in addition to increasing the amount of virus that traverses the epithelial
barrier, MPA may also induce the recruitment of target cells to the genital epithelium, potentially facilitating acquisition. These results, combined with the fact that an estimated 100 million women use MPA-based injectable hormones worldwide and that it is particularly popular in areas where HIV rates remain high (Hel, Stringer, & Mestecky, 2010), underscores the need to better understand the inherent risks of DMPA and to identify effective but safe alternatives to progestin-based contraceptives.

Our results also support the notion that a “window of vulnerability” exists during the menstrual cycle 7-10 days following ovulation, which puts women at greater risk for acquiring HIV than at other times. During this time, aspects of the innate, humoral, and cell-mediated immune systems are suppressed by a P4-dominated microenvironment, which coincides with the recruitment of potentially infectable cells and upregulation of co-receptors essential for viral uptake (C. R. Wira & Fahey, 2008). Further evidence of this comes from a recent study which showed that all 8 of 22 cervical tissue explants found to support productive HIV infection were collected from women during the secretory phase of the menstrual cycle, which corresponds to peak P4 levels (Saba et al., 2013). Our results showed that uptake and transcytosis of HIV-1 by primary GECs was enhanced in the presence of physiological concentrations of P4, including those measured during the secretory phase of the menstrual cycle (Abbassi-Ghanavati et al., 2009; Kratz et al., 2004; Stricker et al., 2006). These results emphasize the importance of using barrier methods during the later stages of the menstrual cycle when P4 levels rise and susceptibility to HIV-1 is likely increased.
Our results suggest that HIV entry into primary GECs from the UGT occurs in a non-canonical fashion primarily via endocytosis. In spite of HIV uptake into GECs, no productive infection was measured including an absence of early reverse transcription products. This varies from recent work published by Micsenyi et al., which found that cervical epithelial cells were productively infected by HIV-1 (Micsenyi et al., 2013), however, in their study, immortalized cell lines were used. Most of the studies which support productive infection of either vaginal, cervical or uterine GECs have been done on transformed (RL95-2, HEC1A, ECC-1, ME180) or immortalized cell lines (Ect-1, VK2, End-1) (Asin et al., 2003; Berlier et al., 2005; Furuta et al., 1994; Micsenyi et al., 2013; Phillips et al., 1994; X. Tan et al., 1993) which arguably have altered physiological characteristics making them less physiologically representative as primary human cell cultures. Only one study has thus far measured productive HIV infection in primary GECs from the UGT and even though Asin et al used the same ex-vivo GEC transwell culture model used by our group (Asin et al., 2004), our study used validated assays that were highly sensitive and specific (Vidricaire & Tremblay, 2005; Wong, Balachandran, Ostrowski, & Cochrane, 2013) to measure productive infection, and in agreement with our findings, several groups have been unable to measure productive infection within primary human GECs, even with doses as high as 250 ng/mL of p24 antigen (Dezzutti et al., 2001; Greenhead et al., 2000; Wu et al., 2003). Although we, and others, have shown that HIV can be transcytosed through GECs including primary human cells (Belec et al., 2001; Bobardt et al., 2007; Bomsel, 1997; Carreno et al., 2002; S. Gupta et al., 2013; Hocini et al., 2001; Kinlock et al., 2014; Saidi et al., 2007;
Stoddard et al., 2009), we are the first to show P4 and MPA-based regulation of transcytosis through primary GECs. Furthermore our results suggest that in the absence of canonical receptors, which mediate fusogenic entry, non-canonical receptors on primary GECs may result in endocytosis and transcytosis in primary GECs. Our preliminary results indicate that neutralization of heparan sulphate moieties on the cell surface partially blocks GEC HIV uptake (*results not shown*).

Lastly, our previous and current results imply that interactions between HIV and GECs may vary under inflammatory or hormone-enriched conditions. Previously, we found primary human columnar epithelial cells of the gut and genital tract directly interacted with HIV-1 gp120 leading to the production of an array of proinflammatory cytokines (Nazli et al., 2010). Among these cytokines was TNF-α, which facilitated disruption of the epithelial barrier, causing bacterial and viral translocation across the epithelium (Nazli et al., 2013). In these studies, HIV was in constant contact with epithelial cells which themselves induced potent inflammatory responses, including TNF-α levels in excess of 1 ng/mL (Nazli et al., 2010). Thus, in the presence of a high viral load and inflammation, paracellular leakage may be the preferred mode of crossing the barrier. In contrast, our current results suggest that in the presence of MPA, small amounts of virus can still cross the barrier by transcytosis, despite a lack of inflammation. The actual cellular mechanism which is responsible for enhanced transcytosis in the presence of MPA still needs to be elucidated, including determination of the steroid receptor used by MPA to exert its effect, since both the progesterone and glucocorticoid receptors have been implicated in MPA-mediated signaling (Hapgood & Tomasicchio, 2010),
whether certain cell-surface receptors are differentially regulated by sex hormones or MPA and whether certain cellular factors directly play a role in facilitating/inhibiting transcytosis.

Our study provides a putative mechanism as to how MPA could enhance susceptibility to HIV. We showed that in the presence of MPA, despite a lack of inflammation and presence of inoculum doses, HIV transcytosed across the genital epithelium and increasingly infected HIV target cells. These results provide valuable knowledge to HIV researchers regarding the early events of HIV transmission in the FGT, which remains a highly controversial, poorly understood, yet important area of HIV-1 research. These results also provide new information regarding the safety of certain hormonal contraceptive formulations, which may have considerable effects on public health measures regarding women's health.
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FIGURE LEGENDS

Table 1 – List of primers and fluorogenic probes used in this study.

Figure 1 – Effect of female sex hormones on uptake of HIV-1 in columnar GECs. Primary endometrial (A-D) and endocervical (E, F) GECs were grown to confluency on transwell inserts in the presence or absence of E2 (10^{-9}M), P4 (10^{-7}M) or MPA (10^{-9}M) and exposed to HIV-ADA (A, E) or HIV-IIB (B, F) at 2ng/mL of p24 antigen. Two hours later, GECs were disrupted and HIV p24 antigen was measured by ELISA. Alternatively, primary endometrial GECs were exposed to HIV-ADA (C) or HIV-IIB (D) and four hours later after which the inoculum was removed, the cells were washed with PBS and replenished with fresh media. At 24-hours post-exposure, primary GECs were disrupted and p24 antigen was measured by ELISA. In all experiments, following the removal of the inoculum the cells were briefly washed cold 1% acetic acid in 0.5 M NaCl for 1 minute in order to remove virus attached to the cell-surface. Statistical significance was evaluated by one-way ANOVA with a Bonferroni post-test to compare all pairs of columns. Data shown represents the mean ± SEM of three separate experiments. All groups were compared to GECs infected in the presence of no hormone (NH + HIV). *p<0.05

Figure 2 – Dose response of females sex hormones and MPA on GEC HIV uptake. Primary endometrial GECs were grown to confluency on transwell inserts in the presence or absence of various concentrations of E2 (A), P4 (B) or MPA (C)
and exposed to HIV-ADA at 2ng/mL of p24 antigen. Two hours later, GECs were disrupted and HIV p24 antigen was measured by ELISA. Statistical significance was evaluated by one-way ANOVA with a Bonferroni post-test to compare all pairs of columns. Data shown represents the mean ± SEM of three separate experiments. All groups were compared to GECs infected in the presence of no hormone (NH + HIV). *p<0.05, **p<0.001

**Figure 3 – HIV is taken up by endocytosis.** Primary GECs from the URT were grown to confluency on transwell inserts in the presence or absence of hormones. Cells were pre-treated with or without the endocytosis inhibitor Dynasore (A) for 30 minutes. Pre-treatment was removed; the cells were washed and subsequently exposed to HIV-ADA at 2ng/mL of p24 antigen. Four hours later, the inoculum was removed; cells were washed with PBS and replenished with fresh media. At 24-hours post-exposure, primary GECs were disrupted and HIV p24 antigen was measured by ELISA. Statistical significance was evaluated by one-way ANOVA with a Bonferroni post-test to compare all pairs of columns. Data shown represents the mean ± SEM of three separate experiments. All treated groups were compared to their untreated counterparts. *p<0.05, **p<0.001. Primary endometrial GECs were exposed to HIV-1 for 4 hours, after which the cells were disrupted and prepared for electron microscopy (B). Results show images taken 5,000x (i), 20,000x (ii) and 60,000x (ii inset). Chronically infected H9 T-cells were used as a positive control in electron microscopy experiments and images shown were taken at 5,000x (iii), 20,000x (iv) and 60,000x (iv inset). Black arrows indicate HIV-1. Lastly, we
performed confocal microscopy to examine the off-target effects of Dynasore (C). At 24 hours post exposure, GECs pre-treated with Dynasore were fixed in 4% PFA and confocal microscopy was performed targeting the ZO-1 tight junction protein. As a positive control, cells were treated with TNF-α (100 ng/mL) for 24 hours and subsequently fixed and stained for confocal microscopy. For each experiment, confocal microscope settings for image acquisition and processing were identical between control and treated monolayers and 3 separate, random images were acquired and analyzed for each experimental condition. Images are presented en face and are representative of one of three separate experiments.

Figure 4 – Effect of female sex hormones on HIV replication in primary human GECs. Primary human GECs grown in the presence or absence of sex hormones were exposed apically to HIV-ADA or HIV-IIIB at 10⁵ IVUs for 4 hours. The inoculum was removed, the cells were acid treated, washed and subsequently replenished with fresh media. At 24 or 48 hours post-exposure, the cells were TRIzol disrupted and RNA and DNA were collected. HIV DNA integration (A) was assessed using a two-step, Alu-Gag TaqMan real time PCR assay. Extracted RNA from primary GECs was used to measure RNA splicing by real time RT-PCR (B). Primers were designed that detected unspliced, single spliced and multi spliced RNA transcripts. Lastly, DNA was collected for the purpose of measuring HIV reverse transcription (C). TaqMan real time PCR was performed using primers and fluorogenic probes targeted against early (strong stop DNA), intermediate (Env) and late (Gag) reverse transcription products. In all experiments, chronically infected H9 T-cells were used as a positive
control and uninfected GECs were used as negative controls. Relative differences in product expression between samples were visualized using gel electrophoresis. Data shown represents the results of at least four separate experiments.

**Figure 5 – Role of female sex hormones and MPA on apical recycling and transcytosis primary GECs.** Primary GECs from the URT were grown to confluency on transwell inserts in the presence or absence of E2 (10^{-9}M), P4 (10^{-7}M) or MPA (10^{-9}M) and exposed to HIV ADA (A and C) or HIV IIB (B and D) at 2ng/mL of p24 antigen. Four hours later, the inoculum was removed; the cells were washed with PBS and replenished with fresh media. At 24-hours post-exposure, apical (A and B) and basolateral (C and D) supernatants were collected and HIV p24 antigen was measured by ELISA. *p<0.05, **p<0.01.

**Figure 6 – Primary human GECs transcytose infectious virus, which results in enhanced infection and replication in T-cells.** To measure the effect of Dynasore on apical recycling or transcytosis, cells were pre-treated with or without dynasore and at 24-hours post-exposure, apical (A) and basolateral (B) supernatants were collected and HIV p24 antigen was measured via ELISA. To determine whether basolateral virus was a result of transcytosis and/or paracellular leakage (C), primary GECs from the URT were grown to confluency on transwell inserts in the presence of E2 (10^{-9}M), P4 (10^{-7}M) or MPA (10^{-9}M), or in hormone naive conditions (NH) and were pre-treated with colchicine or a neutralizing TNF-α prior to being exposed to HIV-ADA at 2ng/mL of p24 antigen. Four hours later, the inoculum was
removed; cells were washed with PBS and replenished with fresh media. At 24-hours post-exposure, primary GECs were disrupted and HIV p24 antigen was measured by ELISA. Furthermore, at 24 hours post-exposure, basolateral supernatants were collected, serially diluted and placed onto TZM-b1 indicator system to measure infectious HIV (D). Lastly, primary GECs were grown to confluency +/- hormones and exposed to HIV-IIIB for 4 hours. After which, the inoculum and basolateral supernatants from each transwell were removed. GECs were washed with PBS and fresh hormone-supplemented media was added to the apical side. The transwell was then transferred to a new well containing 5x10^5 uninfected Jurkat T-cells and incubated for 7 days. Supernatants from the basolateral chamber (containing the T-cells) were collected and p24 antigen was measured using an ELISA. Controls included uninfected co-cultures and a culture containing only epithelial cells and No T-cells (E). Statistical significance was evaluated by one-way ANOVA with a Bonferroni post-test to compare all pairs of columns. Data shown represents the mean ± SEM of three separate experiments. *p<0.05, ***p<0.001.

**Table 2 – Proinflammatory cytokine and chemokine profiles for primary GECs exposed to female sex hormones and HIV.** Supernatants were collected from cultures of primary GECs grown in E2, P4, MPA or hormone naïve conditions and exposed to HIV, and were analyzed for multiple cytokines and chemokines using a 64-Plex assay. Proinflammatory cytokines and indicated by # and chemokines are indicated by *. Statistical significance (p<0.05) was evaluated by one-way ANOVA.
with a Bonferroni post-test to compare all pairs of columns. Data shown represents the mean ± SEM of three separate experiments. All groups were compared to GECs infected in the presence of no hormone (NH + HIV). Green and red numbers indicate statistically significant suppression and upregulation, respectively, relative to controls.
<table>
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<th>Primer Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
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<td><em>Unspliced RNA</em></td>
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<td>5'-CTG AAG CCC GCA CAG CA-3'</td>
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<td><em>Single spliced RNA</em></td>
<td>5'-GGC GGC GAC TGG AAG AAG C-3'</td>
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<td><em>Multi-spliced RNA</em></td>
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<td>5'-AGT CTC TCA AGC GGT GGT-3'</td>
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<tr>
<td><em>RPL13a RNA</em></td>
<td>5'-GGC TGA AGC CTA CCA GAA AG-3'</td>
<td>5'-CTT TGC CTT TCG CTT CCG TT-3'</td>
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<tr>
<td><em>Alu &amp; Gag M661</em></td>
<td>5'-TCC CAG CTA CTC GGG AGG CTG AGG-3'</td>
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<td><em>M667 &amp; AA55</em></td>
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<td>5'-FAM-AAC GAG ACC CTG TCT CAA AA-(BHQ)-3'</td>
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</tbody>
</table>
Figure 2

A.

B.

C.
Figure 3

A. 

![Graph showing bar chart with treatments and comparison between untreated and Dynasore groups.]

B. 

![Micrographs showing different samples labeled as i, ii, iii, and iv.]

C. 

![Images of media, Dynasore, and TNF-α samples.]
Figure 4

A.  

B.  

C.  

Unspliced, 101bp  
Single Spliced, 145bp  
Multi Spliced, 176bp  
RPL13a, 157bp  

Strong Stop  
Env  
Gag  
RPL13a
Figure 5

A.

B.

C.

D.
Figure 6

A. P24 Antigen (Percent of inoculum)

B. P24 Antigen (Percent of inoculum)

C. P24 Antigen (Percent of inoculum)

D. Infectious Viral Units/mL

E. P24 Antigen (pg/mL)
**Table 2**

<table>
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<tr>
<th>Analyte Name</th>
<th>NH + HIV</th>
<th>E2 + HIV</th>
<th>P4 + HIV</th>
<th>MPA + HIV</th>
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<tr>
<td>TNF-α*</td>
<td>305.4 (+ 73.47)</td>
<td>760.6 (+ 196.3)</td>
<td>556.1 (+ 228.5)</td>
<td>14.22 (+ 2.316)</td>
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<td>IL-15*</td>
<td>16.78 (+ 0.8810)</td>
<td>19.57 (+ 1.034)</td>
<td>20.55 (+ 2.064)</td>
<td>13.44 (+ 0.4046)</td>
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<td>IL-1α*</td>
<td>142.8 (+ 30.04)</td>
<td>149.9 (+ 17.52)</td>
<td>90.71 (+ 29.66)</td>
<td>1968 (+ 274.3)</td>
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<td>IL-1β*</td>
<td>2.648 (+ 0.2722)</td>
<td>3.045 (+ 0.2001)</td>
<td>2.390 (+ 0.2684)</td>
<td>2.328 (+ 0.02750)</td>
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<tr>
<td>IL-1RA*</td>
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<td>163.0 (+ 3.592)</td>
<td>79.78 (+ 4.724)</td>
<td>90.88 (+ 0.8202)</td>
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<td>IL-6*</td>
<td>6940 (+ 1912)</td>
<td>10000 (+ 0)</td>
<td>2319 (+ 112.6)</td>
<td>88.89 (+ 0.4939)</td>
</tr>
<tr>
<td>G-CSF*</td>
<td>2950 (+ 79.82)</td>
<td>7369 (+ 1596)</td>
<td>6708 (+ 2493)</td>
<td>511.9 (+ 41.97)</td>
</tr>
<tr>
<td>GM-CSF*</td>
<td>770.7 (+ 132.7)</td>
<td>1431 (+ 143.4)</td>
<td>988.7 (+ 313.8)</td>
<td>68.10 (+ 2.369)</td>
</tr>
<tr>
<td>IL-8*</td>
<td>7237 (+ 883.0)</td>
<td>3620 (634.9)</td>
<td>11406 (1371)</td>
<td>13552 (443.4)</td>
</tr>
<tr>
<td>IP-10*</td>
<td>258.1 (+ 82.93)</td>
<td>213.2 (+ 33.37)</td>
<td>358.3 (+ 102.1)</td>
<td>678.7 (+ 54.77)</td>
</tr>
<tr>
<td>MCP-1*</td>
<td>1908 (+ 126.1)</td>
<td>4414 (+ 1333)</td>
<td>9321 (+ 687.6)</td>
<td>11738 (+ 1738)</td>
</tr>
<tr>
<td>MIP-1α*</td>
<td>42.90 (+ 6.525)</td>
<td>47.68 (+ 1.412)</td>
<td>33.46 (+ 4.934)</td>
<td>68.08 (+ 7.516)</td>
</tr>
<tr>
<td>MIP-1β*</td>
<td>15.18 (+ 1.385)</td>
<td>15.00 (+ 0.2771)</td>
<td>15.03 (0.4618)</td>
<td>37.33 (4.345)</td>
</tr>
<tr>
<td>MIP-2β/GRO*</td>
<td>18024 (+ 2792)</td>
<td>27288 (+ 4585)</td>
<td>18451 (+ 446.7)</td>
<td>775.0 (+ 91.37)</td>
</tr>
<tr>
<td>RANTES*</td>
<td>36.30 (+ 7.408)</td>
<td>103.2 (+ 13.30)</td>
<td>73.72 (+ 26.18)</td>
<td>208.4 (+ 136.4)</td>
</tr>
<tr>
<td>Eotaxin-1*</td>
<td>24.99 (+ 0.8703)</td>
<td>27.59 (+ 1.875)</td>
<td>20.48 (1.200)</td>
<td>21.88 (+ 0.2949)</td>
</tr>
<tr>
<td>Eotaxin-2*</td>
<td>51.91 (+ 1.076)</td>
<td>53.39 (+ 1.757)</td>
<td>55.56 (+ 5.610)</td>
<td>109.7 (+ 6.635)</td>
</tr>
<tr>
<td>Fractalkine*</td>
<td>849.1 (+ 152.3)</td>
<td>73.10 (+ 10.18)</td>
<td>1072 (+ 179.6)</td>
<td>1638 (+ 584.6)</td>
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Chapter 6 – Discussion
Although women constitute half of all people living with HIV/AIDS globally, little is known about the early events of HIV-1 infection in the FGT, where 40% of all new infections are established each year. Inflammation has been associated with increasing HIV replication and facilitating HIV pathology, but it is unknown what role it plays with respect to HIV infection in the FGT. Two principal genital tract factors associated with initiating or regulating inflammation include sexually transmitted co-infecting microbes, such as HSV-2 and *N. gonorrhoeae*, and female sex hormones. Previous studies showed that GECs respond to *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* and HSV-2 with an array of cytokines and chemokines, including TNF-α, IL-6 and IL-8 (Drannik et al., 2013; Fichorova et al., 2001; Fichorova et al., 2006; H. Li et al., 2009; H. Liu et al., 2013; S. J. Rasmussen et al., 1997; Triantafilou et al., 2014). With respect to female sex hormones, although limited primary GEC data is available, studies suggest that E2 induces inflammatory responses (J. V. Fahey et al., 2008; Lesmeister et al., 2005) whereas data on P4 and MPA-mediated responses is limited and conflicting (Africander et al., 2013; Govender et al., 2014; Lesmeister et al., 2005; Patel et al., 2013; C. R. Wira et al., 2014).

Although it has been postulated that sexually transmitted co-infections and female sex hormones can regulate inflammatory responses and thus, impact HIV infection, the underlying mechanisms of these interactions is incompletely understood. The work undertaken in this thesis determines and characterizes how GEC innate inflammatory responses to co-infecting microbes and female sex hormones impact HIV infection and replication in the FGT.
HSV-2 is a common HIV co-infecting agent, and as such, we were interested in characterizing the GEC innate immune responses against HSV-2 and to determine the viral factors that influenced these responses. In response to HSV-2, GECs upregulated innate immune responses consisting of elevated proinflammatory cytokines and chemokines in addition to biologically active IFN-β. Furthermore, our results showed that these responses required potent viral HSV-2 replication and that proinflammatory cytokine and chemokine responses were enhanced in the absence of the HSV-2 vhs protein. Thus, we found that GECs are uniquely sensitive to HSV-2 and responded by producing proinflammatory innate responses.

Next, we determined whether GEC innate immune responses to bacterial and viral co-infecting microbes enhanced HIV-1 replication in T-cells. As part of our work, we identified that common sexually transmitted co-infecting microbes (HSV-1, HSV-2, N. gonorrhoeae) and the TLR ligands FimH (TLR-4), flagellin (TLR-5), and poly (I:C) (TLR-3), representative of various bacterial and viral STIs, directly induced HIV-LTR promoter activation in T-cells. Other studies have previously identified that co-infecting microbes can also directly induce HIV replication in macrophages or T-cells (A. Chen et al., 2003; Ding et al., 2010; Golden et al., 1992; Schafer et al., 1996). With respect to our hypothesis, we found that supernatants collected from GECs exposed to co-infecting microbes or TLR ligands also indirectly induced HIV-LTR promoter activation in T-cells. Production of TNF-α, IL-6, IL-8 and MCP-1 was elevated in GECs exposed to co-infecting microbes. Interestingly, when NFκB and AP-1 signalling pathways were impaired in T cells, both direct and indirect HIV-LTR activation was abrogated. Thus, our studies suggest that co-
infections indirectly increase HIV replication in T-cells via the induction of GEC inflammation.

Next we examined a translational aspect of the aforementioned studies by examining whether blocking inflammatory pathways, using the broad anti-inflammatory compound curcumin, could provide prophylactic or therapeutic protection against HIV. We identified that curcumin pre-treatment protected the genital epithelial barrier against HIV-1-mediated disruption and inflammation, prevented the gp120-mediated upregulation of chemokines by GECs that recruit HIV target cells to the FGT, blocked GEC innate inflammatory responses and indirect activation of the HIV-LTR promoter in T-cells and decreased both HIV-1 and HSV-2 replication. Together, our results suggest that by blocking inflammatory pathways in primary human GECs, using curcumin, we could decrease inflammation, protect the mucosal barrier against HIV and decrease HIV replication in T-cells and HSV-2 replication in GECs.

Lastly, we explored whether female sex hormones regulated HIV-1 infection and replication in primary human GECs and whether inflammation was an important component of this regulation. We hypothesized that GECs grown in the presence of P4 and in particular MPA, would upregulate proinflammatory molecules such as TNF-α, IL-6 and IL-1α, that would facilitate HIV uptake and result in productive infection of primary human GECs, whereas E2 would decrease the levels of inflammatory molecules, thus protecting primary human GECs from HIV infection/replication. Our results suggest that this hypothesis was partly incorrect. Although we measured a significant increase in IL-1α production by GECs exposed
to HIV and MPA, relative to hormone naïve comparators, MPA broadly suppressed proinflammatory cytokines, including TNF-α and IL-6. In contrast, proinflammatory cytokines were generally upregulated by infected GECs grown in E2, though only RANTES was statistically upregulated relative to hormone-naïve controls. Despite the lack of induction of inflammation, we observed that P4 and in particular MPA increased uptake and transcytosis of HIV-1 across GECs, but that GECs were not productively infected, regardless of viral tropism (R5 vs X4) or presence of sex hormones. However, the enhanced transcytosis under the influence of MPA did result in increased infection of HIV target cells. Since GECs grown in an MPA-rich environment were most adept at transcytosing HIV-1, it can be inferred from our results that P4 and MPA mediate HIV transcytosis across primary human GECs independently from a proinflammatory state.

The results summarized in this thesis have significantly improved our understanding of how GECs interact with HIV. With respect to inflammation, past studies stopped short of identifying that co-infections induce inflammation, and inflammation is associated with increasing HIV replication, and speculating that therefore, co-infections must increase HIV replication by inducing inflammation. For instance, a recent study published by Rollenhagen et al reported greater levels of HIV-1 reverse transcription, DNA integration, RNA expression and virion release in HIV-1/HSV-2 co-infected ectocervical tissues compared with HIV-1 only infected tissues (Rollenhagen, Lathrop, Macura, Doncel, & Asin, 2014). In that paper, they discussed that HSV-2-mediated inflammation could be a means by which this enhancement may have taken place, but the study failed to explore this mechanism.
To our knowledge, we are the first to provide a mechanism by which GEC STI-mediated inflammation may increase HIV replication, specifically in the FGT. Our results provide evidence that GEC inflammation, although important to recruiting immune cells and neutralizing microbes to the genital tract (Ferreira et al., 2014; Kaushic et al., 2010; Nguyen et al., 2014; C. R. Wira, Fahey, et al., 2005), may also play a pathogenic role in the context of HIV by inducing replication in T-cells.

Previous in vitro studies have shown that curcumin can directly inhibit HIV replication in susceptible cells (Gandapu et al., 2011; C. J. Li et al., 1993), inhibit HIV-LTR activation (Barthelemy et al., 1998; Ferreira et al., 2011; Gandapu et al., 2011; C. J. Li et al., 1993), block HIV integrase and protease activity (Burke et al., 1995; Mazumder, Raghavan, Weinstein, Kohn, & Pommier, 1995; Sui, Salto, Li, Craik, & Ortiz de Montellano, 1993), enhance HAART activity (Riva et al., 2008), inhibit acetyltransferases needed for post-translational modification of HIV DNA (Balasubramanyam et al., 2004), inhibit histone deacetylase/NFκB activation (H. S. Zhang, Ruan, & Sang, 2011) and gp120-mediated neurotoxicity in rats (Guo et al., 2013). Previous studies have also shown that curcumin can block HSV-1 and HSV-2 replication in epithelial cell lines (Bourne, Bourne, Reising, & Stanberry, 1999; Kutluay, Doroghazi, Roemer, & Triezenberg, 2008; Zandi et al., 2010). Our work in primary GECs further expands and supports these studies by showing, for the first time, that by preventing the gp120-mediated induction of GEC cytokines, such as TNF-α, curcumin protects the mucosal epithelial barrier. Furthermore, not only are we the first to describe the mechanism by which co-infection mediated inflammation indirectly induces HIV replication in T-cells, we are also the first to
show that by blocking inflammatory pathways in primary GECs, you can prevent indirect induction of HIV replication.

We are the first, to our knowledge, to describe the putative cellular mechanism of how female sex hormones or hormonal contraceptives regulate HIV acquisition in the context of GECs. Previous studies identified the relationship between acquisition of HIV-1 and physiological levels of different endogenous and exogenous sex hormones but did not identify the cells or molecules that may be involved in this pathway. Our study used primary human GEC cultures, grown specifically in the presence of physiological levels of E2, P4 and MPA and attempted to explain how these hormones influenced the interactions between GECs and HIV and whether other components, mainly inflammation, played a role in regulating these interactions. Based on these results, we can infer that GECs from the UGT, such as endocervical or endometrial epithelial cells, are capable of capturing HIV-1 and that in the presence of certain hormonal environments (P4 or MPA), women may be at higher risk for acquiring HIV-1 infection.

**Implications of This Study**

Our epithelial cell hormone results provide context on the mechanism by which HIV is acquired in the FGT. There is tremendous controversy regarding whether HIV is acquired in the LGT, where post-coital microabrasions or specialized cells such as DCs expressing DC-SIGN may facilitate transmission, or whether the virus enters the body in the UGT. While we did not directly compare LGT GECs like vaginal epithelial cells and UGT GECs, our study did show that both endocervical and
endometrial GECs were capable of non-productively taking up HIV-1 and transcytosing it. Other studies have shown that SIV may preferentially invade through the endocervix of the UGT (Q. Li et al., 2009) and that HIV-1 can penetrate both intact human cervical columnar and squamous epithelial barriers to depths where potential target cells reside (Carias et al., 2013). Furthermore, since there is a rich population of CD4+ T-cells that reside in the cervical transformation zone (Pudney et al., 2005), there is increased likelihood that HIV may cross in the UGT. There is also evidence that in rhesus macaques the ovary may be one of the hot spots for viral entry (Hope, 2013). Thus, our results support the view that acquisition could take place in the UGT, via transcytosis across the columnar epithelium of endocervix and that female sex hormones and MPA may significantly influence this process.

Others had previously shown that HIV transcytosed through GECs, but these experiments were mostly performed on transformed or immortalized epithelial cells (Belec et al., 2001; Bomsel, 1997; Bomsel et al., 1998; Carreno et al., 2002; S. Gupta et al., 2013; Hocini et al., 2001; Kinlock et al., 2014; Saidi et al., 2009; Saidi et al., 2007; Tudor et al., 2009) but no study took the role of female sex hormones into consideration. One previous study reported that X4-tropic HIV-1 transcytosed through primary endocervical GECs with poor efficiency, with approximately 0.02% of the inoculum getting through (Bobardt et al., 2007). In comparison, we find that approximately 0.25-2.5% of HIV-ADA and 4-10% of HIV IIIB is transcytosed through primary endometrial GECs of the UGT. These differences are likely not explained by differences in inoculum dose since the aforementioned study exposed their cells to a
dose 2.5 times greater than ours. Two possible explanations for the differences observed include the use of different viral strains between studies, as well as the fact that their study was performed on endocervical cells and ours was performed on endometrial GECs.

While our results provide a cellular mechanism by which acquisition takes place in the UGT, it does not rule out infection in LGT; it is likely that HIV-1 has multiple ways of entering. From an evolutionary standpoint of successful infection, there may be multiple, means by which HIV may enter the body. Therefore, future modalities that attempt to impair the sexual transmission of HIV-1 should focus on the entire FGT. For instance, mucosal vaccines should be equally concerned with eliciting strong neutralizing antibody responses in the vagina as well as in the cervix, uterus and even ovaries, as unpublished work from Dr. Tom Hope’s lab at Northwestern University suggests that following sexual exposure SIV may reach that far into the UGT (Hope, 2013).

Our finding that women using progestin-based formulations, such as MPA, may be at greater risk for acquiring HIV from an infected partner fall in line with a recent clinical study by Heffron et al (Heffron et al., 2012). In this study, nearly 3,800 serodiscordant couples from seven African nations were prospectively followed and investigators found that the risk of acquiring HIV from an infected male partner was twice as high among women who used injectable hormonal contraceptives, such Depo-Provera, which contains MPA. These results have tremendous significance from a public health point of view. It has been estimated that more than 100 million women use MPA-based injectable hormones worldwide
and this form of contraception is particularly popular in developing nations where rates of HIV-1 remain high (Hel et al., 2010). Injectable hormones are cheap, have higher adherence rate and empower women who generally may not have the ability to negotiate their reproductive or sexual rights (Hel et al., 2010). MPA has been identified as a contraceptive with potentially unwanted properties, including bone mineral density loss (Wooltorton, 2005) and increased risk of acquiring STIs, such as HIV-1 and HSV-2 (Kaushic et al., 2003). However, contraceptive drugs are crucially important for reducing maternal and child mortality rates. Herein lies the dilemma: do the significant benefits of MPA outweigh its risks? This question has garnered significant attention and discussion regarding hormonal contraceptives, subsequently leading to calls from WHO and UNAIDS for more research on hormonal contraceptives and how they affect risk for HIV-1 infection (UNAIDS, 2011a). Our results add to the growing breadth of literature that suggests that clinicians should be looking for alternatives to Depo-Provera. Unfortunately, while a the large majority of studies have focused on Depo-Provera, very few studies have thus far attempted to evaluate other safe, affordable and effective contraceptives for women to use. Therefore, future studies need to take into consideration that in addition to identifying a problem, a solution must be found since millions of women rely on safe and affordable contraceptive techniques.

One of the most informative aspects of this work was identifying the role indirect inflammation could play in enhancing HIV infection in the FGT and even more uniquely that curbing inflammation could have a multitude of beneficial effects. As such, our results suggest that to stop the burden of co-infection mediated
enhancement of HIV, you may need more than just conventional antiviral or antibiotic treatments. Although many studies have identified a relationship between STIs such as herpes, gonorrhea, syphilis and chlamydia with increasing HIV infection, replication or accelerating disease progression, therapeutic or prophylactic antivirals or antibiotics, have for the most part, failed to have an impact on HIV (C. Celum et al., 2010; Kaul et al., 2004; D. H. Tan, Murphy, Shah, & Walmsley, 2013). Our results show that in response to intact co-infecting microbes, GECs induce potent inflammation that can indirectly drive HIV-LTR promoter activity in T-cells. Furthermore, we observed that PAMPs, including bacterial FimH and flagellin, might be sufficient to induce potent inflammatory responses that indirectly drive HIV replication. These results suggest that intact or actively dividing microbes may not be needed in order to enhance HIV replication and that microbial antigens may be sufficient to induce inflammatory responses. Thus, conventional antiviral or antibiotic therapies may not be sufficient to reduce co-infection mediated induction of HIV because allowing a co-infecting virus or bacteria to enter the body may be sufficient to induce inflammation that drives HIV replication. Future studies should investigate whether the addition of anti-inflammatory compounds like curcumin to conventional antimicrobials like antibiotics may have a beneficial role in reducing the unwanted inflammation that contributes to HIV infection and replication.

Our results indicate the promising potential of anti-inflammatory compounds such as curcumin, which protect the mucosal barrier and/or limit inflammation, unlike current ARV therapies that target infection or replication. Inflammation may contribute to the acquisition or spread of HIV-1 infection, as well as contribute to
the sequelae of disease associated with chronic HIV infection. Thus a paradigm shift toward limiting inflammation in the genital tract and/or systemically, may have a significant impact on HIV infection and disease progression. This is particularly true since our results showed that pre-treatment with curcumin prevented disruption of the mucosal barrier by HIV-1 gp120 and abrogated the concomitant upregulation of the proinflammatory cytokines TNF-α and IL-6, suggesting that curcumin may have significant benefits with respect to reducing HIV acquisition, abrogating the genesis of immune activation and blocking the inflammatory milieu associated with HIV amplification.

**Limitations**

While these results provide valuable information regarding the early events of HIV pathogenesis in the FGT and the safety of hormonal contraceptives, there are a number of limitations to our study that must be taken into consideration. Firstly, our primary GEC model is an *ex vivo* model and as such it does not take into account the complex interactions that take place in the tissue environment as it is composed of a single cell type. Although we have attempted to use a primary GEC model that would most closely mimic *in vivo* settings, the reality is that there are a number of *in vivo* factors that may alter our results inside the body including the effects of other cell types, seminal plasma, mucus and commensal bacteria. While it may be more advantageous to perform such studies on pieces of intact genital tract tissue or in small-animal models to gain a more complex view of what’s happening, single-cell models such as the one used in our study are nevertheless important as they are
useful in conducting mechanistic studies and identifying underlying mechanisms that must be understood in order to identify potential targets for therapies.

While we have decided to use hormone levels associated with peak serum concentrations, tissue levels of these hormones may actually exceed that with which we used to perform our studies. We still have an incomplete understanding of hormone concentration levels in the actual genital tract during the menstrual cycle and so much of our work has to be based on hormone levels in the blood. Studies in female pigs have shown that blood collected from arteries proximal to the oviduct and uterus contained sex hormone levels up to 69% greater than those measured in distal blood supply, (i.e. the jugular vein) (Stefanczyk-Krzymowska, Grzegorzewski, Wasowska, Skipor, & Krzymowski, 1998), suggesting that genital tract hormone levels may be higher than serum blood levels since sex hormone are produced in the vicinity. Also, in vivo, E2 and P4 are not found in isolation and usually both hormones are present throughout the cycle, albeit at different concentrations (Marieb, 2012). Thus, our results should be viewed cautiously since they do not take the complexity of hormone cycling into consideration. Future studies examining the role of sex hormones on regulating various immune parameters, such as susceptibility to STIs, will need to develop models where multiple hormones are present, but as previously mentioned, this requires a better understanding of tissue hormone concentrations.

Although many promising effects of curcumin have been observed in patients with various proinflammatory diseases including cancer, cardiovascular disease, arthritis, Crohn’s disease, ulcerative colitis, irritable bowel disease, psoriasis,
diabetes and acute coronary syndrome, atherosclerosis and diabetes (S. C. Gupta et al., 2013), a clinical trial examining the effectiveness of curcumin as an anti-viral agent in 40 AIDS patients failed to find any therapeutic benefits. No evidence of curcumin-associated reduction in viral load was observed over the course of the 8-week trial, however, CD4 cells showed a slight increase in the high-dose group and a consistent decrease in the low-dose group (James, 1996). One of the main reasons why this study failed is likely due to curcumin's poor bioavailability. Curcumin is poorly absorbed, rapidly metabolized/glucuronidated and systemically eliminated following enteral/oral administration (Anand et al., 2007). As a result, numerous efforts have been made to improve curcumin’s bioavailability by altering these features. The use of compounds that can block the metabolic pathway of curcumin is the most common strategy for increasing the bioavailability of curcumin, such as using piperine, a known inhibitor of hepatic and intestinal glucuronidation, which increased the bioavailability of curcumin in healthy human volunteers by 2,000% (Shoba et al., 1998). Other promising approaches to increase the bioavailability of curcumin in humans include the use of nanoparticles (Sasaki et al., 2011) or liposomes (Basnet et al., 2012; Berginc, Skalko-Basnet, Basnet, & Kristl, 2012; Gota et al., 2010). We have established an international collaboration with Dr. Natasa Skalko-Basnet from the University of Tromsø in Norway to evaluate the efficacy of curcumin-loaded liposomes on protecting humanized mice against HIV-1. Furthermore, while we have evidence that curcumin protects the mucosal barrier, our evidence is based on in vitro work and future steps should be performed to examine what effects curcumin has in vivo.
Other reasons why the previous clinical trial with curcumin failed to find any therapeutic benefits could be that the study was conducted on AIDS patients who may have already been past the point where curcumin would have been helpful. Furthermore, the curcumin AIDS study did not examine whether curcumin had any effect on chronically infected individuals who had not reached the clinical stage of AIDS, and whether the compound had any role in decreasing chronic immune activation. The study was conducted at a time when HAART treatment was just starting to become common clinical practice. It remains to be seen whether the anti-inflammatory and replication suppressing roles of curcumin can work in tandem with conventional HAART therapy to provide better outcomes for HIV patients. Furthermore, the curcumin AIDS clinical study was not designed to examine whether curcumin had any prophylactic benefits in preventing HIV infection in the first place and when one considers the results of our study, it suggests that perhaps prophylactic applications of curcumin may be a novel and effective means of preventing HIV infection at mucosal surfaces.

Although we propose blocking anti-inflammatory pathways to protect the barrier, decrease inflammation and thus, abrogate HIV replication, there may be a number of undesired effects from doing so. It goes without saying that some basal level of inflammation is necessary so whenever dealing with anti-inflammatory compounds, those that block unwanted inflammation, while still enabling baseline inflammation may be the most attractive candidates. Interestingly, curcumin pre-treatment blocked the gp120-mediated induction of proinflammatory cytokines; it did not completely abrogate inflammatory cytokine and chemokine production.
Further studies are needed to evaluate curcumin to determine whether there are any off-target effects, for example will curcumin pre-treatment make one more susceptible to other types of infections with non-inflammatory etiologies, and what kind of an impact will it have on antigen presentation, T-cell activation, AMP production, as well as other essential body pathways that require inflammation?

Finally, with respect to using curcumin as a microbicide, one unusual limitation from the study may come from the fact that following curcumin-exposure, GECs are stained brightly yellow for 24-48 hours post-exposure. This raises the question about whether women will want to use a microbicide that leaves behind some unwanted physical effects. In the context of a genital microbicide, this raises questions of comfort/sexual desire for the sex partners involved and may bring attention to the fact that one is using the microbicide, thus affecting privacy and discreetness. While this may seem like a rather trivial issue compared to the benefit of an efficacious microbicide, the issues surrounding compliance for the use of microbicides based on convenience and aesthetics to women is well known, based on previous lessons learned from female condoms, which are highly effective, but rarely used due to issues of discomfort, diminished pleasure and lack of discreetness. (Hillier, 2013). Thus, if curcumin is ever going to be used as an effective genital or possibly rectal microbicide, we will likely need to find a way of quenching its colour without getting rid of its bioefficacy, and we may also want to consider including it in items such as personal lubricants to increase higher user accessibility.

**Future Directions**
These results provide a number of promising directions for future research. Firstly, while we have identified that P4 and MPA regulate GEC uptake of HIV-1, future studies need to explore the mechanism by which this takes place. Past studies have shown that HIV transcytosis across primary endocervical epithelial cells is mediated by cell surface gp340 (Stoddard et al., 2009). Interestingly, previous work from our lab indicates that primary endometrial epithelial cells express gp340. Although we examined transcytosis, we did not describe the cell-surface interactions that take place that are required for HIV transcytosis. Future studies will need to examine whether gp340 is differently expressed under the influence of sex hormones, such as P4 or MPA, as well as whether gp340, or some other non-canonical receptor(s), is/are responsible for mediating entry and transcytosis in our model.

Furthermore, it would be valuable to explore whether MPA exerts its effects through the PR, or whether it utilizes other hormone receptors, such as the GR, which it has been shown to bind to at high affinity in other studies (Africander et al., 2011; Hapgood & Tomasicchio, 2010; Kemppainen et al., 1999; Koubovec et al., 2005; Philibert et al., 1999). In light of the fact that MPA may lead to increased susceptibility to HIV-1, it would also be beneficial to examine whether other progestin-based contraceptives, such as Net-A or combined injectable contraceptives, such as Cyclofem and Lunelle (which are monthly injections of both MPA and estradiol), have similar roles in regulating susceptibility to HIV-1.

One of the more ideal ways to answer this question would be to perform a randomized control trial (RCT) where at-risk women would be divided into
different groups, placed on different hormonal formulations and followed to
determine whether there is a direct relationship between acquisition and
contraceptive use. Conducting an RCT of hormonal contraception, however, would
not be easy. For one thing, it is not clear that it is ethical – certain contraceptives
really are more appropriate for certain women than others. Beyond this, a woman
may very well go and obtain her preferred contraceptive and thus it would be very
hard to assure that the original, randomized groups of contraceptive users actually
continued throughout the duration of the study (C. Morrison, 2013). Furthermore, a
number of confounders exist for such a study that would make data interpretation
difficult. For example, accurate measurements of frequency of sexual activity,
number of partners, infection status of partners, and type of sexual contact are very
difficult to measure accurately – and even more difficult to be confident of
measuring accurately (C. Morrison, 2013). Questions of this kind are probably
subject to “courtesy bias” from study participants. In other words, participants
asked about condom use, for example, or number of sexual partners may feel some
pressure to respond with the “right” answers – that is, the answers they believe the
researchers wish to hear. In such cases, participants may respond that they use
condoms regularly, or only have one sexual partner, even when this is not the case
(Gynuity Health Projects, 2005). In lieu of an RCT, alternatives include observational
clinical studies, or studies in which humanized mice or non-human primates are
used to study effect of hormonal contraceptives on HIV acquisition.

One of the most promising applications of curcumin may be as a genital tract
microbicide against HIV-1 and co-infections, including HSV-2. Curcumin protected
the mucosal barrier, decreased inflammation and blocked HSV-2 replication in primary GECs following direct application to cells in culture. These results suggest that direct mucosal application may prevent the degradation associated with taking curcumin orally or systemically. Based on our observation, an ongoing collaboration with Dr. Keith Fowke at the University of Manitoba will be evaluating the efficacy of time-release vaginal rings containing curcumin for the purpose of preventing HIV-1 infection in women. Ongoing studies in our lab are planning to evaluate the microbicidal potential of curcumin in humanized mice. In this model, we can mucosally expose the mice to curcumin for various time points and then infect with HIV and explore a number of different parameters such as the kinetics of protection, determine a dose-response curve and evaluate how curcumin influences the local immune cell populations, vaginal flora and the reproductive health of the animals. Furthermore, based on the results of our studies, future studies should investigate the role of curcumin on protecting the mucosal barrier against HIV-1 and preventing the immune activation associated with chronic HIV disease.

**Concluding Remarks**

Approximately half of all people living with HIV/AIDS are women and it is estimated that the FGT is the main site of HIV acquisition globally, accounting for 40% of all new HIV infections. In spite of this information, very little is known about the early events of HIV pathogenesis in the FGT and how endogenous or exogenous factors associated with regulating HIV infection *in vivo*, such as female sex hormones, hormonal contraceptives, sexually transmitted co-infections and inflammation,
affect HIV infection and replication at this site. Thus, there is a tremendous need for studies to unravel these questions in order to develop novel, and effective therapies against HIV.

The studies described in this thesis are the first to describe a biological-level explanation of how female sex hormones or hormonal contraceptives regulate HIV acquisition in the context of GECs. Not only does this provide valuable information regarding the early events that take place in the FGT following heterosexual transmission of HIV-1, but it also provides important public health information regarding the safety of hormonal contraceptives such as Depo-Provera and provides information regarding the window of vulnerability during the menstrual cycle that may place women at greater risk for acquiring HIV-1.

Our work has further characterized GEC inflammatory responses to common co-infecting microbes such as HSV-1, HSV-2 and *N. gonorrhoeae* and we are the first to systematically describe a mechanism by which these GEC inflammatory responses can induce HIV replication in T-cells. As a continuation of these findings, we also explored the potential prophylactic and therapeutic benefits of the anti-inflammatory compound curcumin on protecting the genital mucosa against HIV-1. Our work suggests that anti-inflammatory compounds such as curcumin may provide paradigm-shifting benefits that change the way we treat people living with HIV-1 or prevent HIV infection.

As a whole, this work not only provides answers to questions that have been lingering in the HIV medical literature for many years, but it also provides
mechanisms and suggests future steps and applications of the work that may have significant clinical implications.


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Proteins, called envelope proteins, embedded in the outer membrane of the HIV virion bind to receptors on the surface of target cells. T-cells (white blood cells) have CD4 and the chemokine co-receptors CCR5 and CXCR4, to which HIV can bind. Binding of the HIV envelope protein to CD4 and chemokine co-receptors allows the HIV-1 outer membrane to fuse with the cell's outer membrane and the contents of the virus particle to enter the cell. There are two strands of RNA in each HIV-1 virus particle. An enzyme known as reverse transcriptase initiates the formation of double-stranded DNA by copying the sequence of the RNA strands contained in the virus particle. The viral DNA enters the nucleus of the host and becomes integrated into the host's DNA. An enzyme called integrase is key in this process. Once the viral DNA has integrated into the cell's DNA, the cell is infected for the remainder of its life. The integrated viral DNA is now referred to as a provirus. The provirus DNA serves as a template for the creation of new viral RNA via transcription. The host cells own machinery that is normally used for the transcription of human genes is co-opted by the virus to create new viral RNA molecules. The newly formed viral RNA moves out of the infected cell's nucleus. The viral RNA carries code for the synthesis of viral proteins and enzymes. The code is translated into protein and enzyme components of new virus particles. Components that are required to build new virus particles, namely viral proteins, enzymes and genetic material (viral RNA) move to the cell's outer membrane where they accumulate and assemble in the form of a bud. A variety of host cell proteins are recruited to assist in virus assembly. Host-cell proteins cut the virus bud from the cell's outer membrane, thereby releasing a new virus particle. During and after assembly and release, a viral enzyme called protease cuts the HIV polypeptide chains at several positions, in a process called maturation, to make the finished components of the new, infectious, virus particle.
Appendix B - The Menstrual Cycle

Stimulated by gradually increasing amounts of E2 in the follicular phase, menses slows then stops, and the lining of the uterus thickens. Follicles in the ovary begin developing under the influence of a complex interplay of hormones. Approximately mid-cycle, 24–36 hours after the luteinizing hormone (LH) surges, a follicle releases an ovum, or egg, in an event called ovulation. After ovulation, the remains of the dominant follicle in the ovary become the corpus luteum, which primarily functions in producing large amounts of P4. Under the influence of P4, the endometrium (uterine lining) changes to prepare for potential implantation of an embryo to establish a pregnancy. If implantation does not occur within approximately two weeks, the corpus luteum will involute, causing sharp drops in levels of both P4 and E2, leading to the shedding of the uterine lining and menstruation.
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