IMMUNE RESPONSES TO SEMINAL TGF-BETA 1

THE ROLE OF SEMEN TRANSFORMING GROWTH FACTOR BETA 1 IN MODULATING IMMUNE RESPONSES DURING HIV-1 INFECTION

By

JESSICA KATHERINE KAFKA, B.Sc.

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"The highest reward for a person's toil is not what they get for it, but what they become by it."

– John Ruskin

--- DESCRIPTIVE NOTE ---

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AUTHOR: Jessica Katherine Kafka, B.Sc. in Microbiology (University of Guelph)

SUPERVISOR: Charu Kaushic, PhD

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--- ABSTRACT ---

Thirty five million people are currently living with HIV-1 today with women accounting for half of infected individuals globally. Sexual transmission is the main route of HIV transmission with approximately 40% of HIV infections occurring when the mucosal lining of the female genital tract (FGT) is exposed to HIV in semen from an infected male partner. Seminal plasma (SP), the fluid portion of semen, is a complex fluid which plays an immunomodulatory role in the FGT for successful conception, largely due to its high concentrations of TGF-β1. Several factors in SP from HIV-uninfected men have been shown to either inhibit or enhance HIV infection in target cells, however it is not clear how SP from HIV infected men would modulate genital epithelial cells (GECs), the first cells that encounter HIV in the FGT. The overall goals of this thesis were to compare inflammatory and regulatory cytokine concentrations in SP from HIV-uninfected and infected men, and subsequently compare GEC cytokine responses following exposure to SP from HIV-uninfected and HIV-infected men. I also investigated how SP and TGF-β regulated cytokine production and barrier function in GECs in the presence of HIV. The results summarized in this thesis demonstrated that HIV infection leads to different cytokine profiles in SP, based on stage of HIV-1 infection. HIV-infected men in acute stage contained higher levels of proinflammatory cytokines in their SP compared to HIV-uninfected and chronically infected men (CI men) which subsequently lead to higher levels of proinflammatory cytokines from GECs compared to CI men. In the follow up to this study we found that active TGF-β, which was found in higher concentrations in SP from CI men and led to decreased inflammatory response from

GECs, was compartmentalized between blood plasma and seminal plasma. Higher levels of active TGF-β in SP correlated with decreased semen viral load and the immune activation marker sCD14 leading us to believe that ART-naive CI men in our cohort were naturally controlling their immune activation status, as active TGF-β levels were lower in ART-treated men. Short-term exposure of GECs to SP from CI men or TGF-β at comparable concentrations to SP protected the GEC barrier against HIV by decreasing inflammatory cytokines and preventing tight junction breakage. However, long-term exposure to TGF-β in the presence of HIV further increased inflammation in GECs suggesting a biphasic role for TGF-β in the FGT. This body of work summarized in this thesis demonstrates for the first time how semen from HIV-infected men modulates FGT epithelial cell cytokine responses and barrier function, an important consideration in the design of local therapeutic strategies to protect the FGT against HIV infection.

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I can say with all honesty that getting my PhD has been the most challenging mental endeavour I have experienced to date and has taught me so much about myself and what it means to be a research scientist. I have also learned that to survive graduate school you need to have true grit, which I consider to be my greatest strength. No I'm not talking about the Jeff Bridges movie; I'm talking about getting up after the hard knocks. May it be failed experiments, harsh criticism from your peers, or just an overall hard day.

Remember, *"It isn't about how hard you hit, it's about how hard you can get hit and keep moving forward, that's how winning is done, and that's what makes a true champion."*

-Unknown

- All my love,

Jessica (aka Katie, Fenis)

--- PREFACE ---

The majority of this thesis is prepared in the sandwich format in accordance with the December 2014 edition of 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 3 chapters with each one being an independent study. Chapters 2 and 3 are two published manuscripts, and chapter 4 is unpublished research at the time of thesis submission, and is therefore styled in the standard thesis format. All published manuscripts and unpublished research included in this thesis are written by the author of this thesis, who is also the first author on all included works. The preface of each chapter describes the contributions of other authors to the work as well as the implications of the research. Finally, the discussion section (Chapter 5) summarizes the conclusions of this thesis and draws out the overall implications and limitations of the work.

Unless otherwise stated, HIV and TGF-β refer to HIV-1 and TGF-β1 respectively in this thesis.

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--- LIST OF ABBREVIATIONS AND SYMBOLS ---

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
μ	Micro
Ab	Antibody
AI	Acute Infected
AIDS	Acquired Immunodeficiency Syndrome
AMP	Antimicrobial Peptide
APOBEC3G	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like 3G
ART	Antiretroviral Therapy
BFGF	Basic Fibroblast Growth Factor
BP	Blood Plasma
BST2	Bone Marrow Stromal Antigen 2
CA	Capsid
CI	Chronic Infected
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
EC	Epithelial Cell
EI	Entry Inhibitor
Env	Envelope Glycoprotein
FGT	Female Genital Tract
FITC	Fluorescein Isothiocyanate
FSH	Follicle-Stimulating Hormone
Gag	Group-Specific Antigen
GALT	Gut-Associated Lymphoid Tissue
G-CSF	Granulocyte Colony-Stimulating Factor
GEC	Genital Epithelial Cell
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
Gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HeNe	Helium-Neon (laser)
HESN	HIV-Exposed, Seronegative
HIV	Human Immunodeficiency Virus

ICAM	Intercellular Adhesion Molecule
IFN	Interferon
II	Integrase Inhibitor
IL	Interleukin
IN	Integrase
IVF	In vitro Fertilization
kDa	Kilodalton
LAP	Latency-Associated Peptide
LH	Luteinizing Hormone
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharide
LTNP	long-Term Non-Progressors
LTR	Long Terminal Repeat
MA	Matrix
M-CSF	Macrophage Colony-Stimulating Factor
MGT	Male Genital Tract
MIP	Macrophage Inflammatory Protein
MX2	Myxovirus Resistance 2
NC	Nucleocapsid
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
NLR	NOD-like Receptor
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
pDC	Plasmacytoid Dendritic Cell
Pol	Polymerase
PAP	Prostatic Acid Phosphatase
PI	Protease Inhibitor
PIP3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PMN	Polymorphonuclear Cell
PR	Protease
PR A	Progesterone Receptor A
PR B	Progesterone Receptor B
PRR	Pattern Recognition Receptors
PSA	Prostatic Specific Antigen
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
Rev	Regulator of Expression of Viral Proteins

RT	Reverse Transcriptase
SAMHD1	SAM Domain and HD Domain 1
SDF-1	Stromal Derived Factor 1
SEM	Standard Error of the Mean
SEVI	Semen-Derived Enhancer of Viral Infection
SLPI	Secretory Leukocyte Protease Inhibitor
SMAD	*Drosophila* Similar to Mothers against Decapentaplegic
SP	Seminal Plasma
SDF-1α	Stromal Cell-Derived Factor 1 alpha
Tat	Transactivator of Transcription
TER	Transepithelial Electrical Resistance
TGF-β	Transforming Growth Factor Beta
TGFBR	TGF-β Receptor
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
TRIM5α	Tripartite Motif 5α
UNAIDS	Joint United Nations Programme on HIV/AIDS
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor

--- CHAPTER 1 ---

GENERAL INTRODUCTION AND OBJECTIVES

1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV-1)

1.1.1 Epidemiology

Since its discovery in the early 1980s, the human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), has infected over 60 million people worldwide (Le Tortorec & Dejucq-Rainsford, 2009; UNAIDS, 2013). The latest UNAIDS report stated that globally, an estimated 35 million individuals were living with HIV-1 in 2012 (UNAIDS, 2013). In Canada, approximately 71,000 individuals were HIV-1 positive as of 2011 (PHAC, 2012). The HIV-1 epidemic disproportionally affects sub-Saharan Africa where roughly 25 million people are living with HIV-1; constituting 70% of total HIV infections globally (UNAIDS, 2013). Gender disparity exists with HIV infection as women account for approximately half (52%) of the global population infected with HIV with women ages 15-24 representing the demographic most at risk for acquiring HIV (UNAIDS, 2010). Approximately 70% of all infected women reside in sub-Saharan Africa (Magadi, 2011; UNAIDS, 2010). To date, sexual transmission is the main route of HIV transmission worldwide with 40% of infections occurring when the mucosal lining of the female genital tract (FGT) is exposed to cell-free or cell-associated HIV-1 in semen from an infected male partner (Hladik & McElrath, 2008).

1.1.2 Structure and Genome

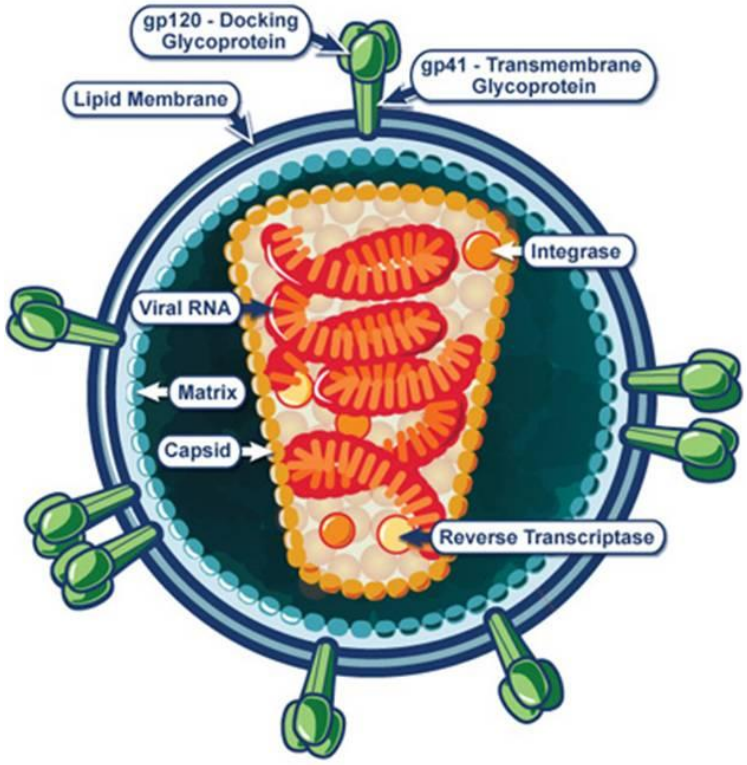
The mature HIV virion is roughly circular, and is ~120 nm in diameter. Its genome is composed of two non-covalently linked, unspliced, positive sense, single-stranded RNA copies encased within a bullet-shaped viral capsid. Proteins important for

viral replication such as the nucleocapsid chaperone protein, reverse transcriptase, integrase and protease are located within the capsid and interact with the viral RNA. Matrix proteins ensure structural integrity of the virion and lie between the capsid and the viral envelope, which is composed of a lipid bi-layer of host cell origin. Embedded within the viral envelope are glycoprotein "spikes" which are composed of the surface protein gp120 and the transmembrane protein gp41.

HIV-1 is the type species of the *Lentivirus* genus in the family *Retroviridae*. The HIV-1 genome encodes the major structural and non-structural proteins common to retroviruses, as well as a number of HIV-specific regulatory and accessory proteins. From the 5' to 3' end, the HIV genome encodes the *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelop glycoprotein) genes, which encode the nucleocapsid, polymerase, and envelope proteins respectively (Freed, 2001). The *gag* gene encodes the gag polyprotein precursor, called $Pr55^{gag}$, which is cleaved by HIV protease into the mature gag proteins MA (p17 matrix), CA (p24 capsid), NC (p7 nucleocapsid), and p6 (Freed, 1998). The *pol* gene encodes the large polyprotein precursor $Pr160^{GagPol}$, which encompasses four enzymes: RT (reverse transcriptase), PR (protease), IN (integrase), and RNase H. The most important of these proteins is the reverse transcriptase, which transcribes the virus' single-stranded RNA genome into double-stranded DNA; a function unique to retroviruses. Lastly, the *env* gene encodes the gp160 polyprotein precursor. Unlike the gag and pol polyproteins which are cleaved by HIV proteases, the env precursor is cleaved by host cellular proteases en route to the cell surface. Cleavage of

gp160 yields the surface membrane glycoprotein gp120 and the transmembrane anchoring glycoprotein gp41.

In addition to the *gag*, *pol*, and *env* genes, the HIV-1 genome also encodes a number of regulatory and accessory proteins. The regulatory proteins Tat and Rev are critical for transcription from the HIV long terminal repeat (LTR) promoter, and for viral RNA transport from the nucleus to the cytoplasm, respectively (Karn & Stoltzfus, 2012; L. Li, Li, Pauza, Bukrinsky, & Zhao, 2005). The accessory proteins Vpu, Vif, Vpr, and Nef are named as such for they are not essential for virus replication, but do contribute to virus spread and pathogenesis (Cullen, 1998; Freed, 2001; Kirchhoff, 2010; L. Li, et al., 2005; Malim & Emerman, 2008).



Courtesy: National Institute of Allergy and Infectious Diseases

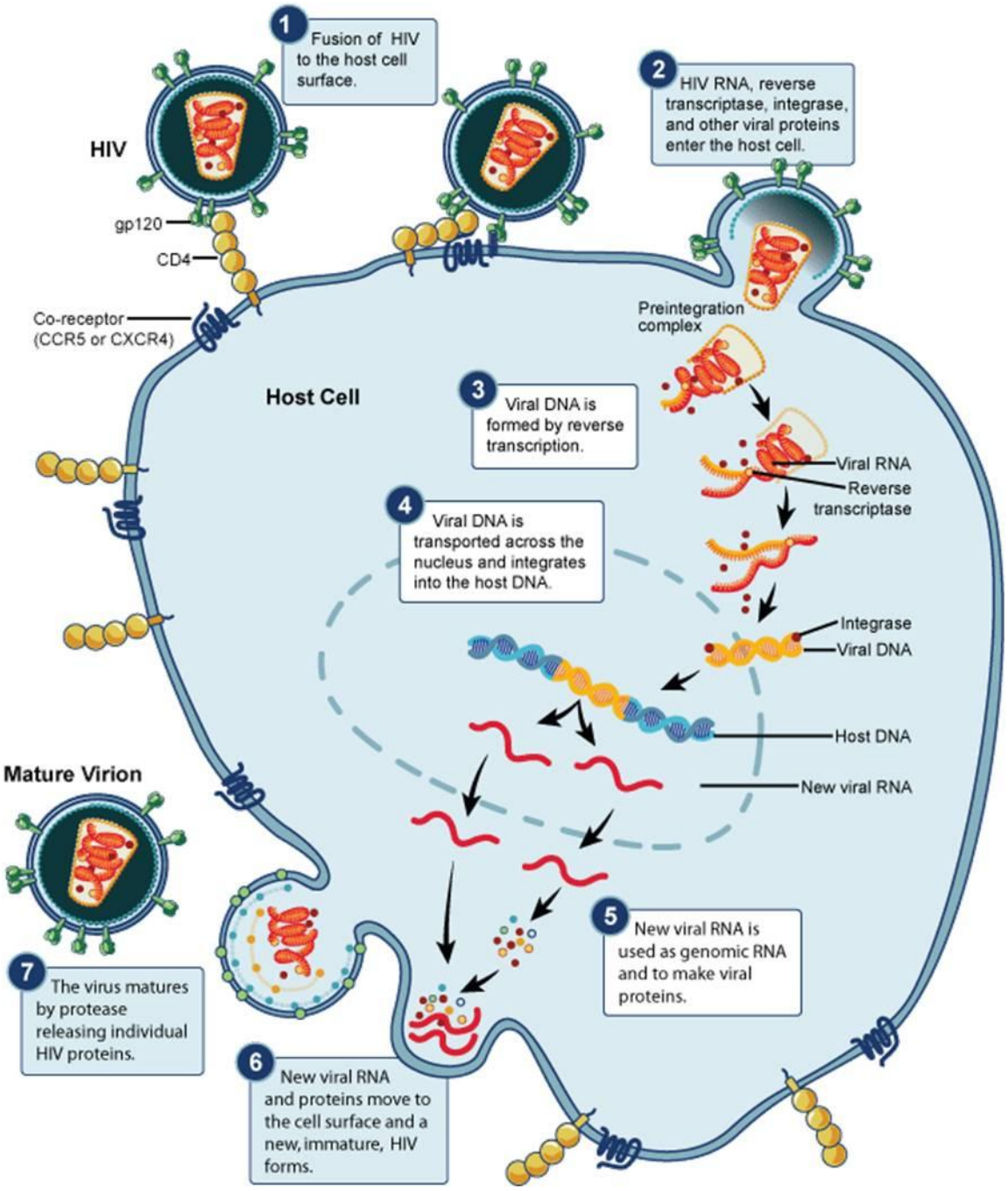
Figure 1: The HIV-1 Virion. HIV is a roughly circular virus with gp120 docking proteins and gp41 transmembrane proteins dotting the surface of the virion's lipid membrane surface. The genetic material of the virus is composed of two single-stranded RNA which is encased within a viral capsid. Viral proteins important for replication, including integrase and reverse transcriptase, are also found within the capsid. Between the lipid membrane and capsid is the protein matrix which provides structural integrity to the virion. Image used with permission from the National Institute of Allergy and Infectious Diseases (NIAID).

1.1.3 Replication Cycle

The replication cycle for HIV can be broadly characterized into the following stages: entry, reverse transcription, integration, transcription, translation, assembly, and budding. Entry of the virus occurs primarily through membrane fusion, or alternatively by endocytosis (Daecke, Fackler, Dittmar, & Krausslich, 2005; Dorosko & Connor, 2010; Pauza & Price, 1988; Wilen, Tilton, & Doms, 2012). The HIV envelope glycoprotein gp120 facilitates entry via binding to CD4 on the surface of helper T cells, macrophages, and dendritic cells (DCs) (Gomez & Hope, 2005). Binding of gp120 with CD4 changes the conformation of gp120 leading to increased affinity for cellular co-receptors CCR5 (macrophage-tropic) or CXCR4 (T cell-tropic), depending on viral tropism (Freed, 2001; Gomez & Hope, 2005). Binding of gp120 to CD4 and co-receptors induces a conformational change in the transmembrane glycoprotein gp41 creating a six helix bundle which completes fusion between host and viral membranes and the release of the viral core into the host cell (Markosyan, Cohen, & Melikyan, 2003). Once in the host cytoplasm, uncoating of the viral core releases the RNA genome as well as viral enzymes important for successful replication. One of the defining features of retroviruses are their ability to convert their single-stranded RNA genomes into double-stranded DNA early post-entry (Freed, 2001). This is facilitated by the reverse transcriptase enzyme

(Telesnitsky & Goff, 1997). Viral DNA along with viral integrase as part of the pre-integration complex is imported into the nucleus where integrase catalyzes the insertion of the linear double-stranded viral DNA into the host cell chromosome (P. O. Brown, 1997; Freed, 2001). The inserted viral DNA, called the provirus, now behaves like a cellular gene and provides a template for the synthesis of viral mRNA that ultimately encode for all of the HIV-1 structural, non-structural, regulatory, and accessory proteins. The HIV LTR promoter is the initiation site for transcription and is the binding site for the viral transcription transactivator protein Tat, host NF-κB, and host RNA polymerases (Freed, 2001). Transcription from the HIV LTR yields a large number of RNAs which can be characterized as either unspliced RNAs (Gag and GagPol polyprotein precursors), partially spliced RNAs (Env, Vif, Vpu, and Vpr accessory proteins), and multi-spliced RNAs (Tat, Rev, and Nef proteins) (Freed, 2001). While most of the viral RNAs leaving the nucleus are multi-spliced, partially spliced and unspliced RNAs are transported from the nucleus with the aid of the viral Rev protein (Pollard & Malim, 1998). Once in the cytoplasm, viral mRNA is translated into polyprotein precursors by host translational machinery, and assembly of progeny virus can begin at the host plasma membrane. Targeting of viral proteins and genome to the plasma membrane and virion assembly is largely mediated by the matrix domain of the Gag polyprotein precursor $Pr55^{Gag}$ and happens within minutes of its synthesis (Freed, 1998; Freed, Orenstein, Buckler-White, & Martin, 1994; Swanstrom & Wills, 1997). Encapsulation of the viral genome is mediated by interactions between "packaging signal" sequences within the two RNA copies and the nucleocapsid domain of Gag (Freed, 1998). The HIV Env glycoprotein gp160 is

synthesized in the rough endoplasmic reticulum and is transported to the cell surface by the secretory pathway (Freed & Martin, 1995). Enroute to the cell surface, the gp160 protein is cleaved into mature gp120 and gp41 subunit glycoproteins by host proteases. At this point, gp41 anchors itself to the membrane and becomes non-covalently associated with gp120. Although not completely understood, it is believed that envelope glycoproteins are incorporated into virions via interactions between the matrix domain of Gag and the cytoplasmic tail of gp41 (Freed, 1998; Murakami & Freed, 2000). Viral maturation commences with the cleavage of Gag and GagPol precursors by viral proteases to generate mature Gag and Pol proteins, and ultimately the formation of the viral capsid "shell" around the viral RNA and nucleocapsid proteins (Freed, 2001). Budding of the virus from the host plasma membrane is the final step in the process of viral assembly and release, and is mediated by "late" domains within the Gag polyprotein (Freed, 2002). Knowledge of how progeny virions are created has lead to the development of anti-retroviral drugs targeting specific steps in the HIV replication cycle. Information on antiretroviral therapy (ART) can be found in section 1.1.5 of this thesis.



Courtesy: National Institute of Allergy and Infectious Diseases

Figure 2: The HIV-1 Replication Cycle. (1) The envelope glycoprotein gp120 embedded in the lipid membrane of the HIV-1 virion binds to the CD4 receptor and CCR5 or CXCR4 co-receptor on the surface of target cells such as macrophages and T cells. (2) Binding of the HIV envelope protein to CD4 and chemokine co-receptors allows the HIV-1 outer membrane to fuse with the host cell outer membrane and allows the contents of the virus particle to enter the cell. (3) An enzyme known as reverse transcriptase initiates the formation of double-stranded DNA by copying the sequence of the two RNA strands contained within the virus particle. (4) The viral DNA enters the nucleus of the host and becomes integrated into the host DNA with the help of the viral integrase enzyme. The integrated viral DNA is now referred to as a provirus. The proviral DNA serves as a template for the creation of new viral RNA via transcription. (5) The host cell machinery that is normally used for the transcription of human genes is 'hijacked' by the virus to create new viral RNA molecules. The newly formed viral RNA then moves out of the nucleus of the infected cell. The viral RNA carries the genetic code for the synthesis of new viral proteins and enzymes. (6) Components that are required to build new virus particles, namely viral proteins, enzymes and genetic material (viral RNA) move to the cell outer membrane where they accumulate and assemble in the form of a bud. Host cell proteins cut the virus bud from the cell outer membrane, thereby releasing a new virus particle. (7) 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 virus particle. Image used with permission from the National Institute of Allergy and Infectious Diseases (NIAID).

1.1.4 HIV-1 Pathogenesis and Disease Progression

Transmission of HIV occurs most commonly at genital tract and rectal mucosal surfaces, and less commonly via infected blood, infection drug use, or vertically from mother to child (Moir, Chun, & Fauci, 2011). HIV infection can be categorized into three stages: acute/early infection, chronic infection/clinical latency, and AIDS. The initial phase of infection, or acute phase, is the time between HIV acquisition until seroconversion (Cohen, Gay, Busch, & Hecht, 2010). Clinically, it is measured as detectible HIV RNA in copies/ml or positive p24 antigen in serum or plasma when there is a negative or indeterminate HIV antibody test (Branson, et al., 2006; Pilcher, Christopoulos, & Golden, 2010). HIV neutralizing antibodies are typically measured in plasma past 3 months post-infection (Cohen, Shaw, McMichael, & Haynes, 2011; Wei, et

al., 2003). Acute infection plasma HIV RNA levels are typically very high (100,000-10,000,000 RNA copies/ml) corresponding to aggressive replication by the virus and increased transmission by the infected individual (Daar, et al., 2001; Hecht, et al., 2002; Little, McLean, Spina, Richman, & Havlir, 1999; Piatak, et al., 1993). At approximately two to four weeks following transmission of the virus, 60-90% of patients with acute HIV infection experience acute retroviral syndrome characterized by "flu-like" symptoms like fever, lymphadenopathy, pharyngitis, skin rash, joint and muscle pain, headache, diarrhea, and other symptoms (Kinloch-de Loes, et al., 1993; Niu, Stein, & Schnittman, 1993; Schacker, Collier, Hughes, Shea, & Corey, 1996). Acute HIV infection is often not recognized by physicians since its symptoms are similar to those of other viral infections such as influenza and infectious mononucleosis.

It is believed that infection arises from a single "founder" virus or infected cell in the majority of infected individuals (Keele, et al., 2008; Salazar-Gonzalez, et al., 2009) followed by rapid migration via draining lymph nodes to the gut-associated lymphoid tissue (GALT) where massive depletion of CCR5+ memory CD4+ T cells ensues in the intestinal lamina propria (Brenchley, et al., 2004; Guadalupe, et al., 2003; Q. Li, et al., 2005; Mattapallil, et al., 2005; Mehandru, et al., 2004). In the absence of ART, blood plasma viremia usually peaks around day 21 post-exposure (Fiebig, et al., 2003; Little, et al., 1999) followed by a spontaneous decline before it reaches a steady state, or viral set point (Moir, et al., 2011). The GALT remains a reservoir for persistent infection throughout the course of disease even after years of ART (Chun, et al., 2008; Sheth, et al., 2008).

Following acute infection, individuals enter into the clinical latency/asymptomatic/chronic stage of HIV infection. Although the virus is still actively replicating, albeit at lower levels than measured during acute infection, little to no symptoms are experienced. An infected individual on ART can live in the clinical latency period for several decades due to the therapy's ability to control their viral load. However, chronically infected individuals also experience persistent immune activation; one of the hallmarks of HIV infection and a major contributor to disease progression (Brenchley, Price, Schacker, et al., 2006). This is confirmed by the absence of immune activation in non-pathogenic SIV infection of sooty mangabeys despite high plasma viremia (Silvestri, et al., 2003). Chronic immune activation involves both the innate and adaptive immune systems and is characterized by increased turnover of immune cells, skewing of lymphocytes towards an activated phenotype, B and T cell exhaustion and senescence, and maintenance of proinflammatory cytokine production (Maartens, Celum, & Lewin, 2014; Moir, et al., 2011; Moir & Fauci, 2014). The main drivers of persistent immune activation include HIV directly activating TLR7 and 8 expressed by plasmacytoid DCs leading to production of IFN-α (A. Meier, et al., 2009), co-infection with viruses such as Cytomegalovirus that induce expansion of activated virus-specific T cells (Maartens, et al., 2014), reduced ratio of Th17 and regulatory T cells (Prendergast, et al., 2010), and the breakdown of the intestinal mucosal barrier causing microbial translocation into the circulation (Brenchley, Price, Schacker, et al., 2006). Lipopolysaccharide (LPS), or endotoxin, is an outer membrane component of gram-negative bacteria and a potent activator of TLR4 (Brenchley, Price, Schacker, et al., 2006; Dedrick & Conlon, 1995). It

can produce inflammatory responses from CD14+ phagocytic cells of the monocyte/macrophage lineage through binding with soluble (s)CD14, a marker of immune activation shown to correlate with disease progression and mortality in HIV-infected individuals (Sandler, et al., 2011). Destruction of the GALT and subsequent leakage of microbial products into circulation is maintained throughout the course of disease (Brenchley, Price, & Douek, 2006).

In the absence of therapy, the average infected individual will experience progressive loss of CD4+ T cells, increased immunodeficiency, and subsequent opportunistic diseases characteristic of AIDS (Lewin-Smith, Klassen, Frankel, & Nelson, 1998). Clinically, AIDS is measured as a blood plasma CD4+ cell count less than 200 cells/mm^3 (Naif, 2013).

Although the above is a typical course of pathogenesis and disease progression, in a minority of infected individuals different courses of infection have been observed. Some HIV-infected individuals, known as long-term non-progressors (LTNPs), remain asymptomatic for many years and maintain high CD4 counts (>500 CD4+ cells/μl) without ART (Piacentini, Fenizia, Naddeo, & Clerici, 2008). An small proportion of infected individuals, called elite controllers, have sustained suppression of viral replication and who have maintained undetectable viral loads (<50 RNA copies/ml) for at least 1 year in the absence of ART (Walker, 2007). Similarly, individuals who maintain a viral load of <2000 RNA copies/ml in the absence of ART are called viremic controllers (Walker, 2007). HIV-exposed, seronegative (HESN) individuals are a group who appear to exhibit natural resistance to HIV. A well known cohort of HESN, the Pumwani sex

workers from Kenya, contains a group of high-risk women who are frequently exposed to HIV but show no evidence of seroconversion, nor symptoms of immunodeficiency (Fowke, et al., 1996). Several studies have focused on the different mechanisms and factors involved in natural resistance to HIV-1 infection including the presence of genetic polymorphisms in the viral co-receptors (CCR5-Δ32), innate and adaptive immune cells with particular phenotypic and functional features, and antibodies and soluble factors that play important roles in defense against HIV infection (Taborda-Vanegas, Zapata, & Rugeles, 2011).

1.1.6 Antiretroviral Therapy

The discovery of antiretroviral therapy in the late 1980s has greatly reduced the morbidity and mortality of HIV-1 infection. In 1987, the first antiretroviral drug approved by the FDA was a nucleoside reverse transcriptase inhibitor (NRTI) called zidovudine (AZT). Antiretroviral therapy was prescribed as either one or two NRTIs until the late 1990s when combination therapy (cART), aka highly active antiretroviral therapy (HAART), was initiated. This regimen, consisting of two NRTIs as well as a new class of antiretroviral called protease inhibitors (PIs), lead to decreased rates of opportunistic illness and death in patients compared to NRTI mono- or ditherapy (Gulick, et al., 1997; Hammer, et al., 1997; Moore & Chaisson, 1999). Today, there are several classes of antiretroviral drugs each targeting a different stage of the HIV replication cycle including nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and integrase inhibitors (IIs). The newest class of drugs to be

introduced are the entry, or fusion, inhibitors (EIs). Maraviroc, a CCR5 co-receptor antagonist, was approved for treatment by the FDA in 2007.

The effectiveness of ART is measured by its ability to achieve undetectable blood plasma viral load, and increases in the CD4+ T cell population in infected individuals. Undetectable semen and cervicovaginal viral loads can also be achieved during effective ART (Leruez-Ville, et al., 2002; Vernazza, et al., 2000; Vettore, Schechter, Melo, Boechat, & Barroso, 2006). In recent years, evidence has emerged that ART can reduce the risk of HIV transmission by as much as 96% (Cohen, Chen, et al., 2011). However, a cause for concern is the detection of intermittent shedding of HIV in semen and cervicovaginal fluid in ART-treated men and women respectively with undetectable blood viral loads (Cu-Uvin, et al., 2010; Ferraretto, et al., 2014; Halfon, et al., 2010; Kovacs, et al., 2001; Lambert-Niclot, et al., 2012; Low, et al., 2014; Marcelin, et al., 2008; Politch, et al., 2012; Sheth, et al., 2009). Possible contributors to genital tract shedding in the presence of ART include the existence of viral reservoirs, such as the blood-testes barrier in the MGT, which can prevent passage of antiretrovirals into infected male sex organs, concurrent sexually transmitted infections (STIs), genital inflammation, and the stage of the menstrual cycle (J. A. Anderson, et al., 2010; P. Gupta, et al., 2000; Le Tortorec & Dejucq-Rainsford, 2009) (Curlin, et al., 2013; Gitau, et al., 2010; Henning, et al., 2010; Politch, et al., 2012).

Despite reported decreases in viral load, the effect of ART on immune activation during chronic infection remains controversial. While some studies showed a decrease in both sCD14 and immune activation (Brazille, et al., 2003; Brenchley, Price, Schacker, et

al., 2006; Lempicki, et al., 2000), other studies reported immune activation did not normalize with ART (Eden, et al., 2007; French, King, Tschampa, da Silva, & Landay, 2009; Hunt, et al., 2003; Lederman, et al., 2011; Mendez-Lagares, et al., 2013).

1.2 THE MALE GENITAL TRACT (MGT)

1.2.1 Anatomy and Cell Composition

The MGT is composed of the penis, testes, prostate and various accessory tubular structures which, under the influence for androgens, function to produce, maintain, and transport sperm and seminal fluid (semen) for reproduction. The majority of organs of the MGT are composed of stratified epithelium with an underlying stroma. The penis is made up of distinct microenvironments comprised of different epithelial and non-epithelial cell types. The external surface of the glans penis is composed of keratinized squamous epithelium which forms a barrier against pathogens provided the skin remains intact, uninflamed, and uninfected. The foreskin is lined by stratified squamous epithelium, with the external surface being more heavily keratinized than the internal surface (McCoombe & Short, 2006; Patterson, et al., 2002). A rich population of immune cells exists in the foreskin such as CD4+ T cells and Langerhans cells in the squamous epithelium as well as T cells, macrophages and DCs in the underlying stroma (Donoval, et al., 2006; Hussain & Lehner, 1995; McCoombe & Short, 2006; Patterson, et al., 2002). The urethral orifice (meatus) is also covered in keratinized squamous epithelium, which abruptly transforms into non-keratinized stratified epithelium in the fossa navicularis (Nguyen, Kafka, Ferreira, Roth, & Kaushic, 2014). Finally, the penile urethra is lined with pseudostratified columnar epithelium along the shaft of the penis. Aside from epithelial cells, the penile

urethra is lined with mucin-producing pseudoglands called the glands of Littre, which secrete a viscous fluid called the pre-ejaculate that provides a lubricant during sexual intercourse as well as a neutralizing agent for residual urine (Nguyen, et al., 2014; Pudney & Anderson, 2011). Abundant numbers of CD4+ and CD8+ T cells, and macrophages are found lining the penile urethra as well as in the lamina propria (Pudney & Anderson, 1995), however DCs appear to be absent in the urethral mucosa (Hussain & Lehner, 1995; Pudney & Anderson, 1995).

The testes are largely made up of seminiferous tubules within which spermatozoa are produced (Bronson, 2011). These tubules connect with the rete testis to the head of the epididymis, and subsequently to the vas deferens. Columnar Sertoli cells lining the lumen of the seminiferous tubules "nurse" the maturing spermatozoa as well as separate the sperm from the systemic immune system via basal tight junctions that form the blood-testes barrier (Bronson, 2011). The lumen between seminiferous tubules contains testosterone and estradiol-producing Leydig cells, macrophages, Natural Killer (NK) cells, and CD8+ T cells (Hedger & Meinhardt, 2000; Hedger, Wang, Lan, Atkins, & Wreford, 1998; Tompkins, Hutchinson, de Kretser, & Hedger, 1998). The main functions of the epididymis are storage, maturation, and transport of spermatozoa. The epididymis is a coiled gland composed of pseudostratified columnar epithelium, intraepithelial CD8+ T cells and, to a lesser extent, CD4+ T cells, as well as a cuboidal basal epithelium (Bedford, 1994).

The seminal vesicles are accessory organs which produce approximately 60% of the total secretions in seminal fluid (Deleage, et al., 2011). These secretions play an

important role in semen coagulation, sperm motility, stability of sperm chromatin, and suppression of the female genital tract (FGT) immune system (Gonzales, 2001). These organs are a pair of single coiled or folded tubes composed of pseudostratified columnar epithelium with CD8+ T cells and resident CCR5+ macrophages found within the epithelial lining as well as in the lamina propria. CD4+ T cells have also been measured in the stroma of seminal vesicles, though at a lesser frequency than macrophages (Deleage, et al., 2011; Le Tortorec, et al., 2008).

The prostate is a walnut-sized accessory organ which, like the seminal vesicles, contributes to the fluid component of semen in the form of simple sugars and enzymes (Wolff, 1995). It is composed of a series of lobes comprised of luminal and basal glandular epithelium embedded in a fibro-muscular stroma (Long, Morrissey, Fitzpatrick, & Watson, 2005). The luminal layer is composed of tall columnar cells responsible for the secretion of prostatic specific antigen (PSA), prostatic acid phosphatase (PAP), and human kallikrein-2 which are secreted into the seminal fluid (McNeal, 1988; Rittenhouse, Finlay, Mikolajczyk, & Partin, 1998). The basal layer of the prostate is composed of cuboidal epithelial cells followed by a basement membrane separating the basal epithelium from the stroma (Long, et al., 2005). Similar to the seminal vesicles, CD8+ T cells are the dominant lymphocyte within the epithelial layer whereas CD4+ T cells, and to a lesser extent B cells, populated the stroma (el-Demiry, et al., 1985).

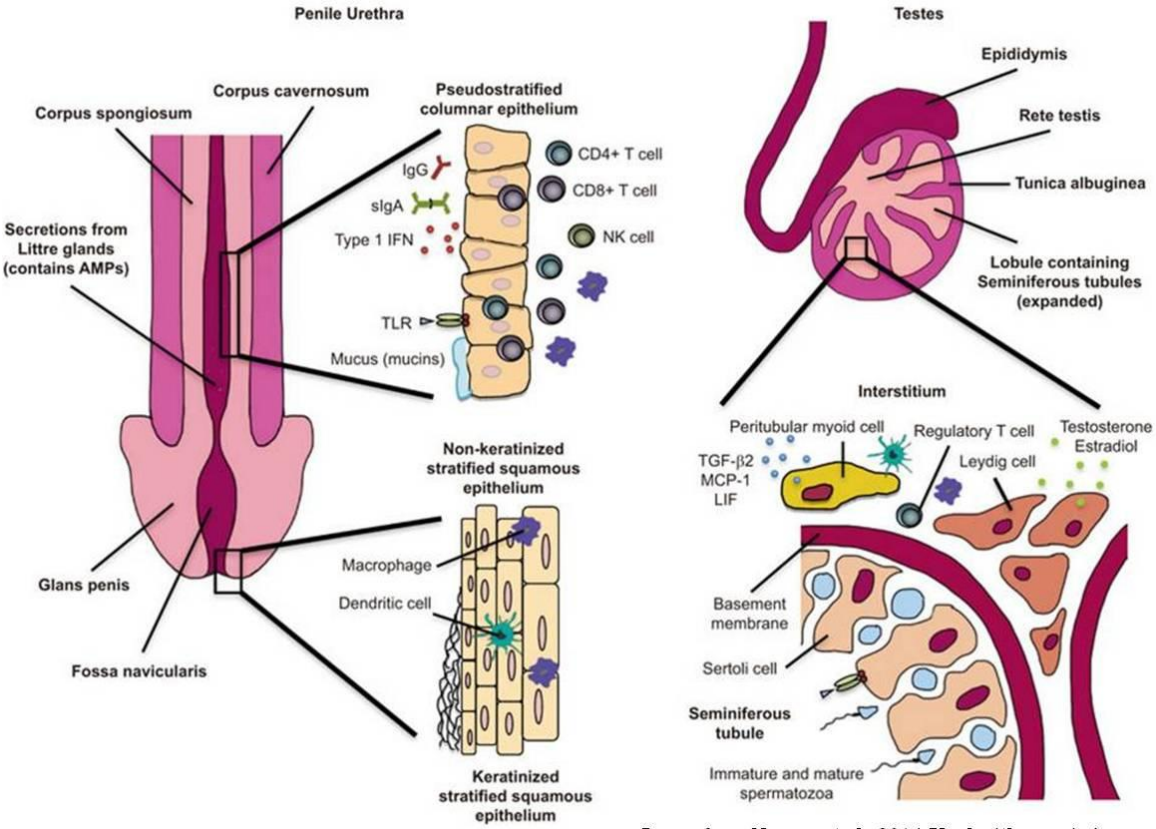


Image from Nguyen et al., 2014. Used with permission.

Figure 3: Anatomical and immunological components of the male genital tract. The MGT is composed of two main parts—the penile urethra and the testes. The opening of the penile urethra transitions from keratinized stratified squamous epithelium to non-keratinized stratified squamous epithelium in the fossa navicularis. As the non-keratinized stratified squamous epithelium enters the shaft of the penis, it transitions into the pseudostratified glandular columnar epithelium, which lines the length of the penile urethra. Within the epithelium of the penile urethra are deep invaginations, known as Littre glands, which provide lubricating pre-ejaculate for sexual intercourse and contribute to innate immunity by producing AMPs. Urethral pseudostratified columnar epithelium is an active immune microenvironment that contains $CD8^+$ and $CD4^+$ T cells, NK cells, dendritic cells and resident macrophages, which have been found to be key targets for HIV infection in the penile urethra. IgG, IgA and IgM immunoglobulins are also secreted from the urethra epithelium, along with Type 1 IFN, mucins and AMPs. The testes are the main site of spermatogenesis, which begins in the rete testes and seminiferous tubules; mature spermatozoa enter the epididymis. Seminiferous tubules, located within the lobules of the testes, are made up of Sertoli cells that are surrounded by a basement membrane. Sertoli cells aid in spermatogenesis but also express TLRs. Within the interstitial space of the seminiferous tubules are Leydig cells, which produce testosterone and estradiol, and PMCs, which regulate testes development and spermatogenesis by secreting TGF-β2, MCP-1 and LIF. Resident $ED2^+$ macrophages, which have an attenuated inflammatory function, are found within the interstitial spaces of the MGT along with regulatory T cells—both cell types contribute to the immunoregulatory microenvironment of the testes. Image and figure legend used with permission from Nature Publishing Group.

1.2.2 HIV-1 Infection in the MGT

HIV infection of the MGT occurs either through vaginal or rectal intercourse with an infected partner (Hladik & McElrath, 2008). Studies have suggested that the foreskin and the glans penis are the two main sites where HIV infection takes place. As mentioned earlier, the inner foreskin contains an abundance of HIV target cells such as CD4+ T cells, DCs, Langerhans cells, and macrophages within the epithelial lining as well as in the underlying stroma with many of these cells expressing CCR5, CXCR4, and CD4 (Hladik & McElrath, 2008; Hussain & Lehner, 1995; Lemos, et al., 2014; McCoombe & Short, 2006; Patterson, et al., 2002). With the foreskin being a main site for HIV infection, circumcision has been implemented in protecting against HIV infection in men. Results of the first randomized controlled intervention trial, the ANRS 1265 Trial, showed a 60% reduction in HIV incidence in the intervention group compared to the control group (Auvert, et al., 2005). Since then, many other studies have shown the effectiveness if circumcision in decreasing HIV infection (Bailey, et al., 2007; Gray, et al., 2007; A. S. Meier, Bukusi, Cohen, & Holmes, 2006; Quinn, 2007; Siegfried, Muller, Deeks, & Volmink, 2009).

The penile urethra in the glans penis contains non-keratinized columnar epithelial cells which, like the inner foreskin, contains intraepithelial and stromal HIV target cells including CD4+ T cells, and macrophages making it another possible site for HIV transmission (Pudney & Anderson, 1995). Detection of *CCR5* and *CXCR4* mRNA by urethral swabs suggests that the urethral mucosa may contain target cells with HIV-1 co-

receptors (McClure, et al., 2005). Additionally, inoculation of macaques with intraurethral SIV resulted in 100% infection (Miller, et al., 1989).

Following transmission and dissemination, several organs in the MGT can become infected with HIV and SIV, which contribute to semen viral load during acute and chronic infection as well as the possibility of a viral reservoir distinct from blood (Le Tortorec & Dejucq-Rainsford, 2009). *In vivo* and *in vitro* studies have shown that the testis can become productively infected with HIV and SIV with resident testicular macrophages, T cells, and germ cells being the main targets for infection (Le Tortorec, et al., 2008; Muciaccia, et al., 1998; Paranjpe, et al., 2002; Roulet, et al., 2006; Shehu-Xhilaga, et al., 2007). HIV infection of the testes creates a roadblock for treatment strategies due to possible reduced availability of ART. The existence of drug efflux pumps on a variety of testicular cell types and the blood-testes barrier may restrict drug access to this site (Choo, et al., 2000; Livni, et al., 2004). Infected intraepithelial and stromal T cells and macrophages have also been detected in the epididymis, seminal vesicles, and prostate of SIV-infected macaques in acute and chronic stages of infection (Le Tortorec, et al., 2008; Shehu-Xhilaga, et al., 2007). Infected intraepithelial lymphocytes may release cell-free and cell-associated virus into the lumen of these organs and subsequently into the seminal fluid.

1.3 SEMEN, SEMINAL PLASMA COMPONENTS, AND HIV-1 INFECTION

1.3.1 Semen Composition

Semen is a heterogeneous fluid from the male sex organs which functions as a support medium for the transport and survival of spermatozoa, as well as induces changes

in the FGT in preparation for pregnancy (Schjenken & Robertson, 2014). It is composed of simple sugars, enzymes, proteins, lipids, immature germ cells, and spermatozoa from the testes, epididymis, prostate, seminal vesicles, and bulbo urethral glands (Deleage, et al., 2011; Doncel, Joseph, & Thurman, 2010; Fung, Glode, Green, & Duncan, 2004; Le Tortorec & Dejucq-Rainsford, 2009; Politch, Marathe, & Anderson, 2014; Sabatte, Remes Lenicov, et al., 2011). Proteomic analysis of human semen has lead to the identification of over 900 different proteins (Fung, et al., 2004; Pilch & Mann, 2006). Several studies have identified a plethora of immunomodulating factors such as immunoglobulins, prostaglandins, growth factors, sex hormones, immune cells, cytokines, and chemokines in human semen (Alexander & Anderson, 1987; D. J. Anderson, et al., 1998; Maegawa, et al., 2002; Moldoveanu, Huang, Kulhavy, Pate, & Mestecky, 2005; Politch, Tucker, Bowman, & Anderson, 2007). The majority of immune cells detected in semen from healthy, HIV-negative men are polymorphonuclear cells (PMNs), followed by substantial numbers of macrophages and CD4+ T cells (Olivier, et al., 2012; Tomlinson, Barratt, & Cooke, 1993; Wolff & Anderson, 1988). CD8+ T cells, B cells, and DCs have also been detected in human semen (Duan, et al., 2014; Olivier, et al., 2012; Tomlinson, et al., 1993; Wolff & Anderson, 1988). IgG and IgA are the predominant immunoglobulins in semen, and IgM is present at very low levels (Moldoveanu, et al., 2005; Politch, et al., 2007). Recent studies showed that semen also contains exosomes which may contain immunoregulatory, and anti-viral factors (Madison, Roller, & Okeoma, 2014; Vojtech, et al., 2014). Human semen also contains a diverse microbiome with *Streptococcus*, *Corynebacterium*, *Staphylococcus*, and

Lactobacillus being among the most prevalent genera (Hou, et al., 2013; Liu, et al., 2014). Semen is the term describing both cellular and fluid components, whereas seminal plasma (SP) refers exclusively to the fluid component of semen.

1.3.2 The Role of Semen in Reproduction

Studies in mammalian species show that SP exerts a profound influence on the FGT to promote reproductive success (Robertson, 2005; D. J. Sharkey, Tremellen, Jasper, Gemzell-Danielsson, & Robertson, 2012). One of the main functions of SP is modulation of the FGT immune system to create a state of immune tolerance to prevent immunological attack at implantation. The main site for semen deposition in women is the anterior vagina near the cervical os (Suarez & Pacey, 2006). Within an hour of deposition of semen into the FGT after intromission, a proinflammatory response characterized by rapid influx of leukocytes; firstly neutrophils are recruited to the lumen, followed by macrophages, T cells, and DCs populating the underlying stroma (Robertson, 2005). Leukocytes are recruited to the area of semen deposition by chemotactic factors present in semen, as well as by chemokines and cytokines secreted by the epithelial lining in response to semen. Research by Sharkey et al., showed that SP induced production of the proinflammatory cytokines and chemokines GM-CSF, IL-6, IL-8, and MCP-1 in ectocervical epithelial cells (D. J. Sharkey, Macpherson, Tremellen, & Robertson, 2007). The leukocyte influx promotes clearance of excess sperm and seminal debris, as well as removes micro-organisms introduced into the FGT during mating (Schjenken & Robertson, 2014).

Tolerance to implantation and development of the allogeneic conceptus involves the modulation of a multitude of immune cells in the FGT including DCs, NK cells, cytotoxic T lymphocytes (CTLs), and T cells. Prevention of fetal rejection is achieved through suppression of cytokine production and effector function of T cells, B cells, NK cells, DCs, and macrophages, but also via the differentiation of DCs and T cells into tolerogenic DCs and regulatory T cells (Tregs) respectively (Remes Lenicov, et al., 2012; Sakaguchi, 2000; Warning, McCracken, & Morris, 2011). Seminal fluid also promotes development of the pre-implantation embryo, specifically blastocyst development, via influencing secretion of embryotrophic cytokines and growth factors, such as GM-CSF, G-CSF, and LIF, by oviduct and uterine epithelial cells (Robertson, Seamark, Guilbert, & Wegmann, 1994; A. M. Sharkey, et al., 1995). Studies in humans, pigs, rodents, and other mammals have identified TGF-β1 as the principle factor in inducing the initial leukocyte response in the FGT (Robertson, Ingman, O'Leary, Sharkey, & Tremellen, 2002; D. J. Sharkey, Macpherson, et al., 2012; K. P. Tremellen, Seamark, & Robertson, 1998). Additionally, prostaglandins, particularly prostaglandin E2 (PGE2), are other potent immunomodulating factors present in seminal fluid. PGE2 promotes IL-8 expression and differentiation of tolerogenic DCs, and suppresses the effector functions of neutrophils and macrophages as well as proinflammatory factors such as SLPI (Denison, Calder, & Kelly, 1999; Remes Lenicov, et al., 2012; Templeton, Cooper, & Kelly, 1978). The cytotoxic effects of NK cells and CTLs are also suppressed by PGE2 (Kalinski, 2012).

Clinical studies in humans and murine studies have demonstrated the importance of SP in pregnancy outcomes. Data from in vitro fertilization (IVF) and other

reproductive technologies have shown the importance of seminal fluid at conception with the absence of SP *in vitro* leading to increases in implantation failure and compromised embryo quality, as well as changes in birth weight and health outcomes for IVF children (Ceelen, et al., 2007; Ceelen, van Weissenbruch, Vermeiden, van Leeuwen, & Delemarre-van de Waal, 2008; Maher, Afnan, & Barratt, 2003; Schieve, et al., 2002; Schjenken & Robertson, 2014). A recent study in a murine model showed that the absence of seminal fluid lead to impaired conception, down-regulation of embryotrophic factors, and subsequently altered growth and metabolic health of male offspring (Bromfield, et al., 2014). With this knowledge in mind, efforts are currently being made to introduce factors induced by SP such as GM-CSF into IVF culture media to improve survival of embryos (Schjenken & Robertson, 2014; Ziebe, et al., 2013).

1.3.3 The Role of Semen in HIV-1 Infection

Semen is the main transmission vector of sexually transmitted cell-free and cell-associated HIV, however the role of semen in modulating HIV transmission is controversial as several studies have suggested that factors normally present in semen may facilitate or inhibit HIV transmission (Doncel, et al., 2010; Sabatte, Remes Lenicov, et al., 2011). One of the ways semen may enhance HIV transmission is by increasing the infectivity of the virus by promoting HIV-target cell interactions. Heparan sulfate moieties on the surface of spermatozoa (Ceballos, et al., 2009), semenogelins (Martellini, et al., 2009; Roan, et al., 2014), and amyloid fibrils formed by amyloidogenic fragments of PAP (aka SEVI) have been shown to facilitate virion interaction with target cells (Munch, et al., 2007; Munch, et al., 2013). *In vivo*, semen-derived enhancer of viral

infection (SEVI) may facilitate the spread of physiologically lower doses of HIV-1 found during sexual transmission (Munch, et al., 2007). Additionally, it has been shown that spermatozoa may capture HIV and transmit the virus to DCs, macrophages and T cells, and that acidic pH enhanced binding of HIV to heparan sulfate on spermatozoa (Ceballos, et al., 2009). Another way semen may facilitate HIV transmission is by increasing secretion of CCL20 from cervical and vaginal epithelial cells (D. J. Sharkey, et al., 2007), leading to the recruitment of LC and Th17 target cells to genital tissues (Berlier, et al., 2006; D. J. Sharkey, Tremellen, et al., 2012). Complement may also enhance HIV infection as opsonisation of HIV enhanced infection of T cells, B cells, PBMCs, epithelial cells, and primary monocyte/macrophage cultures (Bouhlal, et al., 2002; Bouhlal, et al., 2001; Delibrias, Fischer, & Kazatchkine, 2000; Stoiber, et al., 1997). Lastly, semen may enhance HIV replication by promoting the survival of target cells. A study showed that IL-7, a cytokine involved in the maintenance of T cell homeostasis, was abundant in semen and enhanced HIV replication by stimulating the proliferation of T cells, and preventing their apoptosis (Introini, Vanpouille, Lisco, Grivel, & Margolis, 2013).

In contrast, other factors present in semen have been shown to prevent HIV infection. One way this is achieved is by competing with HIV for binding sites on target cells. Such factors include clusterin (Sabatte, et al., 2007; Sabatte, Faigle, et al., 2011), and mucin-6 (Stax, et al., 2009) which have been shown to compete with HIV for binding sites on DC-SIGN on the surface of target cells, which may aid in preventing HIV infection. Although not conclusive, it has been hypothesized by Sabatté et al., that high

concentrations of immunoregulatory factors present in semen may suppress innate and adaptive immune responses to HIV (Sabatte, Remes Lenicov, et al., 2011).

To date, it is unclear what role semen alteration of vaginal pH plays in HIV infection. The healthy vaginal microenvironment is acidic (pH 4.0-6.0), whereas semen pH is slightly alkaline (pH 7.2-7.8). Deposition of semen into the vagina raises the pH of the vaginal mucosa to values of 6.0-7.0 for several hours following sex (Bouvet, Gresenguet, & Belec, 1997; Masters & Johnson, 1961). The role of semen alteration of vaginal pH on HIV infectivity remains unclear as studies have showed that a lower pH enhanced (Connor, 2006), or decreased (Kempf, et al., 1991; Shattock & Moore, 2003) HIV-1 infectivity.

Cell-associated HIV contained in infected immune cells in semen may also play an important role in promoting HIV transmission. It has been suggested that the virus could be protected by external anti-viral factors which can limit its infectivity, making it effectively transmitted to other target cells via viral synapse (Politch, et al., 2014). It is believed that infected macrophages are the main host cell mediators of cell-associated HIV transmission as they are the most abundant HIV host cell in semen outnumbering CD4+ T cells at a ratio of 22:1 in one study (Politch, Mayer, & Anderson, 2009). *In vitro*, both macrophages and CD4+ T cells isolated from semen from HIV-positive men were able to transmit HIV to target PBMCs making them attractive targets for microbicide and vaccine interventions (Quayle, Xu, Mayer, & Anderson, 1997).

1.3.4 Molecular Characteristics of TGF-β

Transforming Growth Factor Beta (TGF-β) is a member of the transforming growth factor beta superfamily which includes over 30 growth factors including activin, anti-mullerian hormone, inhibins, nodals, and bone morphogenic protein (D. A. Lawrence, 1996). TGF-β1, the prototype of the family, shares 74% amino acid sequence homology and protein structure with the other two mammalian isoforms TGF-β2, and -β3 despite being encoded by different genes (Derynck, et al., 1985; Gentry & Nash, 1990; Schlunegger & Grutter, 1992; ten Dijke, et al., 1990). The TGF-β genes encode a 390-412 amino acid inactive heterodimeric polypeptide precursor consisting of an N-terminal 65-80 kDa latency-associated peptide (LAP) subunit, and a C-terminal 12.5 kDa protein dimer which forms the mature TGF-β. The purpose of the LAP is to facilitate transport of TGF-β from the cell as well as keep the protein biologically inert (Lopez, Cook, Deininger, & Derynck, 1992). Plasmin proteolysis, deglycosylation, or acidic conditions are needed to dissociate the LAP from the C-terminal subunit to form the 25 kDa bioactive, mature TGF-β homodimer which is recognized by TGF-β receptors (Khalil, 1999; Lyons, Gentry, Purchio, & Moses, 1990; Lyons, Keski-Oja, & Moses, 1988; Miyazono & Heldin, 1989). Extremes in pH or heat as well as treatment with detergents, urea or chaotropic agents *in vitro* as well as the generation of reactive oxygen species *in vivo* can also dissociate the LAP (Barcellos-Hoff, Derynck, Tsang, & Weatherbee, 1994; P. D. Brown, Wakefield, Levinson, & Sporn, 1990; Munger, et al., 1997; Robertson, et al., 2002). Additionally, conformational changes in the inactive TGF-β precursor can also occur via interactions between LAP domain sequences and the glycoprotein

thrombospondin-1 or the epithelial cell integrin αvβ6 resulting in the exposure of receptor binding sites (Crawford, et al., 1998; Munger, et al., 1999). The αvβ6 integrin is expressed on cervical and uterine epithelium demonstrating that TGF-β can be activated in the FGT (Breuss, Gillett, Lu, Sheppard, & Pytela, 1993; Lessey, et al., 1992).

1.3.5 TGF-β Signalling Pathway

Once activated, the mature TGF-β homodimer forms a binding complex with the TGF-β receptor, which is comprised of two type I (TGFBR1) and two type II (TGFBR2) transmembrane serine/threonine kinase receptors (Weiss & Attisano, 2013; Wrana, Attisano, Wieser, Ventura, & Massague, 1994). Binding is initiated between the mature TGF-β and the TGFBR2, which leads to phosphorylation of the TGFBR1 kinase. The TGFBR1 initiates downstream signalling to the nucleus via activation of a family of intracellular mediators called Smads. TGFBR1 phosphorylates the receptor-regulated Smads2 and 3 (R-Smads), which in turn form a heterodimeric complex with the common-mediator Smad4 (Co-Smad4). The Smad complex accumulates in the nucleus where they regulate gene transcription.

In addition to R-Smads and Co-Smads, a third class of Smad, known as inhibitory Smads (I-Smads), exists which serves as a negative regulator of TGF-β signalling (Massague & Chen, 2000; Weiss & Attisano, 2013). Smad7, the key antagonistic Smad for the TGF-β signalling pathway, inhibits R-Smad activation by forming a more stable interaction with phosphorylated TGFBR1 (Heldin, Miyazono, & ten Dijke, 1997). Thus, no R-Smad complexes with Co-Smads are formed. Smad7 is found predominantly in the nucleus at basal state and exports to the cytoplasm upon TGF-β stimulation (Itoh, et al.,

1998). Expression of Smad7 is increased in response to TGF-β autocrine signals, as well as by cytokines which negatively regulate TGF-β such as IFN-γ, TNF-α and IL-1β which increase Smad7 expression via NF-κB/RelA activation (Bitzer, et al., 2000; Ishisaki, et al., 1999; Nakao, et al., 1997).

SMAD-independent TGF-β signaling pathways have also been reported which include activation of MAP kinase kinases (MKK) and MAP/ERK kinases (MEK) such as the p38, ERK1/2 and JNK pathways (Moustakas, Pardali, Gaal, & Heldin, 2002). Like the SMAD-dependent pathway, SMAD-independent pathways are initiated via the binding of TGF-β to the type II receptor and the formation of the hetero-tetrameric receptor complex. Downstream signaling of the SMAD-independent pathway is carried out through the binding of RhoA, Ras, TAK1, MEK and other mediators to the TGF-β receptor complex resulting in p38, JNK and ERK1/2 transcription factors regulating gene expression in the nucleus.

1.3.6 Expression and Function of TGF-β

TGF-β and its receptors are ubiquitously expressed in most tissues and cells and perform a diverse set of biological functions including cell proliferation, recognition, differentiation, and apoptosis, as well as tissue repair and specification, and developmental processes such as embryogenesis. TGF-β is also known for its potent immunoregulatory functions (Chang, Brown, & Matzuk, 2002; Govinden & Bhoola, 2003; M. O. Li & Flavell, 2008; M. O. Li, Wan, Sanjabi, Robertson, & Flavell, 2006; Wan & Flavell, 2007). Of the three mammalian isoforms, TGF-β1 is predominantly expressed in the immune system with potent immunoregulatory properties (Chang, et al.,

2002; Govinden & Bhoola, 2003; Yoshimura & Muto, 2010). In fact, TGF-β1 deficient mice develop a multi-organ autoimmune inflammatory disease and die within a few weeks of birth (Kulkarni, et al., 1993; Shull, et al., 1992). Multiple cell types can be regulated by TGF-β through the following mechanisms: conversion of naive T cells into Foxp3-positive regulatory T cells, suppression of effector T cell differentiation, inhibition of T and B cell proliferation, suppression of macrophages, DCs and NK cells, and inhibition of IL-2, IFN-γ and IL-4 effector cytokine production (Yoshimura & Muto, 2010). TGF-β is a pleiotropic cytokine with potent immunoregulatory and inflammatory activity depending on the local environment (Sanjabi, Zenewicz, Kamanaka, & Flavell, 2009). While TGF-β exerts potent immunosuppressive properties, such as in the context of IL-10 or PGE2 (Baratelli, et al., 2005; M. O. Li & Flavell, 2008; Robertson, Prins, Sharkey, & Moldenhauer, 2013), it also plays a role in inflammation through differentiation of naive T cells to proinflammatory Th17 cells with the aid of IL-6 (Korn, Bettelli, Oukka, & Kuchroo, 2009; Mangan, et al., 2006; Veldhoen, Hocking, Flavell, & Stockinger, 2006). Additionally, TGF-β in combination with IL-4 promotes the differentiation of IL-9 and IL-10-producing T cells, which lack suppressive function and also promote tissue inflammation (Dardalhon, et al., 2008; Veldhoen, et al., 2008).

Interestingly, TGF-β appears to act in a biphasic fashion with respect to certain biological functions such as angiogenesis and bone formation. An earlier study showed that at low concentrations of TGF-β (100pg/ml-1ng/ml) promoted VEGF and bFGF-induced endothelial invasion, whereas high concentrations of TGF-β (5-10ng/ml) inhibited endothelial cell invasion and capillary lumen formation in an *in vitro* endothelial

cell model (Pepper, Vassalli, Orci, & Montesano, 1993). Osteoblast differentiation by TGF-β has been shown to be time-dependent as initial exposure of osteoblasts with TGF-β stimulated their differentiation, but prolonged exposure inhibited their differentiation (de Gorter, van Dinther, Korchynskyi, & ten Dijke, 2011). Another study in mouse bone marrow cultured showed that low concentrations (10-100pg/ml) TGF-β enhanced production of osteoclast-like cells, but high concentration of TGF-β (4ng/ml) was inhibitory (Shinar & Rodan, 1990). Taken together, these studies suggest that some functions of TGF-β appear to be biphasic and are dependent on concentration and exposure time.

1.3.7 The Role of TGF-β in HIV-1 Infection

HIV infection has been found to increase expression of TGF-β in a variety of tissues with HIV-infected individuals having elevated TGF-β levels in their blood and CSF (Kekow, et al., 1990; Navikas, Link, Wahren, Persson, & Link, 1994). Several research groups have shown that the HIV Tat protein can induce TGF-β expression in different cell types including mitogen-stimulated PBMCs, monocytes, T cells, splenocytes, thymocytes, and astrocytes *in vitro and in vivo* (Gibellini, et al., 1994; Hori, Burd, Kutza, Weih, & Clouse, 1999; Hu, et al., 1996; M. D. Johnson, Kim, Tourtellotte, & Federspiel, 2004; Navikas, et al., 1994; Reinhold, Bank, Buhling, Lendeckel, et al., 1997; Reinhold, Bank, Buhling, Tager, et al., 1997; Sawaya, et al., 1998).

Despite evidence of increased TGF-β expression with HIV infection, the role of TGF-β in HIV infection and pathogenesis is not well defined as studies suggest that TGF-β may inhibit or enhance HIV infection. TGF-β may promote HIV infection by enhancing

expression of surface receptors utilized by HIV such as CXCR4 on macrophages (S. Chen, et al., 2005) and CD169 on mucosal DCs (De Saint Jean, Lucht, Bourlet, & Delezay, 2014), or by increasing Tat-induced HIV LTR activation (Moriuchi & Moriuchi, 2004). It has also been suggested that HIV replication is only partially controlled by HIV-specific activated effector T cells in chronic HIV infection since it may be hindered by the immunosuppressive functions of TGF-β (Lind, et al., 2014; Lotz & Seth, 1993).

Other studies have suggested that TGF-β may prevent HIV infection. According to Poli et al., TGF-β may be a negative regulator of HIV infection in infected monocytes or macrophages as it suppressed HIV expression and replication in chronically infected target cell lines and primary monocytes-derived macrophages, respectively (Poli, et al., 1991). TGF-β has been shown to downregulate expression of the lymphocyte proliferation cytokine IL-7 i*n vitro* suggesting that it may aid in the control of immune activation during HIV infection (G. Lee, Namen, Gillis, Ellingsworth, & Kincade, 1989; Tang, et al., 1997). However, TGF-β-mediated regulation of effector T cell responses protects the host from damage in persistent immune activation, but may also impair effective immune control via suppression of cellular and humoral immune responses (Kekow, et al., 1990; Kekow, et al., 1991; Lind, et al., 2014; Wiercinska-Drapalo, Flisiak, Jaroszewicz, & Prokopowicz, 2004). Interestingly, it has been shown that one of the reasons for generalized T cell activation during HIV-1 and SIV_{mac} infections in humans and macaques is the inability to respond to TGF-β during early infection due to upregulation of the TGF-β pathway inhibitor Smad7; thus preventing the control of virus-driven inflammation (Ploquin, et al., 2006).

1.4 THE FEMALE GENITAL TRACT (FGT)

1.4.1 Anatomy and Cell Composition

The FGT can be sectioned into distinct upper and lower tracts which, under the influence of estrogen and progesterone, function to initiate and maintain pregnancy, respond to sexually transmitted infections, and tolerate commensal organisms, sperm and the semi-allogeneic fetus. The upper FGT is composed of the fallopian tubes, endocervix, and uterus, and is composed of a single layer of columnar epithelial cells (Ferreira, Kafka, & Kaushic, 2014; Kaushic, 2011; Nguyen, et al., 2014). The fallopian tubes, aka oviducts, function to aid in the passage of the oocyte from the ovary to the uterine opening during ovulation. Ciliated columnar epithelium, and peristaltic action by the subserosa layer contribute to the movement of the ovum from fimbrie projections in the oviduct ampulla to the uterus (Ovalle & Nahirney, 2013). Implantation and fetal development occur in the uterus, which is composed of three layers; the outermost layer containing mostly connective tissue is the perimetrium, followed by the smooth muscle-containing myometrium, and finally the mucosal endometrium which faces the lumen (Ovalle & Nahirney, 2013). Separating the uterus from the vaginal canal is the cervix. It contains two anatomic regions: the endocervix which opens into the uterus (internal os), and the ectocervix which opens into the vagina (external os). The transformation zone, where single columnar epithelium transitions to the multilayered squamous epithelium of the endocervix and ectocervix respectively, is an immune cell rich area containing macrophages, DCs, intraepithelial CD8+ T cells, and CD4+ T cells (Pudney, Quayle, & Anderson, 2005). Neighbouring columnar epithelial cells of the upper FGT express tight

junctions which prevent the unwanted passage of microbes and other factors (Blaskewicz, Pudney, & Anderson, 2011; Ferreira, et al., 2014).

The vagina is the female copulation organ and is the other member of the lower FGT along with the ectocervix. It is composed of three layers: the mucosa consisting of rugae of non-keratinized squamous epithelium with lamina propria, the muscularis comprised of smooth muscle, and the connective tissue-containing adventitia (Ovalle & Nahirney, 2013). Since the vagina has no glands, it is bathed in mucus secreted from glands in the cervix. Under the influence of estrogen, the vaginal epithelium secretes glycogen which contributes to the colonization of commensal *Lactobacillus* strains populating the vaginal tract (Rogosa & Sharpe, 1960; Spear, et al., 2014). Lactobacilli utilization of glycogen contributes to acidic pH in the vaginal tract. Although the squamous epithelium of the ectocervix and vagina contains multiple layers as a defence mechanism against the passage of organisms, its superficial layers do not contain tight junctions and can therefore be "leaky", whereas its basal layers actively proliferate, are metabolically active, and have tight junctions (D. J. Anderson, Marathe, & Pudney, 2014; Blaskewicz, et al., 2011). Intracellular lymphocytes such as DCs, CD8+ T cells, and CD4+ T cells are found within the multiple layers of squamous epithelium in the vagina (Hladik & McElrath, 2008). Beneath the epithelial layer of the upper and lower FGT is the lamina propria containing fibroblasts, blood vessels, and immune cells such as macrophages, DCs, natural killer cells, and CD4+ T cells (Nguyen, et al., 2014).

1.4.2 Epithelial Cell Innate Immune Responses in the FGT

Genital epithelial cells (GECs) lining the upper and lower FGT have been described as sentinels of the mucosal immune system in that they are the first responders to a variety of antigenic stimuli including pathogens, semen, and the allogeneic fetus (Nguyen, et al., 2014; Wira, Grant-Tschudy, & Crane-Godreau, 2005). GECs recognize and differentially respond to a series of pathogens via expression of a wide repertoire of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), and NOD-like receptors (NLRs), which in turn leads to messaging to other innate and adaptive immune cells (Nguyen, et al., 2014). Endometrial epithelial cells express TLRs 1 to 9, as well as NOD1 and NOD2 (Wira, Fahey, Sentman, Pioli, & Shen, 2005). Endocervical epithelial cells express TLRs 1 to 3 and 6 (Wira, Fahey, et al., 2005). PRR recognition of pathogens initiates an intracellular cascade resulting in the activation of transcription factors such as NF-κB and the production of cytokines and chemokines such as IL-6, IL-8, TNF-α, SDF-1, MIP-1α, MIP-1β, and RANTES (Nguyen, et al., 2014; Schaefer, Desouza, Fahey, Beagley, & Wira, 2004). GECs can also induce type I IFN responses in response to viral infections (Schaefer, Fahey, Wright, & Wira, 2005). Aside from PRR-mediated induction of cytokines and chemokines, GECs also produce a variety of innate antimicrobial peptides (AMPS) in response to pathogens. AMPS secreted by FGT epithelial cells include α- and β-defensins, calthelicidin, lactoferrin, lysozyme, as well as the serine protease inhibitors SLPI, cystatins, and elafin (Wiesner & Vilcinskas, 2010; Wira, Fahey, et al., 2005; Wira, Patel, Ghosh, Mukura, & Fahey, 2011). The mechanisms involved in the anti-microbial properties of AMPs include inhibition of inflammation, sequestration

of iron required by microbes, and enzymatic digestion of bacterial cell walls (Aboud, Ball, Tjernlund, & Burgener, 2014; Wiesner & Vilcinskas, 2010; Wira, Fahey, et al., 2005). AMP levels and their biological activity can be regulated by the menstrual cycle, changes in pH, and the presence of microbiota and sperm (Nguyen, et al., 2014). The secretion of mucus by cervical glands in the epithelium, although not an active response to pathogens per se, can prevent the interaction and penetration of pathogens with the FGT mucosa (Ferreira, et al., 2014).

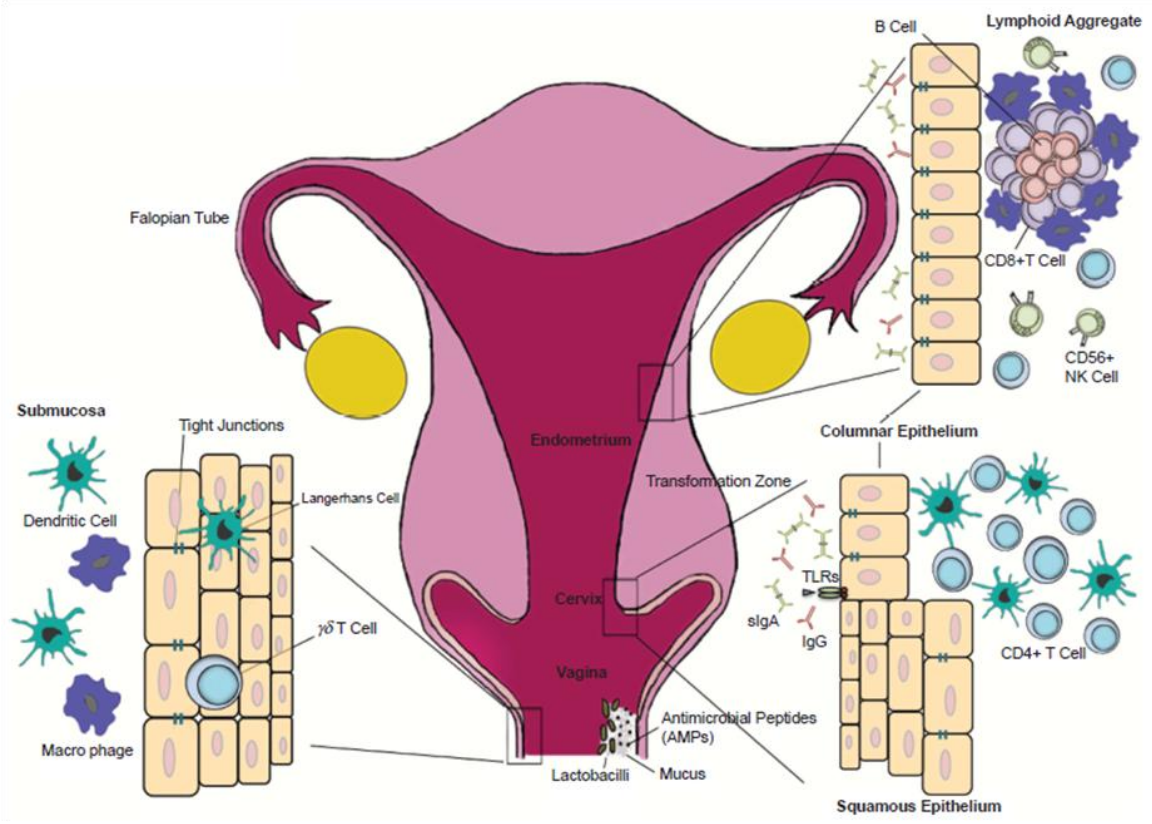


Image from Nguyen et al., 2014. Used with permission.

Figure 4: Anatomical and immunological components of the female genital tract. The FGT consists of an upper (fallopian tubes, uterus and endocervix) and a lower (ectocervix and vagina) tract. The vaginal epithelium has many innate immune-mediated protection mechanisms, such as tight junctions, AMPs and mucus, to neutralize, trap, and prevent the entry of potential pathogens. The vaginal lumen is colonized by commensal bacteria, mainly *Lactobacilli* spp., which help maintain a low pH environment and produce reactive oxygen species. Furthermore, innate immune cells, such as γδ T cells, DCs, and macrophages, are present beneath and between vaginal epithelial cells layer to survey the local environment for danger. The abrupt transition from keratinized squamous epithelial cells of the ectocervix to single columnar epithelial cells of the endocervix represents the transformation zone; this site has an abundance of HIV target cells (DCs and $CD4^+$ T cells) and has been proposed to be one of the major sites of infection. Although traditional mucosal lymphoid structures are not found in the FGT, lymphoid aggregates in the endometrial tissue that are composed of B cells in the inner core and surrounded by $CD8^+CD4^-$ T cells and an outer layer of macrophages have been described. Scattered $CD56^+$ NK cells and $CD4^+$ T cells can be found between lymphoid aggregates. The immune cells and functions of the FGT are regulated by sex hormones that orchestrate cyclical changes with the menstrual cycle. Image and figure legend used with permission from Nature Publishing Group.

1.4.3 The Epithelial Tight Junction Barrier and its Regulation

The epithelial barrier functions to regulate molecular and cellular traffic between neighbouring epithelial cells and providing a physical barrier against pathogen invasion (Blaskewicz, et al., 2011). Three types of cell-cell structural adhesions make up the epithelial barrier: tight junctions, adherens junctions, and desmosomes (Langbein, et al., 2002; Marchiando, et al., 2011). Tight junctions are composed of the peripheral membrane protein zona occluden (ZO), and the transmembrane proteins occludin, junctional adhesion molecules (JAMs) and the claudin family of proteins which make contact across the intercellular space and restrict paracellular movement of molecules and microorganisms (Mitic & Anderson, 1998; Nusrat, Turner, & Madara, 2000). The adherens junctions (zonula adherens) consist of transcellular E-cadherin dimers anchored to the cytoskeleton by α- and β-catenin (Meng & Takeichi, 2009). Adherens junctions connect bundles of actin filaments between cells to form a "continuous adhesion belt"

which is normally located below the tight junctions (Bass-Zubek & Green, 2007; Marchiando, et al., 2011). Desmosomes (macula adherens) form a strong structural framework by connecting keratin filaments between cells (Gorodeski, 2007; Marchiando, et al., 2011). A recent study characterizing the distribution, structure and function of intercellular junction proteins in the FGT showed that classical tight junctions comprise the principle junction proteins between epithelial cells in the endocervix, endometrium and lower layers of the ectocervix and vagina, whereas the uppermost layers of the ectocervix and vagina were devoid of organized intracellular junctions (Blaskewicz, et al., 2011).

The epithelial barrier at mucosal surfaces is dynamically regulated by agents such as calcium concentration, growth factors, cytokines, bacterial and viral products, and in the case of the FGT, sex hormones (Al-Sadi, Boivin, & Ma, 2009; Gorodeski, 2007; Grant-Tschudy & Wira, 2004; Nusrat, et al., 2000; Sawada, et al., 2003; Shen, Weber, & Turner, 2008; Yeo & Jang, 2010). It was previously shown that treatment of mouse polarized uterine epithelial cells with estradiol (E2) significantly decreased transepithelial electrical resistance (TER) within 24 hours of exposure whereas incubation with progesterone had no effect on TER (Grant-Tschudy & Wira, 2004). A number of intestinal pathogens such as astrovirus, rotavirus, *Clostridium difficile*, *Campylobacter jejuni* and enteropathogenic *Escherichia coli* have been shown to increase mucosal permeability by disrupting tight junctions (M. L. Chen, Ge, Fox, & Schauer, 2006; Glotfelty & Hecht, 2012; Moser, Carter, & Schultz-Cherry, 2007; Nava, Lopez, Arias, Islas, & Gonzalez-Mariscal, 2004; Nusrat, et al., 2001).

The influence of HIV on barrier integrity was originally characterized in the intestine where it was thought to cause barrier dysfunction by increasing proinflammatory cytokine production by mucosal T cells (Brenchley & Douek, 2008; Redd, et al., 2009; Stockmann, et al., 1998). Our lab was the first to demonstrate that HIV can have a profound effect on the epithelial barrier in the FGT. We showed that incubation of confluent genital and intestinal epithelial cell monolayers with R5, X4, clinical strains of HIV, or gp120 caused an increase in epithelial barrier permeability as measured by a decrease in TER, leakage of blue dextran dye, virus and bacterial translocation across GEC monolayers, and decreased immunofluorescent staining of tight junction proteins (Nazli, et al., 2010). It was determined that the decrease in epithelial barrier integrity was mediated by TNF-α production by GECs in response to HIV since incubation with TNF-α antibody prevented reduction of TER. Follow-up studies showed that HIV gp120 interacted with TLR2 and -4 with heparan sulfate on the surface of GECs, which initiated downstream signaling of proinflammatory pathways (Nazli, et al.).

Additionally, SP itself is also capable of regulating the epithelial barrier. SP from healthy, uninfected men caused a rapid increase in TER in cervical and endometrial epithelial cell lines (Gorodeski & Goldfarb, 1998; P. Lawrence, et al., 2012). Lawrence et al., also determined that the presence of SP increased the expression and distribution of ZO-1 as well as significantly decreased the number of transmigrated peripheral blood monocytes in the basolateral compartment of the endometrial EC monolayer (P. Lawrence, et al., 2012). Although neither paper determined which factors in SP were

mediating this barrier-maintaining effect, Gorodeski et al., believed that the factor was a labile, low molecular weight lipid (Gorodeski & Goldfarb, 1998).

TGF-β can also regulate the epithelial barrier and appears to function in a site-specific manner. Several studies have shown that TGF-β strengthens the intestinal epithelial barrier by upregulating expression of the tight junction proteins claudin-1 and -4 (Hering, et al., 2011; Howe, Reardon, Wang, Nazli, & McKay, 2005), as well as preventing barrier permeability mediated by IFN-γ and TNF-α (S. Planchon, Fiocchi, Takafuji, & Roche, 1999; S. M. Planchon, Martins, Guerrant, & Roche, 1994), and by the intestinal pathogens *E. coli* O157:H7 (Howe, et al., 2005) and *Cryptosporidium parvum* (Roche, Martins, Cosme, Fayer, & Guerrant, 2000). It has also been shown that TGF-β can increase mRNA and protein expression of the desmosome proteins desmoplakins I and II in bronchial ECs (Yoshida, et al., 1992). In contrast, TGF-β induced disruption of the blood-testes barrier between Sertoli cells in rat testes during spermatogenesis by decreasing expression of occludin, ZO-1 and claudin-11 suggesting TGF-β regulation of barrier integrity is tissue dependent (Lui, Lee, & Cheng, 2001, 2003a, 2003b). Although many studies have outlined the function of TGF-β in the intestinal, lung, and MGT mucosa, it is not clear how TGF-β influences epithelial cells lining the FGT.

1.4.4 HIV-1 Infection in the FGT

Estimates show that approximately 40% of HIV infections are initiated in the FGT (Hladik & McElrath, 2008). Studies in non-human primate models, *ex-vivo* cervicovaginal tissues, and *in vitro* cell cultures have demonstrated that HIV infection can occur in both the upper and lower FGT (Nguyen, et al., 2014). Mechanisms directly

involving the FGT epithelium include micro tears in the squamous epithelium during intercourse, as well as sequestration, direct infection, transcytosis, and paracellular passage of the virus in columnar epithelial cells (Haase, 2011; Nguyen, et al., 2014). Studies in nonhuman primates showed that SIV preferentially crossed the epithelial barrier in the endocervix and established small foci of infection within 48 to 72 hours, followed by local amplification in the genital mucosa before systemic dissemination (Haase, 2011). It is believed that early HIV infection in the FGT occurred primarily in target T cell populations, and to lesser extent DCs, found in the submucosa as well as within the epithelial lining (Hladik & McElrath, 2008). In fact, recent studies have shown that T helper cells expressing α4β7, CCR5, IL-17A and IFN-γ are highly susceptible to HIV infection and are depleted in HIV infected women (Nguyen, et al., 2014).

However, before HIV-1 can establish a productive infection in the FGT, it must first overcome an arsenal of mechanical, chemical, and biological barriers including mucus, AMPs, type I IFNs secreted by both innate immune cells and epithelium, tight junctions between columnar epithelial cells, restriction factors, and vaginal acidic pH. 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).

Cytokines and chemokines found in the FGT can interfere with HIV infection. SDF-1 found in the subepithelial layer of the cervix competitively inhibited X4 strains of HIV (Agace, et al., 2000). As well, MIP-1α, MIP-1β and RANTES produced by CD8+ and CD4+ T cells in the FGT competitively bind to the CCR5 receptor thus preventing infection of R5 strains (Abdelwahab, et al., 2003; Cocchi, et al., 1995; Fahey, Schaefer, Channon, & Wira, 2005). Serine protease inhibitors have also been shown to exert anti-HIV effects indirectly by controlling inflammation (Aboud, et al., 2014).

In contrast, co-infecting STIs can promote HIV infection. STIs such as gonorrhea, syphilis, bacterial vaginosis, candidiasis, and genital herpes have been associated with increased HIV genital shedding, transmission, and susceptibility (Atashili, Poole, Ndumbe, Adimora, & Smith, 2008; Celum, 2004; Corbett, et al., 2002; Freeman, et al., 2006; L. F. Johnson & Lewis, 2008; Mitchell, et al., 2009; Mole, Ripich, Margolis, & Holodniy, 1997; Rotchford, Strum, & Wilkinson, 2000; J. Zhu, et al., 2009). Increased HIV acquisition may relate to local micro-ulcerations due to the pathologies associated with the infection (Plummer, 1998). Additionally, different cells in the FGT upregulate inflammatory responses in response to STIs which can enhance HIV infection. Proinflammatory cytokines have been implicated in enhancing HIV infection at the cellular level as studies of latently infected target cells have shown that the addition of cytokines such as IL-1β, IL-6, or TNF-α increased HIV replication, mediated through activation of the HIV LTR (Chun, Engel, Mizell, Ehler, & Fauci, 1998; Folks, et al., 1989; Poli, et al., 1990). Our lab previously showed that in response to common co-infecting STIs, specifically HSV-1, HSV-2, and *N. gonorrhea,* primary GECs upregulated

proinflammatory cytokines including IL-6, IL-8, MCP-1 and TNF-α, which contributed to indirect induction of the HIV LTR promoter in T cells (V. H. Ferreira, et al., 2011). Recruitment of activated immune cells may also act as targets for HIV (Plummer, 1998). By blocking inflammatory signaling pathways, either with the broad anti-inflammatory compound curcumin or with specific transcription factor inhibitors, induction of proinflammatory cytokines could be blocked suggesting that inflammation may play a major role in the acquisition or spread of HIV infection (Deeks, Lewin, & Havlir; V. H. Ferreira, et al., 2011).

Several studies have indicated that endogenous female sex hormones and exogenous hormonal contraceptives affect HIV infection or disease progression (reviewed in (Kaushic, Roth, Anipindi, & Xiu, 2011). Results from *in vivo* nonhuman primate studies suggest that estrogen protects against SIV infection (S. M. Smith, Baskin, & Marx, 2000) whereas progesterone increases susceptibility (Marx, et al., 1996; Trunova, et al., 2006). In fact, progesterone has been routinely used in laboratories to increase the susceptibility to vaginal SIV infection in female macaques (Poonia, et al., 2006; Veazey, et al., 2003). In contrast, macaques were protected from SIV infection when given either subcutaneous estrogen implants (S. M. Smith, et al., 2000) or topical estrogen cream (S. M. Smith, et al., 2004) prior to intravaginal SIV challenge.

Altered susceptibility of the FGT to HIV infection with female sex hormones occurs through several different mechanisms such as modulation of epithelial thickness (Felding, Mikkelsen, Clausen, Loft, & Larsen, 1992; S. Gupta, et al., 2006; Molander, Milsom, Ekelund, Mellstrom, & Eriksson, 1990; S. M. Smith, et al., 2000; S. M. Smith, et

al., 2004); alteration of vaginal pH (Castelo-Branco, Cancelo, Villero, Nohales, & Julia, 2005; Molander, et al., 1990; P. Smith, 1993; S. M. Smith, et al., 2000); and modulation of the innate and adaptive arms of the mucosal immune system of the FGT (Kaushic, Roth, et al., 2011).

1.5 Rationale, Hypothesis and Thesis Objectives

Heterosexual transmission is the primary cause of HIV infection in developing countries (Shattock & Moore, 2003; UNAIDS, 2013) with semen being the main vector for male-to-female HIV transmission during vaginal sex (Doncel, Anderson, & Zalenskaya, 2014). Our knowledge of the role of semen in the FGT mainly involves how SP modulates the FGT immune system to tolerate sperm and the allogeneic fetus for successful pregnancy (Schjenken & Robertson, 2014). On the topic of how semen influences HIV infection, studies have examined how different components in semen may influence HIV infection in target cells including macrophages and T cells (Doncel, et al., 2010; Sabatte, Remes Lenicov, et al., 2011). However, the epithelial cells that line the genital mucosa are the first cells that semen and HIV encounter. Epithelial cells are exquisitely responsive to various types of stimulation and studies have shown that GECs respond to semen and microorganisms by producing a variety of immune factors. Therefore, it would be important to understand how semen from HIV-infected men modulates female GEC responses since studies have only examined GEC responses and barrier function in the context of healthy, HIV-negative semen, not in the context of HIV infection (Denison, Grant, Calder, & Kelly, 1999; Gorodeski & Goldfarb, 1998; Gutsche, von Wolff, Strowitzki, & Thaler, 2003; P. Lawrence, et al., 2012; O'Leary, Jasper,

Warnes, Armstrong, & Robertson, 2004; D. J. Sharkey, et al., 2007). During heterosexual transmission, infection occurs in the presence of semen from an infected male partner. It is not yet known how SP from HIV-infected men may be different in the concentration and types of cytokines and chemokines present, as well as how they influence cytokine responses in female GECs. SP is known to contain high concentrations of the immunoregulatory factor TGF-β1, whose main function is to promote a tolerogenic environment in the FGT during conception. How such high levels of TGF-β will impact FGT epithelial cell cytokine responses and barrier function in the presence of HIV-1 has not been studied. We know that HIV infection leads to altered proinflammatory cytokine levels and elevated TGF-β expression in blood, but whether HIV infection leads to changes in proinflammatory and immunoregulatory factors in semen needs to be studied. It is also known that one of the characteristics of acute HIV infection is elevated systemic proinflammatory cytokine expression (Cohen, Shaw, et al., 2011; McMichael, Borrow, Tomaras, Goonetilleke, & Haynes, 2010). I therefore asked the question: Does SP from HIV-infected men elicit different responses from female GECs than SP from HIV-uninfected men? And are these differences due to varying concentrations of proinflammatory and immunoregulatory cytokines present in SP from HIV-infected and uninfected men? How do these changes impact FGT cytokine responses and barrier function? The answers to these questions will be important factors in the establishment of HIV-infection in the FGT.

Therefore, my overall hypothesis was that SP from HIV-infected men in acute/early infection will have increased levels of proinflammatory cytokines compared to HIV-uninfected men. The increased proinflammatory cytokines in HIV infected SP will correlate with higher proinflammatory cytokine production from GECs and increased barrier permeability. Furthermore, we predicted that the ratio of proinflammatory cytokines and the immunoregulatory factor TGF-β in SP of HIV infected men at various stages of infection will ultimately influence cytokine production and barrier function in GECs.

The overall objectives of this thesis were to examine immune factors in SP of HIV infected men to and elucidate how SP from HIV-infected men modulated FGT epithelial cell cytokine responses, as well as determine how these responses affected the epithelial barrier function. These objectives were addressed through the following aims:

1. Compare proinflammatory and immunoregulatory cytokine and chemokine concentrations in SP from HIV-uninfected men and HIV-infected men in acute and chronic stages of HIV infection.
2. Compare cytokine and chemokine responses from female GECs exposed to SP from HIV-uninfected and HIV-infected men.
3. Elucidate how SP and TGF-β, an immunoregulatory cytokine found in high concentrations in SP, regulates proinflammatory cytokine production and barrier function in female GECs in the presence of HIV.

--- CHAPTER 2 ---

Endometrial epithelial cell response to semen from HIV-infected men during different stages of infection is distinct and can drive HIV-1 LTR.

Jessica K KAFKA[1], Prameet M SHETH[2], Aisha NAZLI[1], Brendan J OSBORNE[2], Colin KOVACS[3], Rupert KAUL[2], Charu KAUSHIC[1].

[1]Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario, Canada; [2]Department of Medicine, University of Toronto, Toronto, Ontario, Canada; [3]Canadian Immunodeficiency Collaborative, Toronto, Ontario, Canada.

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2.1 PREFACE

This manuscript is an original contribution to knowledge as it shows for the first time that SP contains immunomodulatory components that vary depending on presence or absence of HIV infection as well as the stage of infection. Furthermore, exposure to SP can induce a pro-inflammatory response from GECs that can be moderated by high levels of TGF-β or by blocking the NF-κB pathways in GECs. The inflammatory response of GECs to SP can induce HIV replication in infected cells present in the upper FGT. Thus controlling the pro-inflammatory environment in the FGT may be a key step in controlling HIV replication.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments, and I was responsible for the generation and analysis of the data. Dr. Aisha Nazli provided technical assistance. Drs. Brendan Osborne, Prameet Sheth and Rupert Kaul provided seminal plasma samples and expertise. Dr. Colin Kovacs established the semen cohort at the Maple Leaf Clinic in Toronto. Dr. Charu Kaushic and I wrote and edited the manuscript.

2.2 ABSTRACT

Objective: Although >60% of HIV transmission occurs via semen, little is known about the immune impact of seminal plasma (SP) on HIV susceptibility. Here, we examined: (1) the level of selected immunomodulatory factors in SP from HIV-uninfected and therapy-naive, HIV-infected men in acute (AI) and chronic (CI) stages; (2) the cytokine response elicited by SP in genital epithelial cells (GECs); and (3) whether any GEC response to SP could drive HIV replication in infected T cells.

Methods: A panel of nine cytokines and chemokines was measured in SP from HIV-uninfected and infected men, and in primary GEC cultures following SP exposure. HIV-LTR activation was measured in 1G5 T cells exposed to supernatants from SP-treated GECs.

Results: Pro-inflammatory cytokines and chemokines were present at significantly higher levels in SP from AI men, while TGF-β1 was significantly higher in SP from CI men. Pro-inflammatory cytokine production by GECs was significantly decreased following incubation with SP from CI men. Blocking the TGF-β1 receptor in GECs prior to SP exposure enhanced pro-inflammatory cytokine production. Exposure to SP activated NF-κB in GECs and blocking it significantly reduced pro-inflammatory cytokine production. GEC responses to SP, especially from AI men, significantly activated HIV-LTR activation in 1G5 T cells.

Conclusions: Immunomodulatory factors in SP vary, depending on presence and stage of HIV infection. Exposure to SP leads to NF-κB activation and pro-inflammatory cytokine production while TGF-β in SP may suppress pro-inflammatory cytokine production by GECs. GEC responses to SP can activate HIV-LTR in infected CD4+ T cells.

2.3 INTRODUCTION

Approximately 60-90% of human immunodeficiency virus type 1 (HIV-1) transmission is sexual (Shattock & Moore, 2003), and globally women account for over half of the individuals living with HIV (Organization, 2009). Most women acquire HIV during receptive vaginal sex, when the mucosal lining of the female genital tract (FGT) is exposed to cell-free or cell-associated virus from the semen of their infected partner.

While seminal plasma (SP) is primarily a transport and support medium for spermatozoa during coitus (Aumuller & Riva, 1992), SP can also transform the molecular and cellular environment of the FGT resulting in altered susceptibility to pathogens.

Evidence from *in vitro* studies is equivocal whether semen predominantly inhibits or facilitates HIV infection. Semen-mediated effects which could enhance HIV infection include increased viral attachment to target cells by SEVI amyloid fibrils (Munch, et al., 2007), lowering of pH in vaginal fluid following ejaculation, recruitment of Langerhans cells by CCL20/MIP-3α found in semen (Berlier, et al., 2006; Sabatte, et al., 2007), the presence of heparan sulphate on spermatozoa that could bind HIV (Ceballos, et al., 2009) and complement activation (Bouhlal, et al., 2002). On the other hand, SP inhibited HIV-1 attachment to DC-SIGN, possibly decreasing trans-infection of CD4+ T cells. Additionally, protection of target cells by semenogelin-1 (Martellini, et al., 2009) and reactive oxygen species (Agarwal & Prabakaran, 2005) from SP could also inhibit HIV infection. Semen may also play a dual role by reducing CD4 and CXCR4 expression on T cells while increasing CCR5 expression (Balandya, Sheth, Sanders, Wieland-Alter, & Lahey, 2010). Furthermore, TGF-β1 in human SP, found in one of the highest concentrations in any biological fluid, may play both pro-inflammatory and immunosuppressive roles in the FGT (Robertson, et al., 2002).

In vitro studies have shown that SP can upregulate expression of pro-inflammatory cytokine mRNA in GECs and cell lines (Gutsche, et al., 2003; D. J. Sharkey, et al., 2007), indicating that SP may play an indirect role in determining the outcome of interactions between FGT and HIV. GECs are known to respond potently and

differentially to sexually transmitted pathogens (Wira, Grant-Tschudy, et al., 2005). We recently demonstrated that upper GECs produce an array of pro-inflammatory cytokines in response to HIV exposure, herpes simplex type 2 (HSV-2) and *Neisseria gonorrhoeae* (V.H. Ferreira, et al., 2011) (V. H. Ferreira, et al., 2011). However, the immune impact of SP from HIV-infected men on GECs has not been examined, nor is it clear whether putative SP immunomodulatory factors vary by stage of HIV infection, independent of the HIV RNA viral load (Chakraborty, et al., 2001; Pilcher, et al., 2004).

The objective of the current study was to compare the immunomodulatory factors present in the SP between HIV-uninfected men and HIV-infected, treatment-naive men during the acute (AI) and chronic (CI) stages of HIV infection. We also compared the ability of SP from men in different stages of HIV infection to elicit cytokine responses from primary GECs grown *ex vivo*, and determined whether GEC responses to SP could indirectly influence HIV-1 LTR in infected T cells.

2.4 METHODS

Patient participation

Endometrial tissues were obtained from women aged 30-59 years (mean age 42.9 $\pm$ 7.2) undergoing hysterectomies for benign gynaecological reasons at Hamilton Health Sciences Hospital. The most common reasons for surgery were uterine fibroids and menorrhagia (heavy bleeding). Informed written consent was obtained in accordance with the approval of the Hamilton Health Sciences Research Ethics Board.

HIV-infected, antiretroviral therapy (ART)-naïve men were recruited through the Canadian Immunodeficiency Research Collaborative (CIRC; Toronto, ON, Canada); all

were men who have sex with men (MSM). Uninfected SP was obtained from either heterosexual male volunteers or uninfected MSM recruited through the CIRC. Informed written consent was obtained in accordance with the approval of the Research Ethics Boards at the Mount Sinai Hospital, the University Health Network and at the University of Toronto.

SP Collection and Preparation

Semen from HIV-uninfected and HIV-infected men was collected and SP was isolated as previously described (Osborne, Sheth, Kovacs, Mazzulli, & Kaul, 2011; Sheth, et al., 2005). All study participants had to abstain from sexual intercourse or masturbation for 48 hours prior to sample donation. Semen was collected directly by masturbation into 10 mL of sterile RPMI 1640 (Gibco) containing 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). Seminal plasma was cryopreserved at -80°C after centrifugation to pellet semen lymphocytes and spermatozoa. SP HIV-1 RNA measurements were performed by Versant HIV-1 RNA 3.0 assay (Bayer Diagnostics; lower limit of detection, 50 RNA copies/mL) at Mount Sinai Department of Microbiology-Public Health Lab (Sheth, et al., 2009). Classification of study participants as having low (<5000 RNA copies/ml) or high (>5000 RNA copies/ml) semen viral loads was based on previous studies (Sheth, et al., 2009) (Coombs, et al., 1998). All SP samples were further diluted 1:10 prior to addition to GEC cultures. Semen volume was calculated by subtracting the volume of RPMI (10ml) from the total sample volume. Where total sample volume was less than 10ml, a mean semen volume of 2 ml was used for dilution calculations, based on previous studies (Sheth, et al., 2009).

Primary GEC culture preparation

Detailed protocol for isolation and culture of GECs has been described previously (MacDonald, et al., 2007). Briefly, endometrial tissues were minced into small pieces and digested in an enzyme mixture at 37^{o}C with shaking. GECs were isolated by a series of separations through nylon mesh filters (Small Parts Inc., Miramar, FL, USA), resuspended in DMEM/F12 primary growth medium (Invitrogen, Burlington, ON, Canada) (MacDonald, et al., 2007; Nazli, et al., 2010). and seeded onto Matrigel^{TM} (Becton Dickinson and Company, Mississauga, ON, Canada) coated tissue culture inserts (BD Falcon, Mississauga, ON, Canada). GEC cultures were grown for 5-7 days until confluent monolayers were formed. The confluency was monitored by trans-epithelial resistance (TER) measured by a volt ohm meter (EVOM; World Precision Instruments, Sarasota, FL, USA). Confluent monolayers showing TER values greater than 1000 Ω/cm were used for further experiments. The purity of GEC monolayers was between 95% and 98%, with no trace of any hematopoietic cells (MacDonald, et al., 2007).

Cytokine analysis

SP samples and apical and basolateral supernatants from GECs were analyzed for multiple cytokines simultaneously using the Luminex 100 (Luminex Corporation, Austin, TX, USA) as previously described (Fernandez, Gillgrass, & Kaushic, 2007; Nazli, et al., 2009). Multiplex bead-based sandwich immunoassay kits (Millipore, Billerica, MD, USA) were used to measure concentrations GM-CSF, IL-1α, IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α as per the manufacturer's instructions. Samples were run in duplicate. Quantikine® Human TGF-β1 Immunoassay, and Parameter^{TM} PGE2 Assay

kits (R&D Systems, Inc., Minneapolis, MN, USA) were used to measure active TGF-β1 and prostaglandin E2 (PGE2) respectively, as per manufacturer's instructions.

Exposure of GEC cultures to SP

GEC cultures were exposed to 100µl of SP on their apical surface for 4 hours at 37^{o}C, cells were washed and fresh growth medium added. Incubation with SP concentration >10% and longer exposure times were found to affect the viability of GECs. Control cultures underwent the same exposure protocol with medium alone, without SP. After 20 hours post-treatment, apical and basolateral supernatants were collected and frozen at -80^{o}C. TERs of GEC monolayers were monitored before and after treatment.

Exposure of GEC cultures to TGF-β receptor and signalling pathway inhibitors

GECs were pre-incubated with SB 431542 hydrate (Sigma, Saint Louis, MO, USA), a selective inhibitor of TGF-β type 1 receptor kinases ALK-4, -5, and -7 (reconstituted in dimethyl sulfoxide: DMSO), diluted to 10 µM in PBS for 1 hour at 37^{o}C prior to exposure to SP. To examine the involvement of signalling pathway, the apical surface of confluent GEC cultures were pre-treated for 1 hour with 10 µM of SP600125 (Sigma, Saint Louis, MO, USA) AP-1 inhibitor or 10 µM of PDTC (Sigma, Saint Louis, MO, USA), a selective inhibitor for NF-κB at 37^{o}C and 5% CO_2 (V.H. Ferreira, et al., 2011).

Immunofluorescent staining of activated NF-κB

Following SP exposure, GECs were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with blocking solution (2% goat serum) containing 0.1% Triton X . Primary antibody was rabbit anti-p65/RelA NF-κB (Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by AlexaFluor goat anti-rabbit IgG-FITC detection antibody (Molecular Probes, Eugene, OR). Propidium iodide (Molecular Probes, Eugene, OR) was used for nuclear counterstain. Imaging was done on an inverted confocal laser-scanning microscope (LSM 510, Zeiss, Germany). Standard operating conditions were 63x objective, optical laser thickness 1µm, image dimension of 512 x 512, lasers: argon (450nm) for NF-κB and HeNe (543nm) for nuclear staining. For each experiment, settings for image acquisition were identical between control and treated monolayers.

Measurement of HIV LTR Activity

HIV LTR activity was measured as described before(V.H. Ferreira, et al., 2011). Briefly, apical supernatants from GEC cultures exposed to SP from HIV-uninfected and HIV-infected men were incubated at a 1:1 dilution (vol/vol) with $1x10^6$ 1G5 Jurkat T cells for 24 hours at 37^oC. Following incubation, 1G5 cells were lysed and LTR activation was measured as luciferase activity in relative light units (RLU) using the Stratagene luciferase assay kit (Agilent Technologies, La Jolla, CA, USA) as per manufacturer's instructions.

Statistical methods

GraphPad Prism version 4 (GraphPad Software, San Diego, CA) was used for statistical analysis and graphical representation. Differences in pairs of treatments were

compared using the Student unpaired *t*-test. All *t*-tests were two-tailed, and an alpha value of 0.05 was set for statistical significance. *p*-values for each analysis are indicated in figure legends.

2.5 RESULTS

Semen donor demographics

Semen from 9 HIV-uninfected and 19 HIV-infected, therapy-naive men was used in the study. Of the 9 HIV-uninfected men, 7 were MSM and 2 were heterosexual. Seven of the 19 HIV-infected men were within 6 months of their first HIV-positive test and considered to be in the acute/early phase of infection. At their first HIV positive test, these individuals were either negative or indeterminate by immunoblot. Details of clinical parameters and semen samples are summarized in Table 1. Men who had been infected with HIV for 6 months or longer were considered to be in chronic phase of the infection.

Immunomodulatory cytokine and chemokine levels in seminal plasma

To determine whether different stages of HIV infection were associated with differences in the presence or concentration of immunomodulatory factors in SP, 9 cytokines and chemokines were measured in SP samples from HIV-uninfected, AI and CI men. IL-8 and MCP-1 were present in high concentrations (>1000pg/ml) in all SP samples (Fig.1A). IL-6, IL-8, and MCP-1 levels were elevated in SP from AI men compared to HIV-uninfected and CI men. All cytokines, except IL-1α were elevated in SP from AI men, compared to HIV-uninfected men. IL-1α, MIP-1α, and TNF-α levels were also elevated in SP from CI men compared to HIV-uninfected men.

Active TGF-β1 and prostaglandin E2 (PGE2) levels were also measured since previous studies have shown that SP from HIV-uninfected men contained high concentrations of these immunomodulatory factors (Robertson, et al., 2002). Strikingly, SP from CI men contained a 3-8 fold higher concentration of active TGF-β compared to SP from AI and HIV-uninfected men (Fig. 1A). No differences in SP PGE2 concentrations were observed between the three groups (Fig. 1A).

The data was further analyzed to determine if semen viral load was associated with differences in the concentration of immunomodulatory factors in SP. Based on previous studies, semen viral load of 5000 RNA copies/ml was taken as the cut-off to distinguish between HIV-infected low and high shedders (Sheth, et al., 2009) (Coombs, et al., 1998). IL-6, RANTES, and TGF-β levels were significantly higher in low shedders compared to high shedders and uninfected men.

GEC cytokine responses to SP from HIV-uninfected and HIV-infected men

To evaluate whether GECs respond differentially to SP from HIV-uninfected and HIV-infected men, SP was added to the apical surface of GECs and cytokine production was measured. Induction of cytokines following SP exposure was only observed in the apical compartment. The most striking difference noted was the significant decrease in production of the pro-inflammatory cytokines IL-1α, IL-6 and TNF-α after GEC exposure to SP from CI men, compared to HIV-uninfected and AI men (Fig. 2A). GM-CSF and IL-8 production by GEC was seen to increase in all groups following exposure to SP, although this trend did not reach significance (not shown). SP had no effect on GEC production of MCP-1, MIP-1α, MIP-1β or RANTES (not shown). When GEC cytokine

production was correlated with semen viral loads, no significant trends were observed (not shown). Thus cytokine responses by GECs appear to correlate mainly with stage of HIV-1 infection.

To exclude the possibility that exposure to SP could lead to production of TGF-β by GECs and autocrine regulation of pro-inflammatory cytokines, TGF-β1 production in GEC cultures was measured. While detectable levels of TGF-β were observed, there were no significant differences before or after SP exposure (results not shown).

Effect of TGF-β receptor inhibitor on GEC cytokine production in response to SP

Since the most remarkable difference in GEC responses was decreased pro-inflammatory cytokine responses following exposure to SP from CI men, we evaluated whether this effect was mediated by the higher active TGF-β levels found in SP from this group. Confluent cultures were pre-treated with SB431542, a TGF-β1 receptor inhibitor alone or prior to incubation with SP from HIV-uninfected men and CI men (Fig. 1B). Pre-treatment with TGF-β1 receptor inhibitor prior to exposure to SP significantly increased pro-inflammatory cytokines TNF-α (Fig. 2B, C), IL-1α, IL-6.

SP causes activation and nuclear translocation of the NF-κB in GECs

Since SP consistently increased pro-inflammatory cytokine production by GECs, we examined the intracellular pathways that may be activated in GECs following exposure to SP. Following exposure to SP from HIV-uninfected men, a rapid response in GECs visualized by NF-κB activation and nuclear translocation was seen at 30 minutes post-exposure to SP (Fig. 3A).

To further confirm the involvement of NF-κB signalling pathway in GEC cytokine responses to SP, we pre-treated GEC cultures with inhibitors for NF-κB and AP-1 alone or prior to incubation with SP from HIV-uninfected men. IL-6 and TNF-α expression in GECs was significantly decreased in response to pre-treatment with NF-κB inhibitor (Fig. 3B). No significant differences in GEC cytokine responses to SP were observed following pre-treatment with AP-1 inhibitor (data not shown), suggesting that cytokine induction was via an NF-κB dependent pathway.

GEC response to SP from HIV-uninfected and HIV-infected men can activate HIV-LTR

The distinct pro-inflammatory cytokine response of GECs to SP prompted us to determine whether this could lead to LTR activation in HIV-infected T cells that may be present in the FGT at the same time as GECs are responding to SP. Supernatants from GECs treated with SP from HIV-uninfected, AI and CI men were incubated with 1G5 Jurkat T cells and luciferase activity was measured. Irrespective of the presence of HIV or the stage of HIV disease, supernatants from GECs exposed to SP significantly increased the LTR activity in the 1G5 cells compared to supernatants from untreated GECs (Fig. 4A). Supernatants from GEC cultures treated with SP from AI men increased LTR activity significantly higher compared to GECs treated with SP from HIV-uninfected men and CI men. To determine if high TGF-β1 levels in SP had an effect on HIV LTR activation, supernatants from GEC cultures pre-treated with TGF-β1 receptor inhibitor prior to exposure to SP from CI men were incubated with 1G5 Jurkat T cells. Supernatants from GECs pre-treated with TGF-β1 receptor inhibitor showed a 5.5-fold

increase in LTR activity in the 1G5 cells compared to supernatants from untreated GECs and GECs treated with SP from CI men alone (Fig. 4B).

2.6 DISCUSSION

We have demonstrated that SP from HIV-uninfected and HIV-infected men differs substantially with respect to levels of the pro-inflammatory cytokines as well as the immunoregulatory factor TGF- β, and that these differences were dependent mainly on the stage of HIV infection. Furthermore, depending on the net biological effect of the combination of these pro- and anti-inflammatory factors, SP induced differential immune responses from the GECs of the upper FGT. Specifically, the combination of increased levels of pro-inflammatory cytokines with low levels of TGF-β in SP from AI group resulted in induction of increased pro-inflammatory cytokines by GECs. Consequently, these pro-inflammatory responses activated HIV-LTR to the highest level among the different groups. In contrast, TGF-β levels in CI samples that were 3-8 fold higher, compared to other groups, appeared to suppress the production of inflammatory cytokines by GECs and subsequently led to lower LTR activation. In the SP of the uninfected group, the combination of moderate amounts of pro-inflammatory cytokines and TGF-β resulted in induction of modest cytokine responses from GECs and upregulation of HIV-LTR.

Although previous studies have documented the presence of immunomodulatory factors in SP, to the best of our knowledge this is the first study to examine the difference in cytokine profiles between HIV-uninfected men and HIV infected men at different stages of infection (Penna, et al., 2007; Politch, et al., 2007). A recent study found

significant differences in cytokines in SP from HIV-uninfected men and CI men, with higher semen:blood ratios of GM-CSF, IL-1β, IL-4, IL-6, IL-7, IL-8, and MCP-1 in the latter group (J. A. Anderson, et al., 2010). Since we measured levels of cytokines in SP rather than the semen: blood ratio, it is difficult to compare the results of these two studies directly.

We found significant differences in TGF-β levels in SP of different groups. TGF-β plays an immunomodulatory role both systemically (Lotz & Seth, 1993; Poli, et al., 1991) and in the FGT (Robertson, et al., 2002) and suppresses HIV expression and replication in the chronically infected pro-monocytic cell line U1 (Poli, et al., 1991). Seminal plasma is known to contain one of the highest levels of TGF-β of all biological fluids (Robertson, et al., 2002). That SP of therapy-naive CI men contains 3-8 times higher levels of TGF-β compared to uninfected and AI men has not been reported before. These results suggest that seminal TGF-β is a key immunomodulator in the FGT, and that this effect is greatest in SP from CI men. This was confirmed by the observation that GEC pro-inflammatory cytokine production was upregulated and highest HIV LTR activation was induced by CI SP when the TGF-β receptor was blocked. While up to half of all HIV transmission is thought to occur during the early stages of HIV infection, it has been assumed that the major driver of this increased transmission is the higher semen viral load seen during acute infection. In our study, the correlation of semen viral load was seen only with IL-6, IL-8 and TGF-β1 levels in SP. Cytokine induction in GECs did not correlate with viral load, although this could likely be due to SP dilution. Overall, our

results suggest that the semen immune milieu during acute HIV stages may play an important role in enhancing HIV transmission.

Previous studies have examined the effect of SP from uninfected men on the cells of the upper and lower female reproductive tract *in vitro*. Studies done in mice have shown that SP may play a role in embryo implantation by stimulating release of pro-inflammatory cytokines, particularly GM-CSF by mouse uterine epithelial cells (K. P. Tremellen, et al., 1998). Human SP has also been shown to upregulate mRNA for IL-6, IL-8, GM-CSF, and MCP-1 in primary cervical epithelial cells and vaginal cell lines exposed to 10% SP and similar results were seen in pigs and rodent studies (O'Leary, Jasper, Robertson, & Armstrong, 2006; Robertson, et al., 2002; Robertson, Mau, Tremellen, & Seamark, 1996; D. J. Sharkey, et al., 2007). Our results are consistent with these studies in that SP upregulated the production of a number of different pro-inflammatory cytokines, including IL-1α, IL-6 and TNF-α. However, unlike the mice studies, we did not find a consistent upregulation of GM-CSF by GECs in response to SP, perhaps due to species differences. To the best of our knowledge this is the first study to demonstrate that exposure to SP could alter the microenvironment in the upper female genital tract in a way that could affect local HIV replication and hence host HIV susceptibility.

This study adds to the growing evidence that GECs recognize and respond in unique ways to a variety of stimuli, including pathogenic bacteria, virus and semen itself (Kaushic, 2009). If the responses of the epithelium, the first cells to encounter external stimuli, are predominantly pro-inflammatory this would create a microenvironment in the

genital tract that could facilitate HIV infection or replication of virus already present. Support for this premise comes from our recent studies that demonstrated that GECs respond to HIV surface glycoprotein by production of inflammatory cytokines, including TNF-a, that in turn impair the mucosal barrier and facilitate viral and bacterial translocation into the reproductive tract (Nazli, et al., 2010). More recently, we have reported that GEC responses to other sexually transmitted co-infections, including HSV-2 and *Neisseria gonorrhoeae,* facilitate HIV LTR activation in infected T cells (V. H. Ferreira, et al., 2011). Combined evidence from these studies suggests that an inflammatory microenvironment in the upper female genital tract, whatever the cause, is conducive to HIV infection and replication, and that HIV prevention and therapeutic strategies might benefit from targeting local genital inflammation. An important consideration for future studies would be to examine the role female sex hormones play in modulating GEC responses. HIV levels have been shown to increase in the progesterone-dominant luteal phase (Reichelderfer, et al., 2000), suggesting a role for hormones in regulating a pro-inflammatory microenvironment in the FGT. Studies are currently ongoing to examine the role of female sex hormones on GEC responses.

In conclusion, our results show that SP contains immunomodulatory components that vary depending on presence or absence of HIV infection as well as the stage of infection. Further, exposure to SP induces a pro-inflammatory response from GECs that can be moderated by high levels of TGF-β or by blocking the NF-κB pathways in GECs. The inflammatory response of GECs to SP can induce HIV replication in infected cells

present in the upper FGT. Thus controlling the pro-inflammatory environment in the female reproductive tract may be a key step in controlling HIV replication.

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--- CHAPTER 2 FIGURES ---

Table 1. Details of clinical data and semen samples from ART-naïve HIV-infected men used in the study

Sample ID	Disease Progression	HSV-2 Status	Semen Viral Load (RNA Copies/mL)	Blood Plasma Viral Load (RNA copies/mL)	CD4 Count (cells/mm^3)	Semen Volume (ml)*
OM 189	Acute	-	4,452	182,302	520	12.4
OM 159	Acute	+	45,216	>500,000	740	7
OM 209	Acute	-	1,475,004	124,109	340	11.2
OM 253	Acute	+	187,428	462,535	180	11
OM 264	Acute	+	20,760	N/A	N/A	11
OM 359	Acute	-	378	3,399	375	13.5
OM 5223	Acute	-	8,154	36,681	610	11.5
CIRC 0096	Chronic	+	<300	<50	650	10.5
CIRC 0253	Chronic	-	930	8454	270	10.5
OM 208	Chronic	+	3,396	636	120	16.5
OM 314	Chronic	-	9,474	12,600	560	13
OM 315	Chronic	-	1,260	25,159	540	11.5
OM 363	Chronic	-	1,806	86,385	N/A	12
OM 389	Chronic	+	86,856	N/A	102	N/A
OM 5031	Chronic	-	<300	5,569	260	10
OM 5039	Chronic	+	2,088	9,833	400	10
OM 5170	Chronic	+	106,674	>500,000	210	12
OM 5206	Chronic	-	15,696	32,572	670	12
OM 5210	Chronic	-	12,498	30,122	210	12

HIV uninfected samples: Nine HIV-negative semen samples were used in the study. Seven were MSM volunteers and two were heterosexual volunteers. All nine HIV uninfected volunteers were HSV-2 negative. Semen volumes ranged between 12 ml and 13 ml with an average volume of 12.5ml.

*Semen volume was calculated by subtracting the volume of RPMI (10ml) from the total sample volume. Where total sample volume was less than 10 ml, a mean semen volume of 2 ml was used for dilution calculations, based on previous studies.

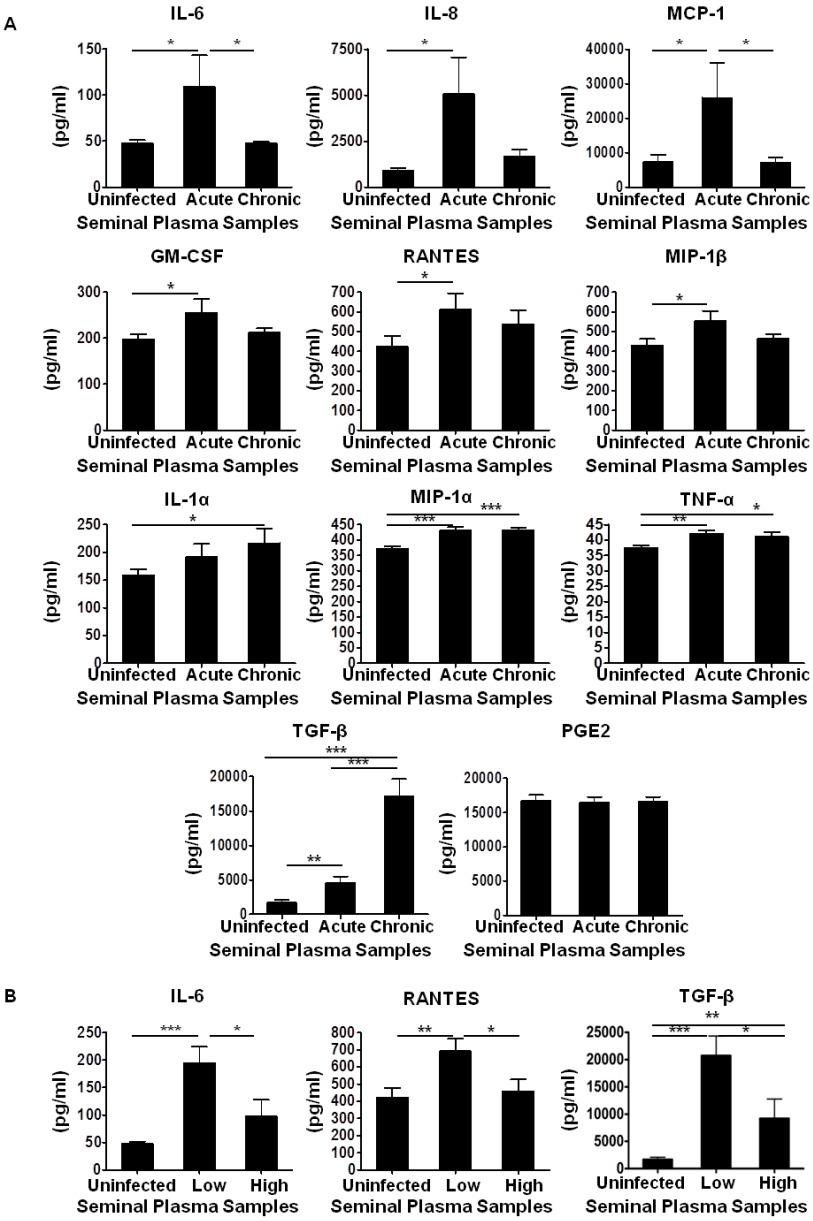

Fig. 1. Measurement of immunomodulatory factors in SP from HIV-uninfected and HIV-infected men. Cytokine, chemokine and PGE2 concentrations in SP samples were assayed by Multiplex bead-based sandwich immunoassay and ELISA. A) Immunomodulatory factors were correlated with stage of HIV infection. HIV-infected men were within 6 months of their first HIV-positive test and considered to be in the acute/early phase of infection. Data is shown from analysis of SP from uninfected (n=9), AI (n=7) and CI (n=12). B) Immunomodulatory factors were correlated with viral load in semen. Semen viral load <5000 RNA copies/ml was considered low and >5000 RNA copies/ml was considered high. Data is shown from analysis of SP from low (n=9) and high (n=10). Cytokine concentrations are presented in neat (undiluted) SP. *P < 0.05; **P < 0.01; *** P <0.001. Bars represent mean and standard error of the mean (SEM).

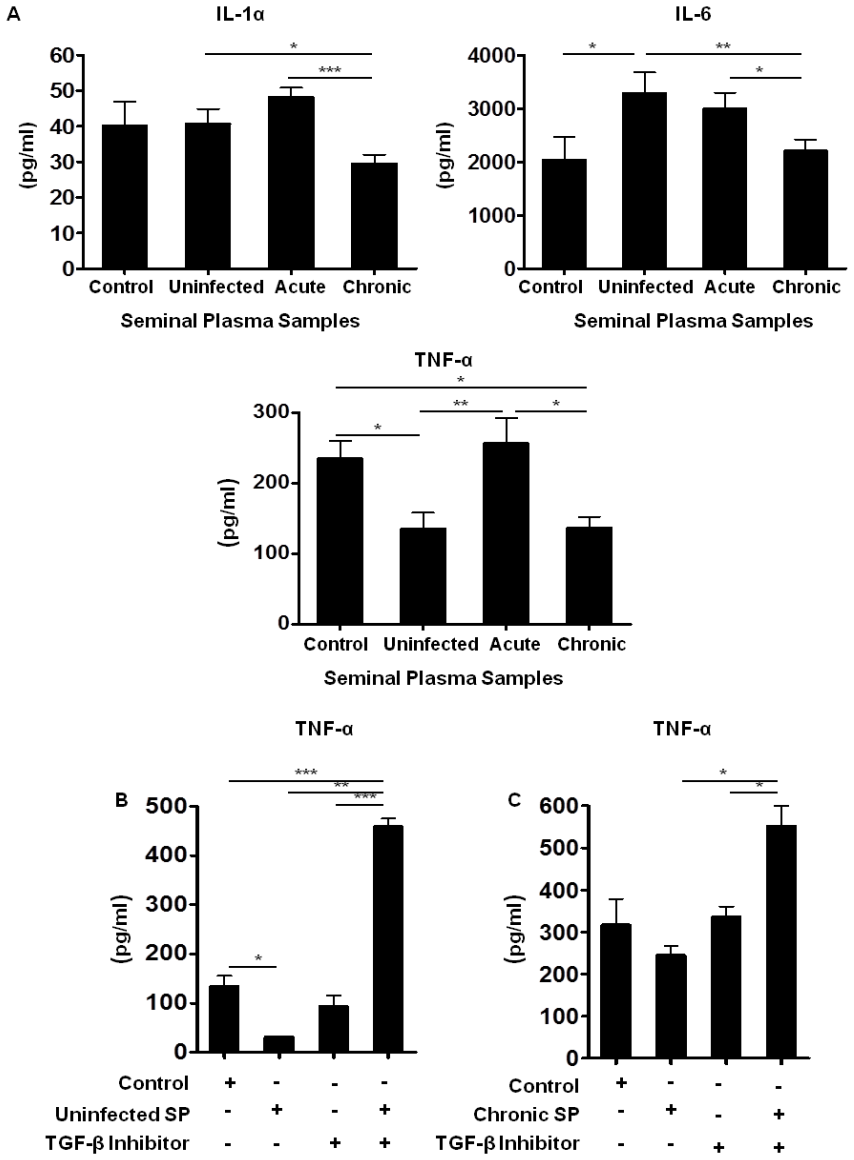

Fig. 2. Effect of SP from HIV-uninfected and HIV-infected men on female GEC cytokine responses. A) Confluent cultures of GECs were incubated with SP from HIV-uninfected men, and AI and CI men. Control represents GECs exposed to growth media alone. Apical and basolateral supernatants were collected and assayed for cytokines and chemokines using a Multiplex bead-based sandwich immunoassay (see Materials and Methods). Duplicate wells were run for each treatment. The experiment was repeated on 4 separate donor endometrial tissues (n=4). B & C) GECs were pre-treated apically with 10μM SB-431542 TGF-β1 receptor inhibitor alone or prior to exposure to 10% uninfected SP (B) or 10% Chronic SP (C). Control represents GECs exposed to growth media alone, without SP or inhibitor. Apical supernatants were collected and assayed using a multiplex bead-based sandwich immunoassay. N=2. *P < 0.05; **P < 0.01; *** P <0.001. Bars represent mean and standard error of the mean (SEM).

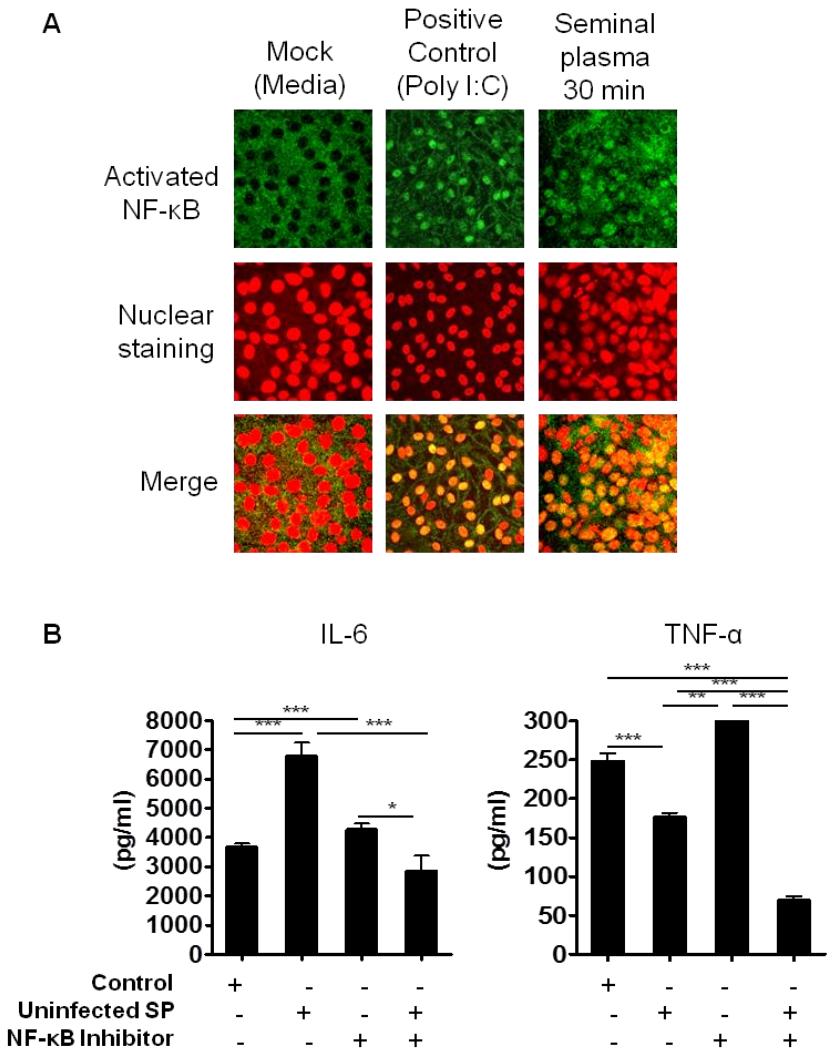

Fig 3. GEC responses to SP from HIV-uninfected men is dependent on NF-κB pathway. A) GECs were treated with SP from HIV-uninfected men for 30 minutes and 1 hour at 37°C, fixed and stained with an anti-NF-κB antibody, a FITC-conjugated detection antibody, and Propidium iodide counterstain. Stained cells were visualized by confocal microscopy (see Materials and Methods). NF-κB staining was observed by the presence of FITC (green) fluorescence and cell nuclei were visualized by Propidium iodide counterstain (red). An overlap of both colours (yellow) indicates nuclear translocation of NF-κB. Figure shown is representative of 3 separate experiments with similar results. Each condition was performed in duplicate. B) GECs were pre-treated with a selective inhibitor for NF-κB (PDTC) alone or prior to being exposed to SP from HIV-uninfected men for 4 hours at 37°C. Control represents GECs exposed to growth media alone, without SP or inhibitor. Apical supernatants were collected and assayed using a multiplex bead-based sandwich immunoassay (see Materials and Methods). Figure shown is representative of 3 separate experiments with similar results. Duplicate wells were run for each treatment. *P < 0.05; **P < 0.01; *** P <0.001. Bars represent mean and standard error of the mean (SEM).

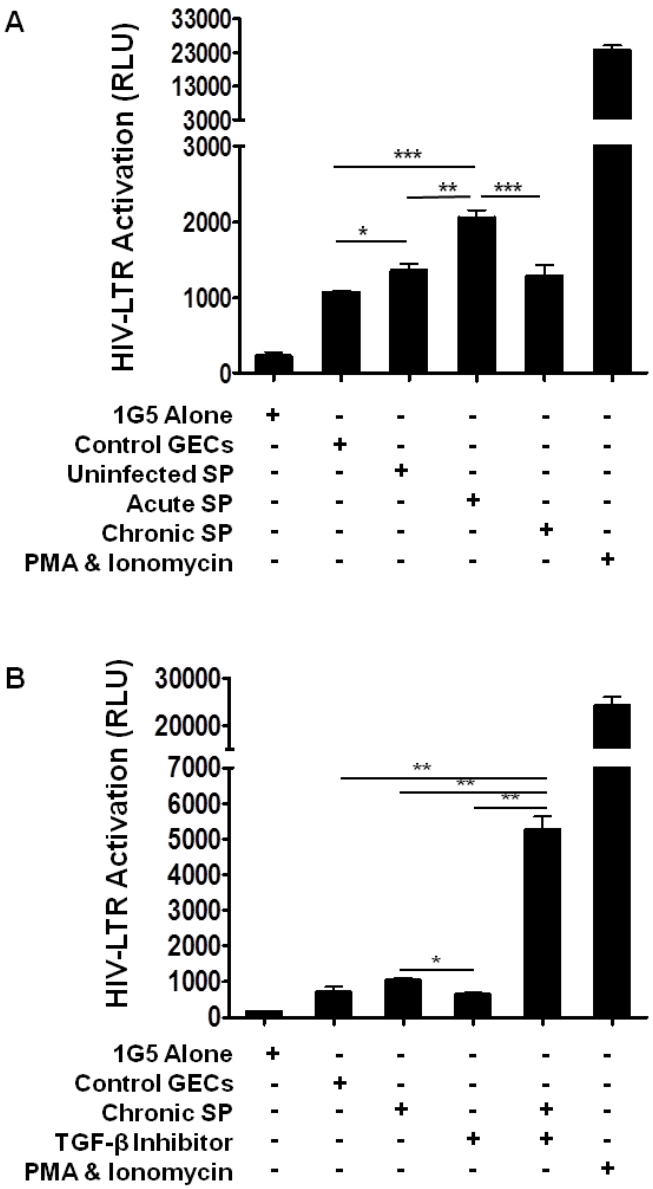

Fig. 4. Supernatants from GECs treated with SP can trigger the HIV LTR. Apical supernatants from A) GECs treated with SP from HIV-uninfected men, AI and CI men and B) GECs treated with TGF-β receptor inhibitor alone or prior to exposure to SP from CI men were incubated with $1x10^6$ 1G5 cells. 1G5 alone and PMA/Ionomycin treatment served as negative and positive controls respectively. Control GECs represents GECs exposed to growth media alone. Cells were lysed and luciferase activity was measured using the Stratagene luciferase assay kit (See Materials and Methods). GEC supernatants and treatments added to 1G5 cells are represented on the x-axis. Figures shown are representative of 2 separate experiments. 1G5 cells were treated in duplicate for each treatment. *P < 0.05; **P < 0.01; *** P <0.001. Bars represent mean and standard error of the mean (SEM).

--- CHAPTER 3 ---

Latent TGF-β1 is compartmentalized between blood and seminal plasma of HIV-positive men and its activation in semen is negatively correlated with viral load and immune activation.

Jessica K KAFKA[1]; Brendan JW OSBORNE[2]; Prameet M SHETH[3,4]; Aisha NAZLI[1]; Sara DIZZELL[1], Sanja HUIBNER[2]; Colin KOVACS[5]; Chris P VERSCHOOR[1]; Dawn M BOWDISH[1]; Rupert KAUL[2]; Charu KAUSHIC[1§].

[1]Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario, Canada; [2]Department of Medicine, Laboratory Medicine and Pathobiology and Immunology, University of Toronto, Toronto, Ontario, Canada; [3]Kingston General Hospital, Kingston, Ontario, Canada; [4]Department of Pathology & Molecular Medicine, Queens University, Kingston, Ontario, Canada; [5]Maple Leaf Medical Clinic, Toronto, Ontario, Canada.

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3.1 PREFACE

The current study showed that TGF-β1 is compartmentalized between blood and semen and its latent form may be co-expressed with sCD14 by activated monocytes/macrophages in BP as a result of HIV infection. Conversion of latent TGF-β1 into its active form could contribute to regulation of semen viral load and local immune activation in the male genital tract, but this regulation depends on the stage of infection.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments, and I was responsible for the generation and analysis of the data. Sara Dizzell and Drs. Aisha Nazli, Chris Verschoor, and Dawn Bowdish provided technical assistance. Drs. Brendan Osborne, Prameet Sheth and Rupert Kaul, and Sanja Huibner provided seminal plasma samples and expertise. Dr. Colin Kovacs established the semen cohort at the Maple Leaf Clinic in Toronto. Dr. Charu Kaushic and I wrote and edited the manuscript.

3.2 ABSTRACT

Problem: Semen is the primary medium for sexual transmission of HIV-1 and contains high concentrations of TGF-β1, but its role in regulating HIV-mediated immune activation is unclear.

Method of Study: TGF-β1 and sCD14 were compared in blood plasma (BP) and seminal plasma (SP) from HIV-uninfected and infected, ART-naive and -treated men and in THP-1 cells following exposure to HIV-1. The relationship between TGF-β1 and sCD14 was determined by Spearman rank correlation.

Results: Active and latent forms of TGF-β1 were compartmentalized between BP and SP. Highest active TGF-β1 levels were present in SP of ART-naïve chronic-infected men and decreased following ART treatment. Latent TGF-β1 was upregulated in BP following HIV infection and highest levels were observed in BP of acute-infected men. Similar expression trends were observed between latent TGF-β1 and sCD14 in BP. A significant negative correlation was observed between active TGF-β1, sCD14, and semen viral load in ART-naive men.

Conclusions: TGF-β1 is compartmentalized between blood and semen, possibly co-expressed with sCD14 by activated monocytes/macrophages in BP as a result of HIV infection. Conversion of latent TGF-β1 into its active form could contribute to regulation of viral load and immune activation in the male genital tract, but depends on the stage of infection.

3.3 INTRODUCTION

Sexual transmission accounts for 60 to 90% of HIV infections globally with semen being the main vector for transmission (Sabatte, Remes Lenicov, et al., 2011; UNAIDS/WHO, 2010). Semen is more than a transport vector of HIV-1, containing a complex array of immune and non-immune factors that may play a role in facilitating or inhibiting HIV transmission (Doncel, et al., 2010; Sabatte, Remes Lenicov, et al., 2011). Measures of semen viral load, cytokines, growth factors, and immune cells can be used as indicators of HIV-1 infection and immune status in the male genital tract (MGT), but they can also have a profound impact on infection via sexual transmission in the female genital tract (FGT).

Early studies have shown that expression of the multifunctional cytokine TGF-β1 can be upregulated by HIV tat *in vitro* in antigen-stimulated PBMCs (Reinhold, Wrenger, Kahne, & Ansorge, 1999), (Kekow, et al., 1990), monocytes (Gibellini, et al., 1994), T cells (Sawaya, et al., 1998), astrocytic glial cells (Cupp, Taylor, Khalili, & Amini, 1993; Sawaya, et al., 1998), and chondrocytes (Lotz, Clark-Lewis, & Ganu, 1994). In seminal plasma (SP), TGF-β1 levels are among the highest concentrations measured in any biological fluid (Robertson, et al., 2002). Studies in rodents and humans have showed that TGF-β1 is mainly synthesized by the seminal vesicles and prostate respectively as well as by resident macrophages in the testis and epididymis (C. Lee, et al., 1999; K. Tremellen & Tunc, 2010; K. P. Tremellen, et al., 1998). The function of TGF-β1 is paradoxical in that it exhibits both pro-inflammatory and immunoregulatory properties, depending on the presence of other cytokines and growth factors in the genital tract microenvironment. When deposited into the FGT, activation of TGF-β1 initially leads to the synthesis of pro-inflammatory cytokines, such as GM-CSF, as well as the recruitment of granulocytes, macrophages and DCs into the epithelium during the "post-mating inflammatory response" (Robertson, 2005). The final outcome of the immune response to SP in the FGT, which is mediated by TGF-β1 and prostaglandin E2, is tolerance to paternal antigens due to the deactivation of macrophages and the suppression of potentially harmful cell-mediated (Th1) immune responses (Ashcroft, 1999; Robertson, et al., 2002). Given that TGF-β1 can induce inflammation or control it, in coordination with other factors present in the microenvironment, it is unclear what role TGF-β1 in semen of HIV-

infected men could play during HIV infection, disease progression, and immune activation.

One of the characteristics of chronic HIV infection is persistent immune activation, which is a hallmark of disease progression (Sandler, et al.). Although the exact etiology of immune activation during HIV infection is unclear, HIV replication is thought to be one of the mechanisms of direct immune activation (Appay & Sauce, 2008). Other indirect mechanisms have been implicated, including increased serum levels of pro-inflammatory cytokines in response to the virus itself (Connolly, Riddler, & Rinaldo, 2005; Valdez & Lederman, 1997), microbial translocation (Brenchley, Price, Schacker, et al., 2006), increased T cell turnover (Hellerstein, et al., 1999), and increased frequencies of T and B cells with activated phenotypes (Hazenberg, et al., 2000). The immune activation marker soluble CD14 (sCD14), produced by monocytes and macrophages, has been found to directly correlate with disease progression and mortality in HIV-infected individuals (Sandler, et al.). Previous work has shown sCD14 present in SP of HIV-negative individuals were at levels comparable to that of blood plasma (BP) (Harris, et al., 2001), however sCD14 in SP of HIV-infected men has not been examined.

We previously demonstrated that HIV-positive, chronically infected antiretroviral therapy (ART)-naive men contained significantly higher levels of TGF-β1 in their SP compared to acutely infected ART-naive and HIV-negative men (Kafka, et al., 2012). However, since this was the first report of alterations in TGF-β1 levels in SP of HIV-infected, ART-naive men, it was unknown if these levels would increase or decrease following ART treatment and how TGF-β1 in SP correlated with its own and sCD14

levels seen in serum. Since sCD14 in serum levels correlate with immune activation, this could provide insight into the role of TGF-β1. In the present study we addressed these questions by measuring TGF-β1 and sCD14 in BP and SP of HIV-infected men and compared them with HIV-negative men and HIV-infected men on ART-treatment. Our results showed that TGF-β1 was present in both latent and active forms and was compartmentalized between BP and SP. Similar expression trends were also observed between latent TGF-β1 and sCD14 in BP. Active TGF-β1 was highest in SP of chronic infected, ART-naive men and decreased following ART treatment. Additionally, significant negative correlations were observed between active TGF-β1, sCD14, and semen viral load in HIV-infected, ART-naive men.

3.4 METHODS

Patient participation

HIV-infected, antiretroviral therapy (ART)-naïve and ART-treated men who have sex with men (MSM), and HIV-negative controls were recruited through the Maple Leaf Clinic (Toronto, ON, Canada). Informed written consent was obtained in accordance with the approval of the Research Ethics Boards at the University of Toronto.

Sample collection and preparation

Paired blood and semen samples were collected from HIV-negative, and HIV-infected, ART-naive and ART-treated men. Details of semen collection have been reported previously (Osborne, Sheth, Kovacs, Mazzulli, & Kaul). All study participants abstained from sexual intercourse or masturbation for 48 hours prior to sample donation. Semen was collected directly by masturbation into 10 mL of sterile RPMI 1640 (Gibco)

containing 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). SP was cryopreserved at -80°C after centrifugation to pellet semen lymphocytes and spermatozoa. BP was collected and cryopreserved after Ficoll density gradient centrifugation at 500x*g* for 25 minutes. BP and SP HIV-1 RNA levels were measured by Versant HIV-1 RNA 3.0 assay (Bayer Diagnostics; lower limit of detection, 50 RNA copies/mL) at Mount Sinai Department of Microbiology-Public Health Lab (Sheth, et al., 2009).

Measurement of TGF-β and sCD14

TGF-β1 and sCD14 levels in BP and SP were measured using the Quantikine® human immunoassay kits (R&D Systems, Inc., Minneapolis, MN, USA) as per manufacturer's instructions. Measurement of active TGF-β1 was conducted in the absence of the acidification step.

THP-1 cell culture

The monocytic cell line THP-1 was cultured at 37^{o}C and 5% CO_2 in a 75 cm^2 polystyrene flask containing RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Gibco, Burlington, Ontario, Canada), 0.05 mM 2-mercaptoethanol (Gibco, Burlington, Ontario, Canada), 2 mM L-glutamine (Invitrogen, Burlington, Ontario, Canada) and 100 U/ml pen/strep (Invitrogen, Burlington, Ontario, Canada).

Treatment of THP-1 cell line with LPS and HIV-1

THP-1 cells (10^6 cells/well) were seeded in a 6-well plate in 3 mL of supplemented RPMI 1640 alone or containing 10 ng/ml of phorbol 12-myristate 13-

acetate (PMA; Sigma-Aldrich, Oakville, Ontario, Canada) for 48 hours. Supernatants were removed, and cells were washed 3 times with sterile PBS before 50 ng/ml of LPS (Sigma-Aldrich, Oakville, Ontario, Canada), HIV-1 ADA (10^5 IU/mL), or both were added in non-supplemented RPMI 1640 for 12 hours at 37^{o}C and 5% CO_2. RPMI 1640 (media) alone served as a negative control. Supernatants were collected and centrifuged at 12,000Xg for 10 minutes to pellet cells before supernatants were added to Quantikine® human TGF-β1 and sCD14 immunoassay kits (R&D Systems Inc., Minneapolis, Minnesota, USA) as per manufacturer's instructions.

Statistical methods

GraphPad Prism version 4 (GraphPad Software, San Diego, CA) was used for statistical analysis and graphical representation. Differences between groups were compared using a non-parametric Mann Whitney U-test for clinical samples or a one-way ANOVA for THP-1 cell line treatment results. The relationship between variables was determined using Spearman rank correlation. The box area of the box-and-whisker plots extends from the 25^{th} and 75^{th} percentiles with the median being the middle line within the box. The whiskers represent minimum and maximum values. Statistical significance was determined as $p<0.05$. R-(correlation coefficient) and p-values for each analysis are indicated in figure legends.

3.5 RESULTS

Semen and blood donor demographics

BP and SP from 22 HIV-negative MSM, 34 HIV-infected, ART-naive MSM and 18 HIV-infected, ART-treated MSM were used in the study. Fourteen of the 34 HIV-infected, ART-naive men were within 6 months of their first HIV-positive test and considered to be in the acute/early phase of infection (acute naive). Men who had been infected for 6 months or longer were considered to be in the chronic phase of infection (chronic naive). HIV-infected, ART-treated men had been on therapy for 1 to 5 years at the time of sample collection. Details of clinical parameters for blood and semen samples are summarized in Table 1.

Active TGF-β1 levels in SP and BP of HIV-negative men, HIV-infected ART-naive men and ART-treated men

Our previous study showed that HIV infection increased active TGF-β1 levels in SP, with the highest levels observed in chronic naïve men (Kafka, et al., 2012). In the current study we increased the SP sample size in our HIV-negative and HIV-infected ART-naïve groups as well as evaluated the levels of active TGF-β1 in BP to determine how they correlated with active TGF-β1 levels in SP. In agreement with our previous results, we observed that active TGF-β1 levels were significantly increased in SP from chronic naïve men compared to HIV-negative men (Fig. 1A). Active TGF-β1 in BP was not measured above the detectable limit of the assay (Fig. 1B), suggesting active TGF-β1 expression is compartmentalized between BP and SP.

To determine the effect of ART treatment on active TGF-β1 levels, SP from ART-treated men was assayed for active TGF-β1 by ELISA. Compared to treatment-naïve chronically infected men, ART treatment significantly decreased active TGF-β1 levels in SP to levels comparable to HIV-negative men (Fig. 1A).

Latent TGF-β1 and sCD14 levels in SP from HIV-negative men, HIV-infected ART-naive men, and ART-treated men

Since active TGF-β1 represents a small fraction of the total TGF-β1 in semen that may be activated when deposited in the female genital tract, we also evaluated how latent TGF-β1 levels in SP changed with different stages of HIV infection. Additionally, we wanted to compare latent TGF-β1 and sCD14 levels to determine the relationship between latent TGF-β1 and chronic immune activation in SP. Higher levels of latent TGF-β1 were measured in SP of HIV-negative men compared to SP of HIV-infected men. Overall, latent TGF-β1 levels were significantly decreased in SP from chronic naive and ART-treated men compared to HIV-negative men (Fig. 2A). Latent TGF-β1 levels were approximately 10- to 60-fold higher than active TGF-β1 levels in chronic naive and HIV-negative men respectively (Fig. 2A). Soluble CD14 was measured in SP of all men, and similar to latent TGF-β1, a modest decrease was observed in sCD14 from chronic naive men compared to HIV-negative men, although this decrease was not statistically significant (Fig. 2B).

Latent TGF-β1 and sCD14 levels in BP from HIV-negative men, HIV-infected ART-naive men, and ART-treated men

Next we examined the relationship between latent TGF-β1 and sCD14 in BP. Interestingly, both latent TGF-β1 and sCD14 had comparable expression trends; BP from acute and chronic naïve men, as well as ART-treated men had significantly elevated latent TGF-β1 and sCD14 levels compared to HIV-negative men, with the highest levels measured during acute infection (Fig. 3A and B).

Upon closer comparison of sCD14 expression between blood and semen compartments, an inverse trend was observed with increased levels in BP and decreased levels in SP following HIV infection and ART treatment compared to HIV-negative men, thus mirroring latent TGF-β1 expression trends and suggesting compartmentalization of both TGF-β1 and sCD14 between blood and semen (Fig. 2B and 3B).

Latent TGF-β1 and sCD14 production by THP-1 cells exposed to LPS and HIV-1

Since we observed simultaneous increased expression trends between latent TGF-β1 and sCD14 in BP following HIV infection, we next decided to determine whether TGF-β1 and sCD14 could be co-expressed by monocytes in response to HIV-1 and/or LPS. Although individual studies have measured TGF-β1 or sCD14 from blood monocytes, none have examined co-expression of these two factors. Monocytic THP-1 cells were stimulated with PMA or directly exposed to LPS, HIV-1, or both, and supernatants were assayed for TGF-β1 and LPS by immunoassay. Overall, exposure to HIV-1 or HIV-1 in combination with LPS significantly increased latent TGF-β1 and sCD14 levels in both PMA-stimulated and non-stimulated THP-1 cells compared to media control (Fig. 4A and B respectively). Exposure to LPS alone did not significantly

increase latent TGF-β1 or sCD14 in stimulated or non-stimulated cells compared to media treatment (Fig. 4A and B respectively).

Relationship between active TGF-β1, semen viral load, and sCD14 in SP from ART-naïve men

Since TGF-β1 is an immunoregulatory cytokine that can control inflammatory processes, and since sCD14 is known to correlate with immune activation during HIV infection, we next evaluated the relationship between active TGF-β1, viral loads and sCD14 in SP from ART-naive men since these men are naturally controlling their disease progression and immune activation status. Overall, a negative correlation was observed between active TGF-β1 and semen viral load in SP from HIV-infected, ART naive men (Fig. 5A). Furthermore, a negative relationship was observed between active TGF-β1 and sCD14 in SP from chronic naïve men, where active TGF-β1 levels were highest, suggesting an immunoregulatory role for active TGF-β1 in the male genital tract (Fig. 5B).

3.6 DISCUSSION

Previously, we have shown that SP from HIV-negative and HIV-infected, ART-naive men differed substantially with respect to levels of the activated form of the immunoregulatory factor TGF-β1 and that these differences were dependent mainly on the stage of HIV infection, with the highest levels of TGF-β1 seen in SP of chronic naive men (Kafka, et al., 2012). In the present study, we confirmed these results and extended them to show that men on ART had lower levels of active TGF-β1 levels in their SP,

comparable to the levels seen in HIV-negative men. Furthermore, we found that active and latent TGF-β1 expression was distinct between blood and semen compartments, both in the presence and absence of HIV infection. Similar expression trends were observed between latent TGF-β1 and sCD14 in BP of all experimental groups, but this trend was not maintained in semen. Interestingly, when we looked into the possible role of active TGF-β1 in regulating local immune activation in the MGT, we found that active TGF-β1 levels in SP were negatively correlated with semen viral load in HIV-infected men and with the immune activation marker sCD14 in chronic naive men which had the highest levels of TGF-β1, suggesting that activated TGF-β1 could play a role in regulating immune activation in the male genital tract.

Early studies have shown that TGF-β1 expression is increased in the presence of HIV-1 viral proteins *in vitro*. While we also saw increase in latent TGF-β1 in the BP, this increase was not observed in SP. Furthermore, we also observed distinct compartmentalization of active TGF-β1 between BP and SP. Previous studies have shown that HIV-1 in semen can arise from multiple local reservoirs within the MGT (J. A. Anderson, et al.; Pillai, et al., 2005). The idea of a local viral reservoir in the MGT compared to peripheral circulation is supported by observations of higher viral loads in semen than in blood in a subpopulation of ART-naive men (Tachet, et al., 1999), the persistence of viral shedding in semen despite effective ART (Halfon, et al.; Marcelin, et al., 2008; Sheth, et al., 2009), local cytokine repertoires conductive to an activated immune microenvironment and virus replication [27], and diversity of discordant drug resistant (Byrn & Kiessling, 1998; Eron, et al., 1998; Ghosn, et al., 2004; Kroodsma,

Kozal, Hamed, Winters, & Merigan, 1994), population(Ping, et al., 2000; T. Zhu, et al., 1996) and phylogenetic markers(Byrn & Kiessling, 1998; Delwart, et al., 1998; Diem, et al., 2008; P. Gupta, et al., 2000; Paranjpe, et al., 2002; Pillai, et al., 2005). Although the exact sources of HIV in the MGT are still unclear, several *in vivo* and *in vitro* studies have shown that the seminal vesicles, testis, epididymis, and prostate can be productively infected with HIV-1 (Deleage, et al., 2011; Le Tortorec, et al., 2008). Other studies suggest that HIV infection more likely occurs outside of the testes in the seminal vesicles and prostate since vasectomy does not prevent virus in semen (D. J. Anderson, et al., 1991; Krieger, et al., 1998). Since TGF-β1 is synthesized in the seminal vesicles and the prostate (Robertson, 2005; Robertson, et al., 2002), we believe that the observed compartmentalized expression of latent TGF-β1 between BP and SP in our cohort may be in response to a distinct HIV reservoir in the MGT which may be controlled by TGF-β1 in its activated form since we also observed a significant negative correlation between active TGF-β1 and semen viral load.

To the best of our knowledge no one has examined the relationship between sCD14 and TGF-β1 in either BP or SP. Soluble CD14 is produced by the liver as well as monocytes/macrophages in response to LPS (Goyert, et al., 1988; Ziegler-Heitbrock & Ulevitch, 1993), and has been shown to be elevated during HIV infection in blood (Sandler, et al.). Previous work has shown that sCD14 is present in healthy HIV-negative human SP at levels comparable to those in BP, and can be synthesized in the seminal vesicles and testes (Harris, et al., 2001). We observed a strong similarity in expression trends between latent TGF-β1 and sCD14 in BP from HIV-negative men, ART-naive men

and ART-treated men in our cohort. We also observed a modest similarity in the SP compartment. These observations could be explained by a common immune cell, such as monocytes/macrophages, being responsible for co-expression of sCD14 and latent TGF-β1 in blood. Our results showed that THP-1 cells, which are monocytic in origin, produced increased levels of latent TGF-β1 and sCD14 following exposure to HIV-1. Based on our results, we suggest that the common source for both sCD14 and TGF-β1 could be the monocyte/macrophage population present in peripheral circulation and are activated in response to HIV-1 infection. The co-expression and coordinated upregulation of sCD14 and latent TGF-β1 in BP following HIV-1 infection also suggests that latent TGF-β1 could also serve as a surrogate marker for chronic immune activation in HIV-infected individuals. The conditions under which TGF-β1 could become biologically active and whether it directly regulates immune activation in response to HIV-1 remains to be determined. However, it is interesting to note that a significant negative correlation was observed between active TGF-β1 and semen viral load in HIV-infected men, as well as sCD14 and active TGF-β1 in SP from chronic naive men. Since the highest levels of active TGF-β1 were observed in SP from chronic naive men, this would suggest that should latent TGF-β1 become activated, it could play a role in regulating chronic immune activation and infection seen by decreased levels of sCD14 and semen viral load, but this regulation would be dependent on the stage of HIV infection. Other studies have shown that one of the main immunoregulatory functions of TGF-β1 is suppression of monocyte/macrophage activation (Kekow, et al., 1990; Werner, et al., 2000), which may result in a lower secretion of sCD14 in response to high levels of active TGF-β1 during

chronic stages of infection in SP. The factors in the local microenvironment of the MGT which could convert latent TGF-β1 to its active form, especially in chronic ART-naive men, remain to be elucidated. However, Anderson et al suggested that the MGT, even in HIV-negative men, is constitutively in an immune activated state which is exacerbated with HIV infection (J. A. Anderson, et al., 2010), which would provide an optimal environment for TGF-β1 to exert its immunoregulatory function.

The current study compared sCD14 levels in BP and SP from HIV-infected, ART-naive and ART-treated men. Soluble CD14 has been observed to be increased as a result of HIV infection and in earlier studies ART treatment was shown to decrease immune activation and sCD14 (Amirayan-Chevillard, et al., 2000; Brazille, et al., 2003; Brenchley, Price, Schacker, et al., 2006; Lempicki, et al., 2000; Papasavvas, et al., 2009). However, more recently a number of studies have shown that HIV-infected individuals have elevated levels of immune activation during untreated disease and that these levels do not normalize with ART (Burdo, et al., 2011; Eden, et al., 2007; French, et al., 2009; Hunt, et al., 2003; Lederman, et al., 2011; Mendez-Lagares, et al., 2013; Neuhaus, et al., 2010). In agreement with these studies, we also observed no significant decreases in sCD14 in ART-treated men. One recent longitudinal study evaluated the effect of long-term ART on sCD14 levels compared to ART-naive men and HIV-negative controls. Similar to our study, HIV infection increased levels of sCD14 compared to uninfected controls; however initiation of ART neither reduced nor normalized sCD14 compared to ART-naive subjects (Mendez-Lagares, et al., 2013). Similar to sCD14 in our study, men on ART had increased levels of latent TGF-β1 in their BP compared to ART-naive men,

further suggesting that latent TGF-β1 could serve as another correlate of immune activation that may persist despite the presence of ART.

Although the current study examined how TGF-β1 levels in BP differ with the presence of ART, an important consideration for future studies would be to examine the role of SP from ART-treated men on FGT epithelial cell responses. Our previous study compared FGT epithelial cell cytokine responses following exposure to SP from HIV-uninfected, and HIV-infected, ART-naive men in different stages of infection (Kafka, et al., 2012). We found that SP from chronic naïve men contained higher levels of active TGF-β1 which lead to a significant reduction in pro-inflammatory cytokine production from female GECs. Since the current study showed a reduction in active TGF-β1 in SP from ART-treated men, to levels comparable to HIV-negative men, we hypothesize that exposure to FGT epithelial cells will elicit a pro-inflammatory response comparable to exposure to SP from HIV-negative men.

In conclusion, our results showed that TGF-β1 is compartmentalized between blood and semen and its latent form may be co-expressed with sCD14 by activated monocytes/macrophages in BP as a result of HIV infection. Conversion of latent TGF-β1 into its active form could contribute to regulation of semen viral load and local immune activation in the MGT, but this regulation depends on the stage of infection.

3.7 ACKNOWLEDGEMENTS

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--- CHAPTER 3 FIGURES ---

Table 1: Details of clinical data, semen samples, and blood samples from HIV-negative men, HIV-infected ART-naive men, and ART-treated men used in the study.

	Acute Naïve	Chronic Naïve	ART	HIV Negative
Study Number (n)	14	20	18	22
Age	35 (26-59)	37 (24-50)	48 (29-74)	NA
% HSV-2 Positive	43%	40%	50%	NA
CD4 Count (cells/mm^3)	530 (180-960)	445 (120-890)	535 (140-960)	NA
Blood Viral Load (HIV-1 RNA copies/mL)	36681 (2262-500000)	9144 (636-500000)	<50	NA
Semen Viral Load (HIV-1 RNA copies/mL)	11154 (300-187428)	2742 (300-106674)	<300	NA
Active TGF-β (ng/ml; Blood)	<0.005	<0.005	<0.005	<0.005
Active TGF-β (ng/ml; Semen)	4.06 (1.09-38.03)	7.16 (2.21-41.84)	4.72 (0.965-12.11)	3.29 (0.259-13.20)
Latent TGF-β (ng/ml; Blood)	4.69 (1.91-7.29)	3.45 (1.65-14.75)	4.65 (2.95-15.79)	1.44 (0.703-5.56)
Latent TGF-β (ng/ml; Semen)	99.98 (31.16-185.64)	74.96 (27.86-134.45)	90.08 (3.13-303.82)	128.15 (31.40-198.12)
sCD14 (ng/ml; Blood)	977 (568-1381)	764 (655-1902)	992 (620-1691)	519 (427-1107)
sCD14 (ng/ml; Semen)	592 (84-3550)	554 (71-1777)	811 (6-1517)	942 (157-1935)

Median values with range in parenthesis.
NA, not applicable; ART, antiretroviral therapy.

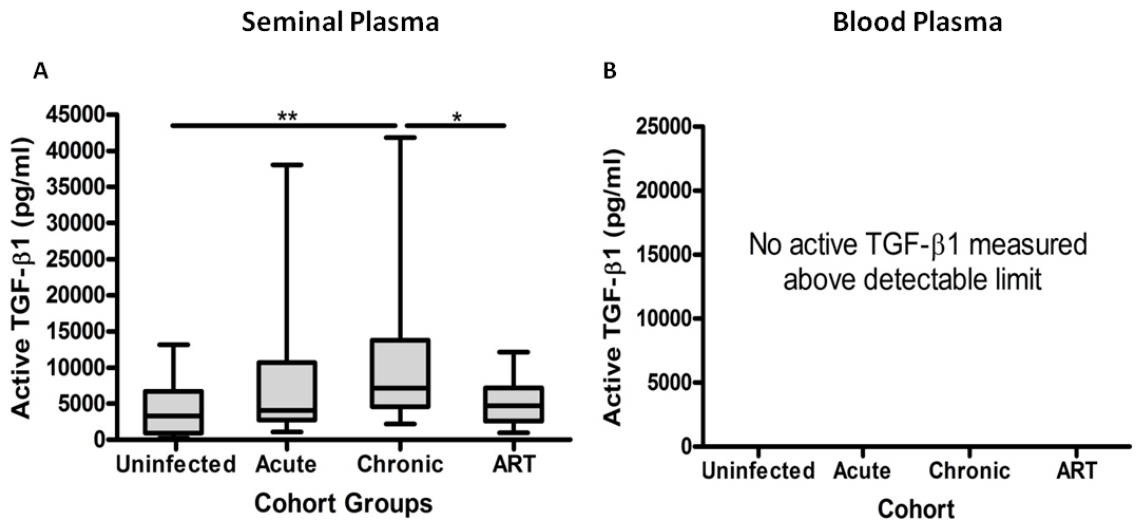


Fig. 1: Active TGF-β1 levels in SP and BP from HIV-negative men, HIV-infected ART-naive men, and ART-treated men. Box-and-whisker plot of active TGF-β1 in SP (A) and BP (B) samples measured by immunoassay. Active TGF-β1 levels were measured by omitting the acidification step in the immunoassay. Mean differences between cohorts were measured by Mann Whitney U-test. BP and SP HIV-negative samples were n=22 paired values; BP and SP acute naive samples were n=14 paired values; BP and SP chronic naive samples were n=20 paired values; and BP and SP ART treated samples were n=18 paired values. Y axis represents active TGF-β1 levels in pg/ml and x axis represents the different cohort groups used in the study. The box plot extends from the 25^{th} and 75^{th} percentiles with the median being the middle line within the box. The whiskers represent minimum and maximum values. *=$p<0.05$, **=$p<0.01$.

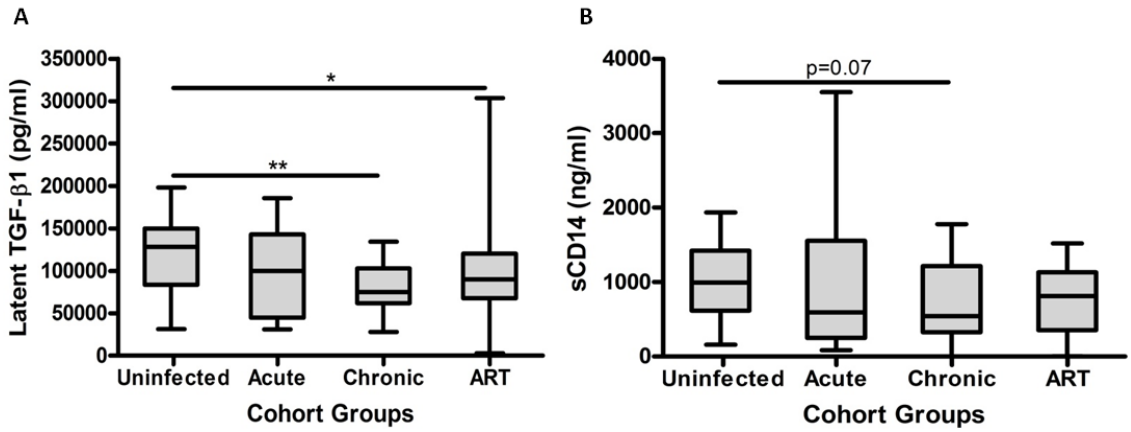


Fig. 2. Latent TGF-β1 and sCD14 levels in SP from HIV-negative men, HIV-infected ART-naive men, and ART-treated men. Box-and-whisker plot of SP samples analyzed for latent TGF-β1 and sCD14 by immunoassay. Latent TGF-β1 was measured by subtraction of active TGF-β1 from total TGF-β1. Mean differences between cohort groups were measured by Mann Whitney U-test. HIV-negative samples were n=14 and 15 for latent TGF-β1 and sCD14 respectively; Acute naive samples were n=10 and 13 respectively; chronic naive samples were n=15 and 17 respectively; and ART treated samples were n=17 and 18 respectively. Y axis represents latent TGF-β1 levels in pg/ml and x axis represents the different cohort groups used in the study. The box plot extends from the 25^{th} and 75^{th} percentiles with the median being the middle line within the box. The whiskers represent minimum and maximum values. *=$p<0.05$, **=$p<0.01$.

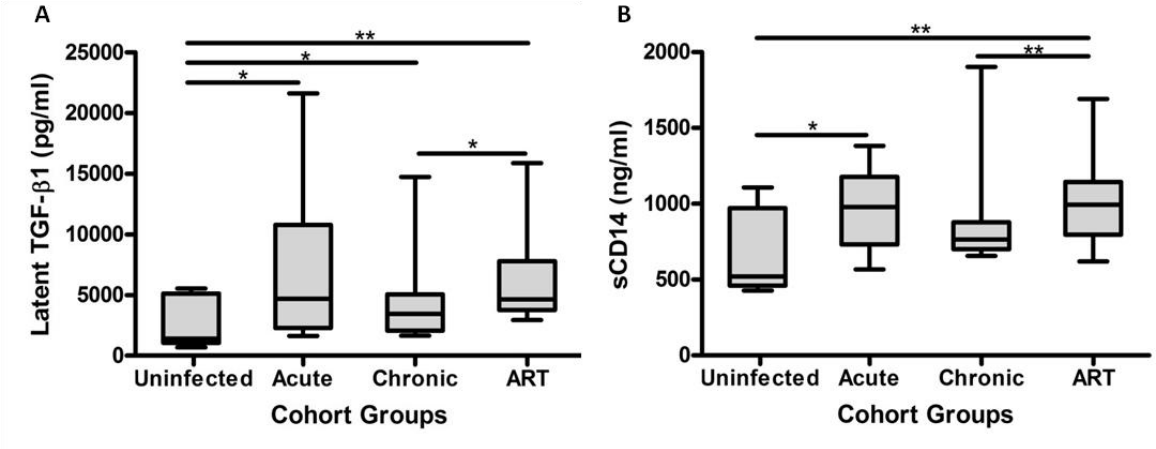


Fig. 3. Latent TGF-β1 and sCD14 levels in BP from HIV-negative men, HIV-infected ART-naive men, and ART-treated men. Box-and-whisker plot of BP samples analyzed for latent TGF-β1 and sCD14 by immunoassay. Latent TGF-β1 was measured by subtraction of active TGF-β1 from total TGF-β1. Mean differences between cohort groups were measured by Mann Whitney U-test. HIV-negative samples were n=9 and 11 for latent TGF-β1 and sCD14 respectively; acute naive samples were n=13 and 13 respectively; chronic naive samples were n=19 and 20 respectively; and ART treated samples were n=18 and 18 respectively. Y axis represents latent TGF-β1 levels in pg/ml and x axis represents the different cohort groups used in the study. The box plot extends from the 25^{th} and 75^{th} percentiles with the median being the middle line within the box. The whiskers represent minimum and maximum values. *=$p<0.05$, **=$p<0.01$.

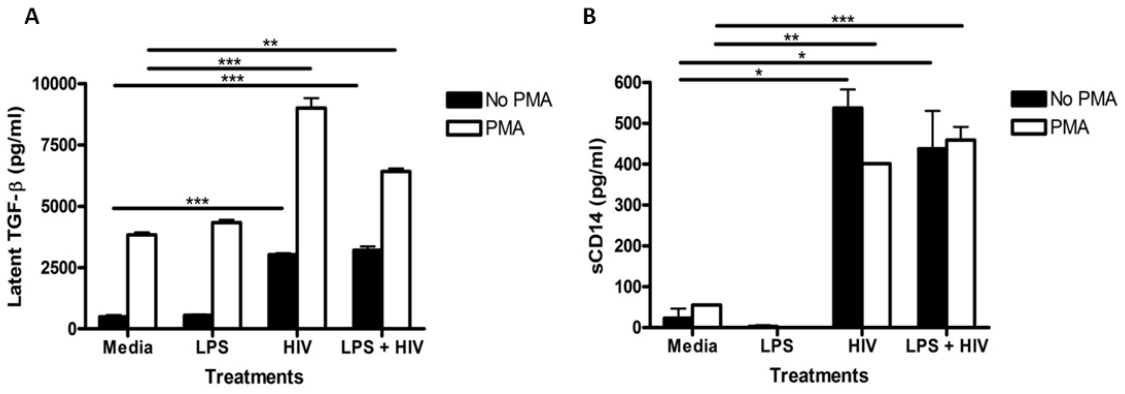


Fig. 4: Latent TGF-β1 and sCD14 production by THP-1 cells exposed to LPS and HIV-1. THP-1 cells were stimulated (white bars; 10 ng/ml PMA) or non-stimulated (black bars; RPMI) for 48 hours prior to being treated with 50 ng/ml LPS, 10^5 IU/ml HIV-1 ADA, or a combination of the two treatments for 12 hours at 37°C. RPMI was used as a media control. Cells were pelleted by centrifugation at 12,000 *xg* for 10 minutes prior to measurement of latent TGF-β1 (A) and sCD14 (B) in cell supernatants by immunoassay as per manufacturer's instructions. Data shown represents one of two separate experiments with similar results. All conditions were performed in triplicate. Y axis represents concentrations of latent TGF-β1 (A) and sCD14 (B) in pg/ml and the x axis represents the different experimental treatments. Differences in treatments were measured using one-way ANOVA. Significance shown is relative to the respective media group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$. Bars represent mean and standard error of the mean (SEM).

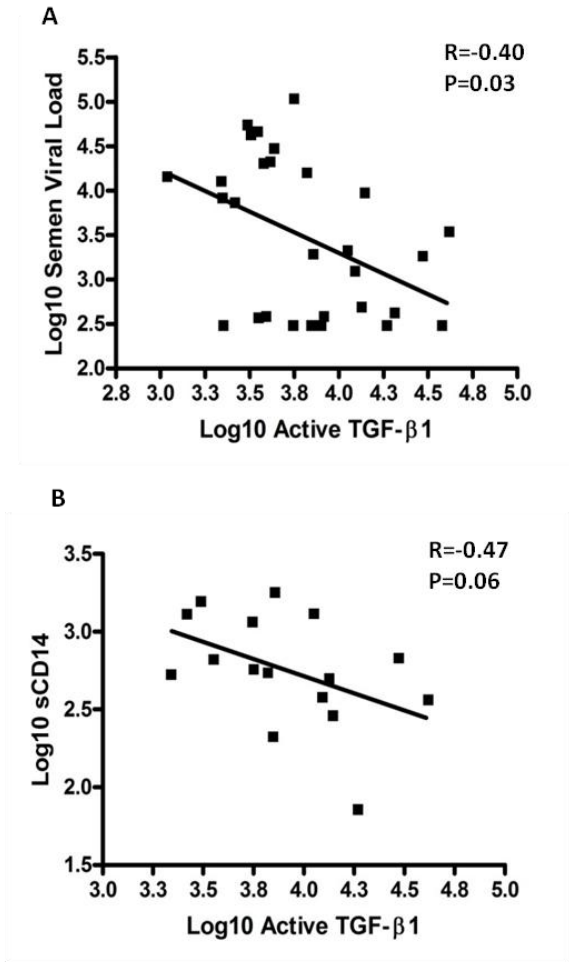


Fig. 5: Relationship between active TGF-β1, semen viral load, and sCD14 in SP from ART-naïve men. Active TGF-β1, semen viral load and sCD14 values were log10 transformed to normalize the data, and then their relationship was determined using Spearman rank correlation test. Latent TGF-β1 and sCD14 correlation was carried out with 16 paired groups, and latent TGF-β1 and semen viral load correlation was carried out with 29 paired groups. Y axis represents log10 semen viral load (A) and log10 sCD14 (B) values, and the x axis represents log10 active TGF-β1 values. R value represents the correlation coefficient with a negative r value and slope representing a negative correlation between groups.

--- CHAPTER 4 ---

Examining the role of seminal plasma and TGF-β1 on HIV-mediated epithelial cell barrier disruption in the female genital tract

4.1 PREFACE

The experiments described in this chapter were designed, and data was interpreted by me and Dr. Charu Kaushic. I performed the experiments and analyzed the data. Sara Dizzell generously provided primary GECs used in some of these experiments.

4.2 RATIONALE

Globally, women are disproportionately more susceptible to HIV infection compared to men with approximately 76% of HIV positive individuals aged 15 to 24 being women (Nicolosi, et al., 1994; UNAIDS, 2010). For this reason, it is important to examine the initial events which occur during sexual transmission of HIV in the FGT. Since HIV is introduced into the FGT in the context of SP, it is essential to understand how SP may influence the ability of HIV to establish an infection at mucosal surfaces. Although it has been previously shown in our lab that HIV-1 can disrupt the tight junction barrier between confluent polarized genital epithelial cells (GECs) grown in monolayers via the upregulation in the production of TNF-α (Nazli, et al., 2010), it is not known how SP may influence this process. Previous studies provide some indication that exposure to SP may increase TER, based on studies done with SP from HIV uninfected men, in cervical epithelial cell lines (Gorodeski & Goldfarb, 1998; P. Lawrence, et al., 2012). However these studies did not examine what factor(s) in SP could be responsible for this effect or how this effect was mediated. The immunoregulatory factor TGF-β1, which is found in high concentrations in SP, has been previously shown to play an important role in the maintenance of the intestinal tract epithelial cell barrier in the presence of intestinal pathogens (Howe, et al., 2005; Roche, et al., 2000). However, it is not clear whether

TGF-β in SP can regulate barrier function in the FGT in presence of HIV. Based on this information, *we hypothesize that SP will protect the epithelial cell barrier in FGT in the presence of HIV. This protective effect is likely mediated by TGF-β, which is present in SP, by downregulation of TNF-α production by GECs which is produced in response to HIV.*

The following chapter describes unpublished research examining how SP and biologically active TGF-β influence upper GEC barrier integrity in the presence of HIV. The results section on page 116 as well as figure 6 were taken from a 2013 published paper by Nazli et al., in the Journal of Immunology for which I was a second co-author (Nazli, et al., 2013). The results and figures from this publication were used with permission from the journal.

4.3 METHODS

Primary GEC Cultures

The detailed protocol for isolation and culture of GECs has been described previously (Kaushic, Nazli, Ferreira, & Kafka, 2011). Briefly, endometrial tissues were minced into small pieces and digested in an enzyme mixture at 37^{o}C with shaking. GECs were isolated by a series of separations through nylon mesh filters, resuspended in DMEM/F12 growth medium and seeded onto Matrigel^{TM} coated tissue culture inserts. GEC cultures were grown for 5-7 days until confluent monolayers were formed. Confluency was monitored by trans-epithelial resistance (TER) measured by a volt ohm meter (World Precision Instruments). Confluent monolayers showing TER values greater than 1000 Ω/cm were used for further experiments.

GEC Exposure to SP

To compare TER between GECs treated between uninfected and HIV-infected SP, confluent primary endometrial ECs were exposed in duplicate to DMEM/F12 growth media (UnT), pooled SP from 5 HIV-negative men, or pooled SP from 5 HIV positive chronically infected ART-naive men (Chronic SP) for 4 hours at 37^{o}C. The 5 pooled SP samples from HIV-negative men contained 259, 331, 388, 1474, and 1672 pg/ml of active TGF-β and were all negative for HSV-2. No other clinical information on the HIV-negative men is available. Clinical information on the 5 pooled SP samples from HIV-infected men is outlined below.

Table 1: Clinical Information and Active TGF-β Concentrations in Pooled SP Samples from HIV Positive Chronically Infected ART-Naive Men

	Active TGF-β (pg/ml)	CD4+ T cell Count (cells/mm3)	Semen VL (RNA copies/ml)	Age	HSV-2 Status
S1	7073	290	300	24	Negative
S2	7238	460	1890	50	Negative
S3	8010	530	300	34	Negative
S4	12440	700	1224	27	Negative
S5	13474	890	480	32	Negative

All SP samples were diluted to 10% in DMEM/F12 prior to being added to GECs. The outlined semen exposure conditions, as previously conducted in chapter 2, were chosen to mimic *in vivo* short term exposure to semen during intercourse while ensuring the least amount of cytotoxic effects to the epithelial cells. The strength of the GEC monolayer was measured using a volt-ohmmeter with readings taken with a chopstick electrode at time 0h prior to treatment and 4 hours. Changes in TER between 0 and 4

hours were graphed as percent pretreatment (TER at 4h/TER at 0h x 100=% pretreatment TER).

To examine TNF-α concentrations and barrier effect of GECs exposed to SP from HIV-infected men in combination with HIV, confluent primary endometrial ECs were exposed in duplicate to DMEM/F12 growth media (UnT), pooled 10% chronic SP, pooled 10% chronic SP mixed with 10^5 IU/ml HIV-1 IIIB, or 10^5 IU/ml of HIV IIIB alone for 4 hours at 37^{o}C. Apical supernatants were collected and assayed for TNF-α via the R&D Systems DuoSet TNF-α ELISA Kit as per manufacturer's instructions (R&D Systems, Cat#DY210). GECs from the same experiment were also fixed and assayed for ZO-1 tight junction protein (Figure 5.4.2b) using the immunofluorescent staining protocol described below.

GEC Exposure to TGF-β1

To examine the effect of TGF-β, with or without HIV, on cytokine production in GECs, confluent primary endometrial ECs were exposed in duplicate to DMEM/F12 growth media (UnT), 1ng/ml TGF-β1, 10^5 IU/ml HIV-1 IIIB, or 1ng/ml TGF-β1 mixed with 10^5IU/ml HIV-1 IIIB for 4 or 24 hours at 37^{o}C. Apical supernatants were collected and assayed for TNF-α and IL-6 either via the R&D Systems ELISA kit or a custom Multiplex kit from Eve Technologies Inc depending on the experiment. Recombinant human TGF-β1 from R&D Systems was used in these experiments (R&D Systems, Cat#240-B) at a 1ng/ml concentration which was comparable to the average concentration of active TGF-β measured in our chronic SP samples.

To examine different doses of TGF-β on TNF-α production in GECs, individual wells of primary endometrial ECs from the same tissue were exposed in duplicate to DMEM/F12 growth media (UnT), 1ng/ml of TGF-β1, or 10ng/ml of TGF-β1 for 24 hours at 37^{o}C. Apical supernatants were collected and analyzed for TNF-α via the R&D Systems TNF-α ELISA Kit. Additionally, individual wells of primary endometrial ECs from the same tissue were exposed in duplicate to DMEM/F12 growth media (UnT), 10^5 IU/ml HIV-1 IIIB alone or in combination with 0.1, 1, or 10ng/ml TGF-β1 for 24 hours at 37^{o}C to compare different doses of TGF-β in combination with HIV on TNF-α production in GECs. Apical supernatants were collected and analyzed for TNF-α via the R&D Systems TNF-α ELISA Kit.

GEC Exposure to HIV gp120 and SP from Acute Infected Men

To examine TNF-α production in GECs exposed to HIV gp120 in the context of SP, confluent endometrial ECs were exposed for 24 hours to uninfected SP spiked with 0.1, 0.2, or 0.3 mg/ml recombinant gp120 (Immunodiagnostics Inc.), uninfected SP, or infected SP alone. Recombinant gp120 (0.1 mg/ml) was used as a positive control for comparison. Apical supernatants were collected and analyzed for TNF-α via the R&D Systems TNF-α ELISA Kit, and cells were fixed for immunofluorescent staining of ZO-1 tight junctions. Confluent endometrial ECs were also exposed for 24h to pooled SP samples from three AI subjects (acute SP), acute SP combined with recombinant gp120 (0.1 mg/ml), acute SP combined with recombinant gp120 (0.1 mg/ml), and anti-gp120 Ab (35 mg/ml; Polymun Scientific) or acute SP with recombinant gp120 (0.1 mg/ml) and

isotype control Ab (35 mg/ml; Southern Biotechnology). DMEM/F12 growth media (Mock) or recombinant gp120 (0.1 mg/ml) were used as controls.

Immunofluorescent Staining for ZO-1 Tight Junction Protein

Following treatment, GEC monolayers were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with blocking solution (0.1% Triton X-100, 5% goat serum and 5% BSA) for 30 minutes. Rabbit anti-human ZO-1 primary antibody was diluted (2 ug/ml) in blocking solution and added to GEC monolayers for 1 hour at room temperature. Following removal of primary antibody and washing with PBS, FITC Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1.5ug/ml) was added for 1 hour at room temperature. Propidium iodide was used for nuclear counterstain. Imaging was done on an inverted confocal laser-scanning microscope. Standard operating conditions were 63x objective, optical laser thickness 1µm, image dimension of 512 x 512, lasers: argon (450nm) for TJ proteins and HeNe (543nm) for nuclear staining. For each experiment, settings for image acquisition were identical between control and treated monolayers. Confocal images were converted to JPEG picture files using the LSM 5 Image Browser program.

Statistical Analysis

Statistical analysis and graphical representation of data was conducted using GraphPad Prism v.4 software. Analysis of three or more means was conducted using a one-way ANOVA and when an overall statistically significant difference was observed ($p<0.05$), the Bonferroni post test was conducted to compare pairs of treatments.

4.4 RESULTS AND FIGURES

SP from HIV-negative men and HIV-positive, chronically infected men increased transepithelial electrical resistance (TER) in GECs

Since it was previously shown that SP from HIV-uninfected men can increase TER in cervical cell lines (Gorodeski & Goldfarb, 1998; P. Lawrence, et al., 2012), we wanted to determine whether SP from HIV-infected men differed in its ability to regulate TER in primary GECs. Confluent endometrial epithelial cells were exposed for 4 hours to SP from HIV-uninfected and HIV-infected men in chronic stages of infection. Growth media was used as a control to determine baseline TER in the absence of any treatments. TER was taken using a volt-ohmmeter prior to SP exposure at time 0 and 4 hours post-exposure and analyzed as percent pretreatment TER. SP from both HIV-uninfected and chronically infected men significantly increased TER compared to media control with the greatest increase in TER observed with SP from chronically infected men (Figure 1).

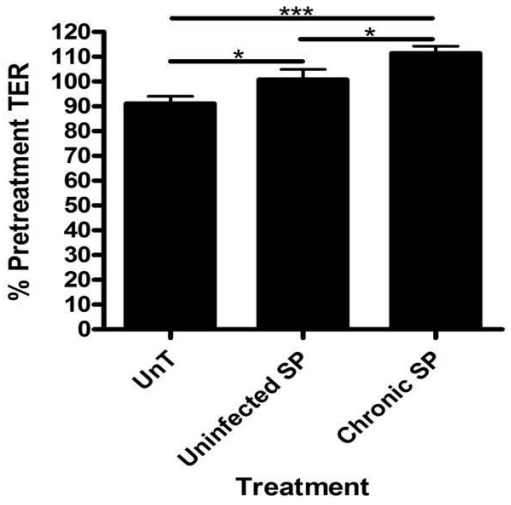


Figure 1: Percent pretreatment TER of GECs exposed to SP from HIV-uninfected and chronically infected men. Primary endometrial ECs were exposed to 10% SP collected from HIV-uninfected men, chronically infected men, or untreated growth media control (UnT) for 4 hours. TER readings were taken at time 0h and 4h, and analyzed as percent pretreatment TER. *$p<0.05$, ***$p<0.001$.

SP from HIV-positive, chronically infected men decreased HIV-mediated TNF-α production and prevented ZO-1 barrier disruption in GECs

Our previous work demonstrated that HIV exposure to GECs lead to a significant increase in TNF-α and disruption of tight junction barrier integrity (Nazli, et al., 2010), therefore we next examined how SP influenced this process. In agreement with results from Nazli et al., HIV alone significantly increased TNF-α production in our GECs after 4 hours of exposure. When HIV was added in the presence of SP from chronically infected men, TNF-α production was not significantly increased, but was comparable to growth media control. SP exposure alone also showed similar TNF-α production to cells exposed to media (Figure 2a).

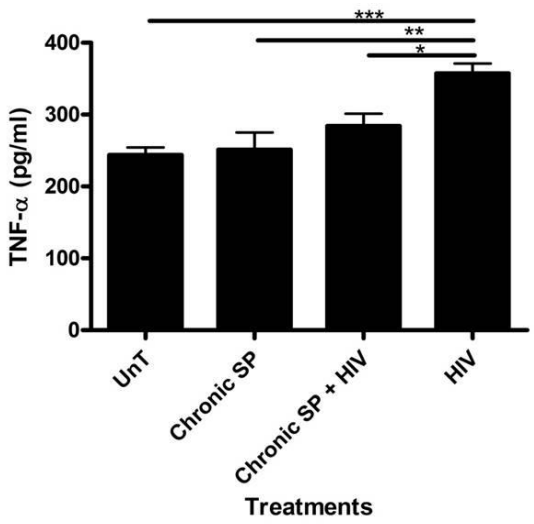


Figure 2a: TNF-α production in GECs treated with HIV in the presence of seminal plasma. Primary endometrial epithelial cells were exposed to 10^5 IU/ml of HIV-1 IIIB for 4 hours alone or mixed with 10% SP from HIV-positive chronically infected men. Growth media (Untreated; UnT) was used as a negative control to determine baseline TNF-α levels. Apical supernatants were collected and analyzed for TNF-α by commercial ELISA. *p<0.05, **p<0.01, ***p<0.001.

Additionally, we examined the effect of SP on HIV-mediated disruption of tight junction proteins. Following treatment with either growth media, HIV, SP, or HIV in combination with SP, the tight junction protein ZO-1 was examined via confocal microscopy on fixed cells stained with fluorescent anti-ZO-1 antibody. GECs exposed to growth media or SP exhibited an intact ZO-1 barrier resembling an intact chicken wire

pattern. In contrast, the ZO-1 barrier was disrupted in cells exposed to HIV-1, as described before (Nazli, et al., 2010). When HIV was added with SP, the disruption in ZO-1 observed with HIV alone was prevented (Figure 2b).

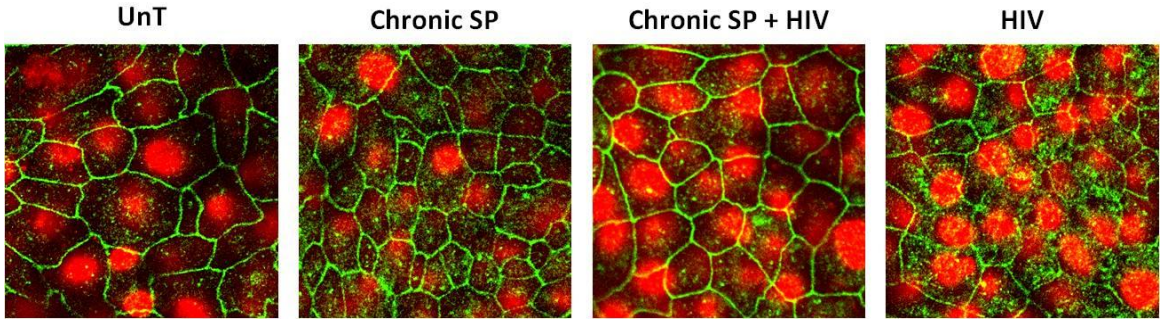


Figure 2b: Fluorescent microscopy of ZO-1 tight junction protein between GECs exposed to HIV and SP. Confluent primary endometrial epithelial cells were exposed to 10^5 IU/ml of HIV-1 IIIB for 4 hours alone or mixed with 10% SP from HIV-positive chronically infected men. Growth media (Untreated; UnT) was used as a negative control to determine baseline TNF-α levels. After the removal of supernatants, cells were fixed with 4% paraformaldehyde and stained with anti-human ZO-1 primary antibody followed by Alexa Fluor 488-conjugated secondary antibody (green stain). Cells were counterstained with propidium iodide nuclear stain. Mounted cells were visualized for ZO-1 by confocal microscopy with a magnification of 1260x.

TGF-β1 regulates TNF-α production and barrier integrity in GECs in the presence of HIV-1 in a manner similar to SP from HIV-positive, chronically infected men

My previous paper demonstrated that SP with the highest concentration of TGF-β lead to reduced proinflammatory responses in GECs (Kafka, et al., 2012). Since SP was able to prevent HIV-mediated increases in TNF-α production and barrier permeability in our GEC model (Figure 2); we next determined whether this effect could be due to the presence of the immunoregulatory factor TGF-β. The same primary GEC culture was exposed to SP from chronically infected men or TGF-β1 at comparable levels to active TGF-β1 measured in the SP samples used in this experiment, either alone or in the presence of HIV for 4 hours. As in the previous section, SP from chronically infected

men prevented HIV-mediated increases in TNF-α production. In the same culture, TGF-β treatment in the presence of HIV-1 also decreased TNF-α production in GECs compared to HIV alone (Figure 3).

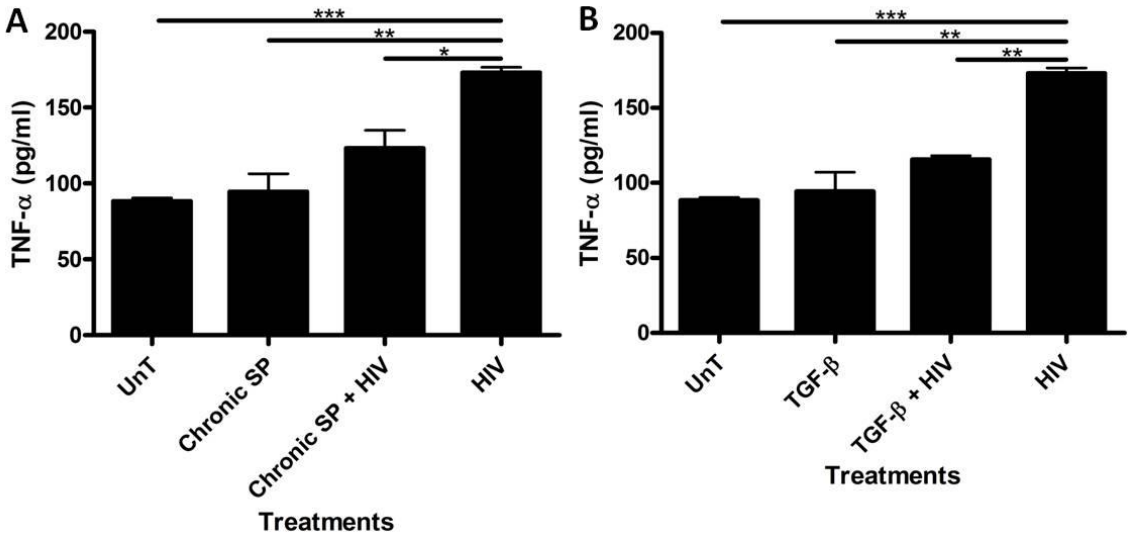


Figure 3: Comparison of TNF-α responses from GECs exposed to HIV in the presence of seminal plasma or TGF-β. Primary endometrial epithelial cells from the same culture were exposed to 10% SP from HIV-positive chronically infected men (A; chronic SP) or 1ng/ml of TGF-β1 alone (B) or in the presence of 10^5 IU/ml HIV-1 IIIB for 4 hours. Growth media was used as a negative control (UnT). Apical supernatants were collected and analyzed for TNF-α by commercial ELISA. *p<0.05, **p<0.01, ***p<0.001.

TGF-β1 prevents HIV-mediated increase of IL-6 in GECs

TNF-α has been shown previously to directly increase barrier permeability in response to pathogens or during pathologic conditions such as IBD (Capaldo & Nusrat, 2009). In addition to TNF-α, other cytokines have also been shown to decrease barrier integrity including IL-6 (Capaldo & Nusrat, 2009). Thus, we next determined whether TGF-β was able to prevent HIV-mediated increase in IL-6 in our GEC model in a manner similar to TNF-α. Primary GECs were exposed to HIV in presence of TGF-β at

comparable concentrations to active TGF-β measured in chronic SP samples, for 4 hours. Similar to TNF-α, IL-6 was significantly increased when GECs were exposed to HIV alone, whereas TGF-β treatment alone did not increase IL-6 above baseline levels. However, TGF-β treatment prevented HIV-mediated increase in IL-6 (Figure 4).

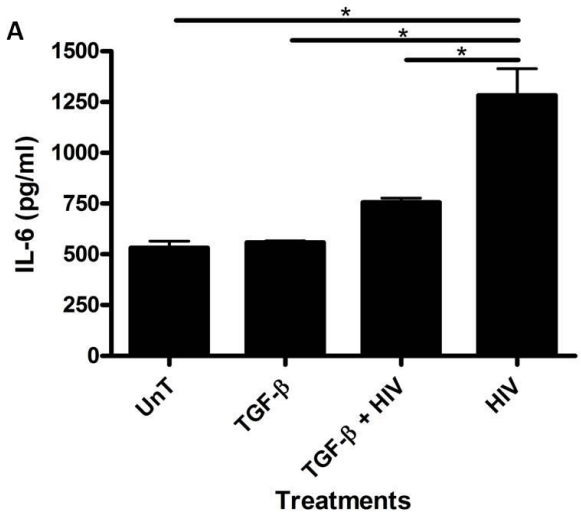


Figure 4: IL-6 production from GECs exposed to TGF-β and HIV. Primary endometrial epithelial cells were exposed to 1ng/ml of TGF-β1 alone or in the presence of 10^5 IU/ml HIV-1 IIIB for 4 hours. Growth media was used as a negative control (UnT). Apical supernatants were collected and analyzed by Eve Technologies, Inc. for IL-6 by custom multiplex assay. *$p<0.05$, **$p<0.01$.

TGF-β1 regulation of GEC TNF-α response depends on exposure time and is concentration dependent

TGF-β is an immunomodulatory cytokine that has been shown to have both anti- and proinflammatory activity based on the local microenvironment and presence of other factors (Sanjabi, et al., 2009). For example, TGF-β play an inflammatory role in combination with IL-6 as it contributes to the differentiation of Th17 cells (Korn, et al., 2009; Mangan, et al., 2006). In contrast, TGF-β can be immunoregulatory when working

synergistically with prostaglandins or IL-10 to induce differentiation of regulatory T cells (M. O. Li & Flavell, 2008; Robertson, O'Leary, & Armstrong, 2006). Since long term HIV infection is known to correlate with chronic immune activation, we decided to determine if TGF-β would enhance or inhibit the inflammatory effect of HIV on GECs when it is present for a longer time of 24 hours. We first examined how long term exposure to TGF-β in the context of HIV would influence TNF-α production in GECs. Since previous experiments were done with 4 hour exposure, we added TGF-β to GEC monolayers for 24 hours in combination with HIV and examined TNF-α production by ELISA. Prolonged exposure of GECs to TGF-β in the context of HIV significantly increased TNF-α production beyond levels measured by HIV exposure alone suggesting that long-term exposure of GECs to TGF-β enhanced the proinflammatory effect of HIV (Figure 5a).

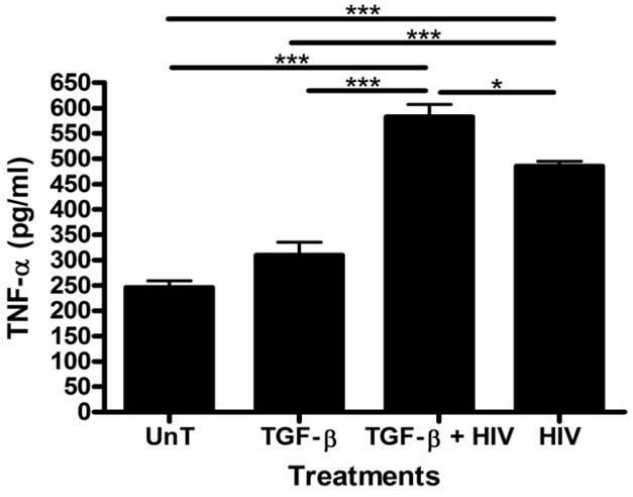
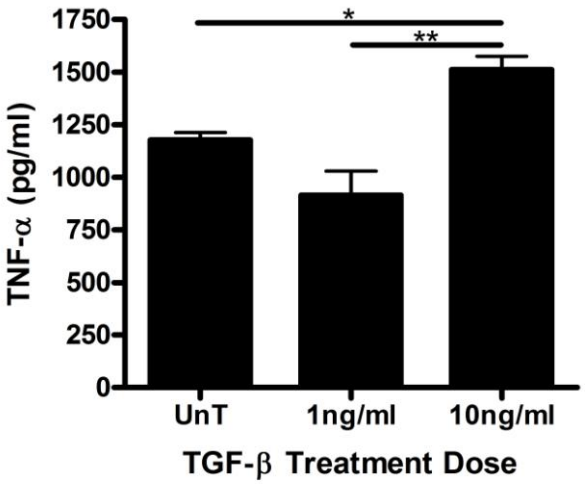


Figure 5a: TNF-α production in GECs treated with TGF-β and HIV for 24 hours. Primary endometrial epithelial cells were exposed to 1ng/ml of TGF-β1 alone or in the presence of 10^5 IU/ml HIV-1 IIIB for 24 hours. Growth media was used as a negative control (UnT). Apical supernatants were collected and analyzed for TNF-α by commercial ELISA. *$p<0.05$, ***$p<0.001$.

TGF-β has been previously shown in other biological systems to be biphasic in that low and high concentrations of TGF-β have lead to contrasting responses during processes such as bone development and angiogenesis (de Gorter, et al., 2011; Pepper, et al., 1993; Shinar & Rodan, 1990). To determine if this biphasic characteristic of TGF-β

also leads to contrasting TNF-α response in FGT epithelial cells, we exposed GECs to different concentrations of TGF-β with or without HIV and compared their TNF-α production. Separate experiments were conducted in which varying concentrations of TGF-β were incubated without (Figure 5b) or with HIV (Figure 5c) for 24 hours. In the absence of HIV, no significant differences in TNF-α were observed in GECs treated with growth media or 1ng/ml of TGF-β, however 10ng/ml of TGF-β significantly increased TNF-α production compared to 1ng/ml TGF-β or media control (Figure 5b).

Figure 5b: TGF-β dose comparison on TNF-α production in GECs. Primary endometrial epithelial cells were exposed to 1 or 10ng/ml of TGF-β1 or growth media control for 24 hours. Apical supernatants were collected and assayed for TNF-α production by commercial ELISA. *p<0.05, **p<0.01.



Long term exposure of GECs to HIV plus TGF-β resulted in significant differences in TNF-α production depending on the dose of TGF-β. When 0.1ng/ml of TGF-β was added to GECs with HIV for 24h, TNF-α production was significantly lower compared to HIV alone, but remained significantly higher than baseline levels (Figure 5c). In previous experiments, 1ng/ml of TGF-β on its own did not significantly change TNF-α production compared to baseline (Figure 5b), however when HIV was present a significant increase in TNF-α compared to baseline was observed (Figure 5c). On its own,

10ng/ml of TGF-β increased TNF-α production in GECs (Figure 5b), however this concentration of TGF-β with HIV lead to a more significant increase in TNF-α levels compared to baseline and HIV alone to levels comparable to 1ng/ml TGF-β dose with HIV (Figure 5c).

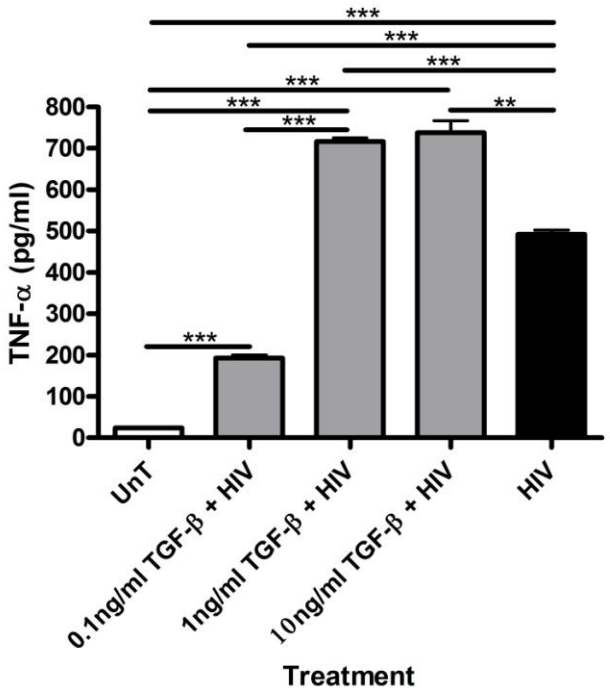


Figure 5c: TNF-α production in GECs co-treated with HIV and differing concentrations of TGF-β. Primary endometrial epithelial cells were exposed to 10^5 IU/ml of HIV-1 (IIIB) alone or with 0.1, 1, or 10ng/ml of TGF-β1 for 24h. Growth media was used as an untreated control (UnT). Apical supernatants were collected and assayed for TNF-α production by commercial ELISA. **p<0.01, ***p<0.001.

Upregulation of TNF-α production by GECs in response to gp120 added to SP

Our lab previously demonstrated that the HIV surface glycoprotein gp120 was the key mediator in the GEC-induced TNF-α response and subsequent barrier disruption in the FGT (Nazli, et al., 2010). Since HIV interacts with FGT epithelial cells in the context of SP, our next step was to examine the ability of gp120 to activate proinflammatory

pathways in the presence of SP from HIV-uninfected and infected men on primary FGT epithelial cells. Confluent monolayers of primary GECs were exposed to 10% uninfected SP or uninfected SP containing known amounts of recombinant gp120 protein. The dilution of SP was based on our previous studies, so that SP did not compromise the viability of GECs (Kafka, et al., 2012). Recombinant gp120 protein added alone without SP was used for comparison. Results indicated that GECs responded to gp120 present in SP in a dose-dependent manner, inducing significant upregulation in levels of TNF-α production, similar or slightly higher than the levels seen with gp120 alone (Figure 6a). Furthermore, when GECs were exposed to HIV-infected SP, TNF-α was upregulated by two-fold compared to uninfected SP exposure (Figure 6a). Because infected SP contains other proinflammatory cytokines that in turn induce inflammatory responses from GECs (Kafka, et al., 2012), we next determined whether addition of gp120 to acute-infected (AI) SP, which contained the maximum amount of inflammatory cytokines, would have an additive effect on the induction of TNF-α from primary GECs. Confluent primary GEC monolayers were exposed to pooled SP from AI men in the presence or absence of exogenous recombinant gp120 with or without a gp120-neutralizing antibody. Mock infection, recombinant gp120 alone, or pooled SP from AI men containing exogenous recombinant gp120 and SP containing isotype control antibody were used for comparison. Although both pooled SP from AI men and gp120 alone increased TNF-α production from GECs, exposure to a combination of pooled SP with recombinant gp120 produced an additive effect on induction of TNF-α from GECs (Figure 6b). Incubation with anti-gp120 antibody in addition to infected SP and recombinant gp120 reduced TNF-α

production to comparable levels observed with pooled SP alone (Figure 6b). The direct effect on the epithelial barrier was seen by a significant disruption of tight junctions by exposure to both uninfected SP spiked with recombinant gp120 and infected SP (Figure 6c). These results indicate that gp120 can induce inflammatory responses in the presence of SP. Further, other proinflammatory factors present in infected SP can add to the effect of gp120 on barrier disruption.

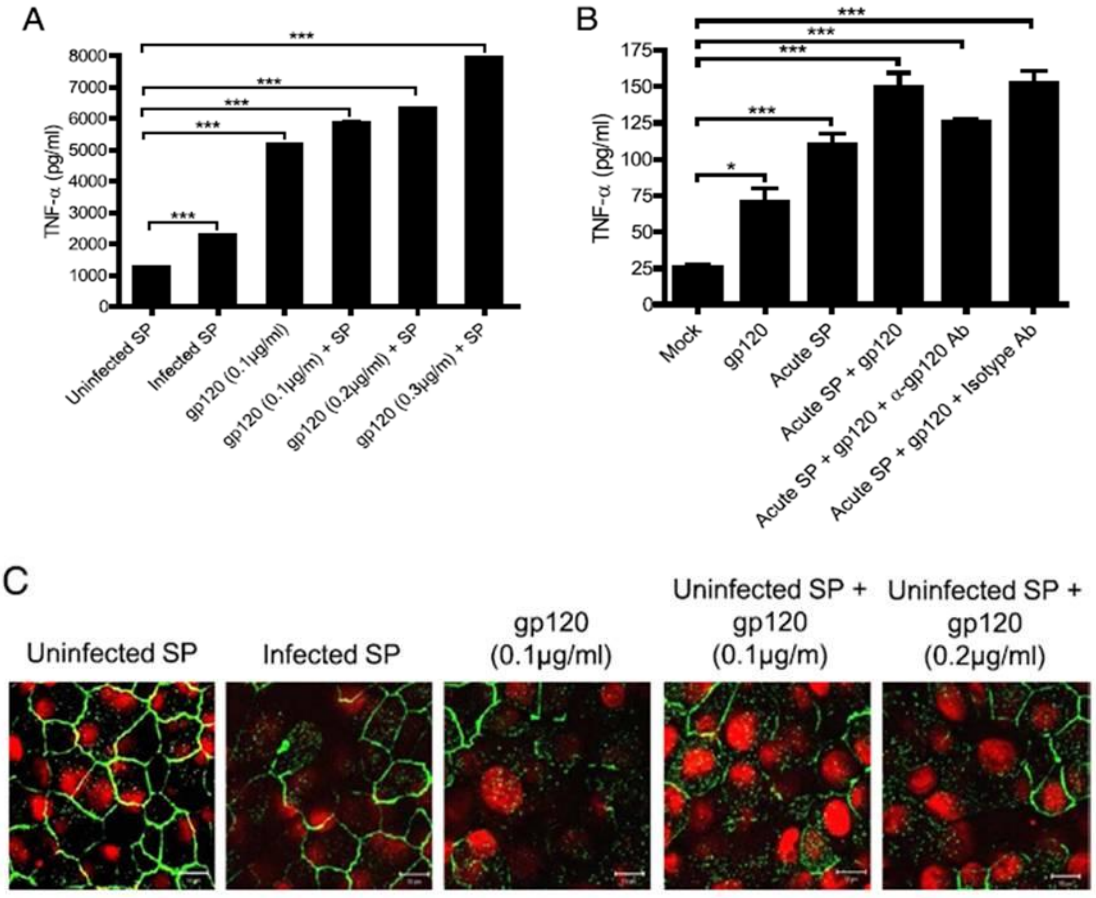


Figure 6: TNF-α production and epithelial barrier disruption in response to gp120 present in SP samples. (A) Primary GECs were exposed for 24 h to SP collected from uninfected or HIV-1–infected individuals or to uninfected SP spiked with known concentrations of gp120. TNF-a production was measured in supernatants using TNF-α ELISA. Bars represent mean ± SEM of triplicate cultures run for each condition from the same tissue sample. Data shown are representative of two separate experiments with similar results. **(B)** Primary GECs were exposed for 24 h to media alone (mock infection), gp120, pooled SP samples collected from three separate AI men, pooled SP from AI men with gp120, pooled SP from AI men with gp120 and anti-gp120 antibody, or pooled SP from AI men with gp120 and an isotype antibody. TNF-α production was measured in apical supernatants by ELISA. Data shown represent mean ± SEM. **(C)** GEC monolayers were fixed following treatment with SP samples and stained for ZO-1 tight junction protein via confocal microscopy. Original magnification x1260. *$p<0.05$, ***$p<0.001$. Images in figure 6 were used with permission from the journal (Nazli, et al., 2013).

4.5 DISCUSSION

The current chapter demonstrates for the first time a possible role of SP and the immunoregulatory factor TGF-β on epithelial cell barrier function in the presence of HIV in the FGT. Despite knowing that HIV is introduced into the FGT in the context of SP during coitus, we have limited knowledge of how SP, especially from HIV-infected men, modulates the FGT epithelial barrier. As shown by the first study described in Chapter 2, SP from CI men contained a different cytokine profile characterized by higher levels of TGF-β and lower levels of proinflammatory cytokines compared to AI men and uninfected men which resulted in a decreased inflammatory response from GECs (Kafka, et al., 2012). How different cytokine repertoires from CI, AI and uninfected men contribute to GEC barrier function in the presence of HIV was one of the key goals of the current study, and we anticipated that the balance of proinflammatory and immunoregulatory factors in SP will dictate the cytokine response from GECs. In AI men, the presence of higher levels of proinflammatory cytokines and HIV may counteract the immunoregulatory effect of TGF-β resulting in an increased inflammatory response

from GECs and subsequent barrier disruption. In CI men where higher levels of active TGF-β were often seen in SP compared to proinflammatory cytokines, the more dominant immunoregulatory profile may protect the GEC barrier in the presence of HIV since the GEC response would be less inflammatory.

Our current knowledge of how HIV-uninfected SP modulates GEC barrier function suggests that it plays a protective role as shown by an increase in TER in cervical epithelium (Gorodeski & Goldfarb, 1998; P. Lawrence, et al., 2012), however it was not clear how SP from HIV-positive men could modulate TER in FGT ECs. In agreement with my hypothesis, we demonstrated for the first time that SP from both HIV-uninfected and CI men, in the absence of HIV, strengthened the epithelial barrier in primary endometrial EC cultures as measured by a significant increase in TER compared to growth media alone (Figure 1). Although SP from AI men was not included in the analysis, in experiments where acute SP was added in presence or absence of gp120, we saw a significant increase in TNF-α production and barrier permeability in GECs; an additive effect was also observed for TNF-α when acute SP was added to GECs with increasing concentrations of gp120 (Figure 6a) suggesting that SP from acutely infected men could be disrupting to the epithelial barrier.

Proinflammatory cytokines such as IL-1, IL-6, IFN-γ and TNF-α are known to disrupt the tight junction barrier in the FGT and intestine (Capaldo & Nusrat, 2009). Our lab was the first to show that GECs exposed to HIV-1, in particular its surface protein gp120, experienced increased epithelial barrier permeability as a result of increased production of TNF-α (Nazli, et al., 2010). In this study we examined the role of SP from

CI men, which was shown to strengthen the GEC barrier (Figure 1), in the presence of HIV. SP was added to GECs for 4 hours as described in my first paper to account for the cytotoxicity of SP (Kafka, et al., 2012), as well as mimic short term exposure of GECs to SP following coitus, while still allowing a measureable cytokine response from our GEC cultures. In agreement with my hypothesis, short term exposure of GECs to SP from CI men with HIV significantly reduced TNF-α production in GECs to levels comparable to chronic SP, or growth media control (Figure 2a) as well as maintained the ZO-1 tight junction barrier in the presence of HIV as observed by immunofluorescent staining (Figure 2b). Our results showed that short term exposure to chronic SP reduced TNF-α production and prevented ZO-1 tight junction breakage in the presence of HIV suggesting that the FGT epithelial barrier is protected.

Next, to determine whether this protective effect was mediated by the immunoregulatory cytokine TGF-β present in SP, we compared short term exposure of GECs to either chronic SP with HIV or TGF-β with HIV and found that TGF-β at comparable levels measured in chronic SP also decreased TNF-α production compared to HIV alone (Figure 3). Additionally, short term exposure to TGF-β and HIV also reduced IL-6 production by GECs (Figure 4). Overall, these results suggest a protective effect for GECs in the presence of HIV by short term exposure to TGF-β at levels comparable to chronic SP, as it decreased production of the barrier compromising cytokines IL-6 and TNF-α. Immunofluorescent analyses still needs to be carried out on GECs to confirm that short term exposure of TGF-β protects the ZO-1 tight junction barrier in the presence of HIV. Additionally, mechanisms underlying TGF-β-mediated decrease in proinflammatory

cytokine production from GECs exposed to HIV, such as potential intracellular SMAD pathways which can block inflammation, need to be further examined.

Chronic HIV infection is known to cause persistent immune activation and elevated TGF-β levels in plasma (Brenchley, Price, Schacker, et al., 2006; Navikas, et al., 1994). For this reason we decided to determine if TGF-β would enhance or inhibit the inflammatory effect of HIV on GECs when it is present for longer time and at higher concentrations as TGF-β can have inflammatory or immunoregulatory functions depending on the microenvironment (Sanjabi, et al., 2009). We found that long term exposure (24 hours) of TGF-β in combination with HIV caused a more inflammatory response from GECs beyond what was measured with HIV alone (Figure 5a). Additionally, 24 hour exposure of GECs to higher concentrations of TGF-β (10ng/ml) also significantly increased TNF-α implying that the presence of TGF-β in the HIV-infected FGT microenvironment may exacerbate the inflammatory response of GECs to HIV. These results imply that in the context of persistent local inflammation in the FGT of an HIV-infected woman, continuous exposure of GECs to TGF-β at high concentrations could exacerbate HIV infection and barrier disruption. The continuous production of TGF-β in the FGT microenvironment may be aimed at restoring immune homeostasis during local immune activation (Sanjabi, et al., 2009).

It was previously shown in our lab that HIV gp120, along with heparan sulphate, increased proinflammatory cytokine expression in GECs via activation of the TLR pathway (Nazli, et al., 2013). In this study we determined if gp120 exerted the same effect in the context of SP. Based on my hypothesis that TGF-β in SP would have a

protective effect, one could argue that in the reverse scenario, such as in the presence of greater levels of inflammatory cytokines in AI SP, the protective effect could be blocked or even reversed. Although both SP from HIV-infected men (AI SP) and gp120 individually increased TNF-α production significantly above mock infected control, an additive effect was observed when GECs were exposed to AI SP in combination with gp120, and this effect increased with increasing concentration of gp120 (Figure 6a). Immunofluorescent staining of GECs exposed to gp120 and AI SP also showed the greatest disruption of the EC barrier when GECs were exposed to gp120 with SP (Figure 6c). The addition of gp120 antibody returned TNF-α production to levels comparable to AI SP alone (Figure 6b).

Although this chapter begins to elucidate the role of SP and TGF-β on GEC barrier function against HIV, future studies need to be carried out to fill in a few gaps in the results. For example, when the dose kinetic was carried out for TGF-β illustrated in figure 5b, the lower dose of 0.1ng/ml should have been included in addition to 1.0 and 10.0ng/ml. The inclusion of the 0.1ng/ml dose would allow for consistency between figure 5b and figure 5c which compared TNF-α responses in GECs exposed to HIV and increasing concentrations of TGF-β. A further improvement would be to combine experiments 5b and 5c on the same set of GECs to have a more accurate comparison of how increasing doses of TGF-β influenced barrier function in the absence and presence of HIV. However, this would require a substantial number of GECs, which are often difficult to obtain due to limitations in FGT tissue sizes.

Overall, these studies have aided us in our understanding of the complex and dynamic interactions between semen, HIV, and FGT epithelial cells and how these interactions can modulate the FGT epithelial barrier. The results in this chapter imply that SP from an HIV-infected man can exert different results from GECs depending on accompanying proinflammatory cytokines, how long SP encounters epithelial cells, the presence of HIV, and the concentration of TGF-β present in the environment and how long GECs are exposed to it.

In summary, the current chapter outlines preliminary experiments for the role of SP and TGF-β in the regulation of the FGT epithelial cell barrier. Future experiments examining the mechanism behind the regulation of the GEC barrier by SP, and its different components, would be beneficial in the design of therapeutic strategies to prevent the disruption of mucosal barriers in the presence of inflammatory conditions and/or HIV.

--- CHAPTER 5 ---

DISCUSSION

5.1 Summary and Context of the Research

Heterosexual transmission is the primary mode of HIV infection in developing countries (Shattock & Moore, 2003; UNAIDS, 2013) with semen being the main vector for male-to-female HIV-1 transmission during vaginal sex (Doncel, et al., 2014). What we know mostly about SP is its ability to modulate the FGT immune system, primarily through epithelial cell signalling, to tolerate sperm and the allogeneic fetus for successful pregnancy (Schjenken & Robertson, 2014). We also know that multiple factors in semen from healthy, HIV-uninfected men may differentially influence HIV-1 infection, mainly in target cells including macrophages and T cells (Doncel, et al., 2010; Sabatte, Remes Lenicov, et al., 2011). This information is not known for epithelial cells, which are the first cells HIV encounters in the FGT. Furthermore, we also do not know how SP from HIV-infected men in different stages of infection may influence epithelial cell responses. SP is also known to contain high concentrations of the immunoregulatory factor TGF-β1, whose main function is to promote a tolerogenic environment in the FGT during conception. Yet, we do not know how such high levels of TGF-β will impact GEC cytokine responses and mucosal barrier function in the FGT in the presence of HIV-1.

The studies described in this thesis addressed this area of research where there are significant gaps in information as described above. The studies were designed with three goals in mind. Our first objective was to compare proinflammatory and immunoregulatory cytokine and chemokine concentrations in SP from HIV-uninfected men and HIV-infected men in acute and chronic stages of HIV infection. Secondly, we wanted to compare cytokine and chemokine responses from female GECs exposed to SP

from HIV-uninfected and HIV-infected men. Finally we wanted to elucidate how SP and TGF-β regulated proinflammatory cytokine production and barrier function in FGT epithelial cells in the presence of HIV.

SP contains a complex array of immunomodulatory cytokines, chemokines and growth factors which, when deposited into the FGT, stimulate cytokine and chemokine expression in the FGT epithelial lining, which could result in altered susceptibility to sexually-transmitted pathogens. Previous studies from Politch et al. and Maegawa et al. measured a wide variety of cytokines, chemokines, growth factors, immunoglobulins, and immune cells in semen of HIV-uninfected heterosexual men (Maegawa, et al., 2002; Politch, et al., 2007). These studies measured a wide repertoire of cytokines, chemokines and growth factors including G-CSF, GM-CSF, IFN-α, IFN-γ, IL-1α, IL-1β, IL-2, IL-6, IL-6, IL-10, IL-12, IL-13, IL-17, M-CSF, MIP-1α, MIP-1β, RANTES, and TNF-α as well as PMNs in semen from HIV-uninfected men. High concentrations of TGF-β, IL-7, IL-8, MCP-1, SDF-1α as well as IgG and IgA were also measured in their studies (Maegawa, et al., 2002; Politch, et al., 2007). Although our study did not measure seminal immunoglobulins or immune cells, and we examined a limited repertoire of cytokine/chemokines, we were the first to compare cytokine, chemokine, and growth factor levels between HIV-uninfected, and HIV-infected MSM in acute and chronic stages of infection. In agreement with my hypothesis that SP from HIV-infected men in acute stages of infection should contain higher levels of proinflammatory cytokines we observed higher levels of proinflammatory cytokines and chemokines, particularly IL-6,

IL-8 and MCP-1, in SP from acutely infected men, compared to HIV-uninfected men and chronically infected men.

When deposited into the FGT, SP elicits a dynamic cytokine and chemokine response from GECs. Work conducted in Sarah Robertson's lab and others demonstrated that SP from HIV-negative men induced expression of several proinflammatory cytokines and chemokines in uterine, cervical, and vaginal epithelial cells and explants in various animal models and humans (Denison, Grant, et al., 1999; Gutsche, et al., 2003; O'Leary, et al., 2004; Ochsenkuhn, et al., 2008; D. J. Sharkey, et al., 2007; D. J. Sharkey, Tremellen, et al., 2012). Although these prior studies have shaped our understanding of the initial cytokine and chemokine responses from GECs in response to SP from normal healthy men, our study was the first to examine cytokine and chemokine production in primary endometrial epithelial cells in response to SP from HIV-infected ART-naive men in the acute/early stage of HIV infection (AI men) and in chronic HIV infection (CI men). We observed differences in proinflammatory cytokine production between FGT epithelial cells exposed to SP from AI and CI men, compared to SP from HIV-uninfected men. In agreement with the second part of my hypothesis, we observed a more proinflammatory response from GECs treated with SP from AI men compared to CI men. A unique and somewhat unexpected observation was that high levels of active TGF-β1 were observed more often in SP from ART-naive CI men, compared to AI and uninfected men, and that high levels of active TGF-β specifically reduced proinflammatory cytokine production by GECs. In our later studies, we showed that elevated active TGF-β levels were unique to

ART-naive men, as men who were on ART had lower active TGF-β levels which were comparable to levels measured in HIV-uninfected men.

Since publishing my first paper in AIDS in 2012, it has been cited in 16 articles and reviews mostly examining how SP influences HIV transmission in the FGT. With semen being a complex fluid, with one proteomics study reporting over 900 different proteins (Fung, et al., 2004; Pilch & Mann, 2006), it is not surprising that since my first publication, there have been other articles identifying additional factors in semen from HIV-uninfected and infected men which may impact HIV transmission (Introini, et al., 2013; Lourenco, et al., 2014; Madison, et al., 2014; Olivier, et al., 2013). In one of these studies, IL-7, which has been detected in high concentrations in human semen and is elevated in semen of HIV-infected men, was shown to enhance HIV replication in cervicovaginal tissue and prevent apoptosis of T cells leading to the maintenance of an infected local T cell pool in the FGT (Introini, et al., 2013). Thus, our article as well as others supports the premise that HIV infection leads to the modulation of the semen cytokine and chemokine repertoire which can ultimately influence HIV transmission.

As a follow up to our first study, we next examined the reason for elevated active TGF-β levels in the ART-naive CI men in our cohort. The results from our first study led us to hypothesize that the high levels of active TGF-β measured in ART-naïve CI men was possibly due to these men naturally controlling their immune activation status. As discussed below, in agreement with this premise we indeed observed differences in active TGF-β in therapy–naive CI men and ART-treated CI men in our second study.

One of the characteristics of chronic HIV infection is persistent immune activation, with the monocyte/macrophage immune activation marker soluble CD14 (sCD14) being found to directly correlate with disease progression and mortality in HIV-infected individuals (Sandler, et al., 2011). Previous work showed sCD14 to be present in SP of HIV-negative individuals at levels comparable to that of BP (Harris, et al., 2001), however sCD14 in SP of HIV-infected men had not been examined prior to our study. It was also not clear whether or how levels of TGF-β would correlate with sCD14 in semen and/or BP of chronic infected individuals. Our results demonstrated that active and latent forms of TGF-β1 were compartmentalized between BP and SP. We also observed a significant negative correlation between active TGF-β1 and sCD14, and between active TGF-β and semen viral load in SP from ART-naive men suggesting a possible immunoregulatory role for active TGF-β in ART-naive men in controlling their immune activation status. Given the correlation between TGF-β and sCD14 in semen, we tested the possibility that a common cell type such as activated macrophages could potentially produce both these factors in the presence of HIV and indeed found that to be the case *in vitro*. Although separate studies had previously showed that HIV infection can increase TGF-β or sCD14 production in monocytes/macrophages, our study was the first to show that *in vitro*, HIV infection could lead to co-expression of latent TGF-β and sCD14 from a common cell type, such as activated macrophages. Following publication of this study, a recent article compared cytokine and chemokine profiles in blood and semen from HIV-negative and HIV-positive ART-treated and ART-naive men, and examined their correlation with T cell activation, plasma viral load, and semen shedding (Olivier, et al.,

2014). They found that, similar to our study, semen cytokine profiles were distinct between blood and semen. Additionally, semen had elevated levels of several proinflammatory cytokines as well as activated T cells compared to blood regardless of HIV infection; however HIV infection disrupted the co-regulation of cytokine networks in semen. They also found that G-CSF levels in semen significantly predicted T cell activation and HIV shedding. This article expanded upon my second study by conducting a more comprehensive comparison of inflammatory and regulatory cytokines in blood and semen of HIV-negative and HIV-infected men. They also examined how these cytokine expression patterns predicted T cell activation and HIV shedding to get a better understanding of how local immune activation and semen inflammatory markers may determine HIV transmission risk.

To understand the functional role of TGF-β, we also examined its role in maintaining the mucosal barrier. Previous studies have shown that TGF-β prevented barrier dysfunction in the presence of intestinal pathogens (Howe, et al., 2005; Roche, et al., 2000) and barrier-disrupting cytokines (S. Planchon, et al., 1999; S. M. Planchon, et al., 1994). We know TGF-β is present in high levels in semen, yet the role of semen TGF-β on FGT epithelial barrier function in the context of HIV infection had not been examined. Studies from our lab were the first to show that HIV directly impaired the GEC barrier by producing increased levels of barrier-disrupting cytokines, especially TNF-α (Nazli, et al., 2010; Nazli, et al., 2013). I expanded upon these experiments by examining the effect of HIV on GECs in the context of SP from CI men, or TGF-β alone at concentrations comparable to SP, and found a protective effect of TGF-β on GEC tight

junctions and barrier function. This study was the first to demonstrate that TGF-β prevented HIV-mediated GEC barrier disruption by downregulating TNF-α production, maintaining ZO-1 tight junction proteins, and increasing TER in the presence of HIV with TGF-β. An interesting observation in this study was that when TGF-β was added to GECs in presence of HIV for longer time periods such as 24 hours, TGF-β exacerbated proinflammatory cytokine production in GECs beyond levels measured with HIV alone, suggesting that TGF-β can be either proinflammatory or immunoregulatory in the FGT depending on the exposure conditions. Several studies have demonstrated a biphasic role of TGF-β in various biological systems (de Gorter, et al., 2011; Pepper, et al., 1993; Shinar & Rodan, 1990; Wahl, 2007; Wenner & Yan, 2003). While TGF-β is more commonly known for its immunoregulatory functions (Robertson, et al., 2013; Schon & Weiskirchen, 2014), other studies have demonstrated that TGF-β can also exert an inflammatory role (Mangan, et al., 2006; D. J. Sharkey, Macpherson, et al., 2012) with some articles suggesting, in support of our recent results, that an over-expression of TGF-β can also lead to an exacerbated inflammatory response (Wahl, 1994, 2007).

One of the published studies for which I was a co-author demonstrated that SP from AI men mixed with increasing concentrations of HIV surface protein gp120 lead to significant increases in TNF-α in GECs in a dose-dependent manner, leading to epithelial barrier disruption. This observation is in agreement with my central hypothesis showing that in the context of HIV, SP from men in acute infection increased proinflammatory cytokine production in GECs leading to barrier disruption.

5.2 Study Implications

The overall implication of the body of research completed for this thesis is that the cytokine environment in SP plays an important role in HIV-1 infection in the genital tract. Firstly, men who are in acute/early stages of HIV infection exhibit higher proinflammatory cytokines in their semen which, when deposited into the FGT, elicited a more proinflammatory microenvironment characterised by increased expression of proinflammatory cytokines from GECs. This could create a favorable environment for heterosexual HIV transmission based on the stage of infection of the male partner and the susceptibility profile of the female partner. In the scenario where HIV infection is already present in the FGT, the introduction of SP from AI men would lead to exacerbation of HIV replication via increased activation of the HIV LTR promoter. Our studies also indicate that GECs in the FGT would respond to HIV in a dose dependent manner in the presence of semen, with increased proinflammatory cytokine production in the presence of higher viral load. This heightened proinflammatory response from GECs in the FGT could potentially increase HIV transmission through increased barrier permeability, especially when semen is from a man in the AI stage and contains high viral loads.

In contrast, CI men who are ART-naive, exhibit a different cytokine repertoire in their SP which is characterised by lower proinflammatory cytokines, and higher active TGF-β. This could potentially lead to regulated proinflammatory cytokine responses from FGT epithelial cells compared to SP from AI men. The higher concentration of active TGF-β in SP from chronic ART-naive men appears to be compartmentalized to the MGT and is unique to ART-naive men since men on ART did not exhibit elevated levels of

active TGF-β in their SP. Given that the highest levels of TGF-β were found in CI men in our study cohort who were ART-naive, the presence of high levels of TGF-β in the SP of these men may be an immunoregulatory response to a localized activated immune status in the MGT.

Our previous studies have shown that HIV exposure in the FGT leads to GEC responses characterized by elevated proinflammatory cytokines which in turn could increase barrier permeability and HIV infection. However, the present studies suggest that short-term exposure to semen with high TGF-β levels would protect the mucosal barrier and therefore hinder HIV transmission in the FGT. In contrast, longer exposure to TGF-β in the context of HIV may exacerbate HIV infection since TGF-β combined with HIV for 24 hours, further increased proinflammatory cytokine production from GECs beyond HIV alone.

5.3 Study Limitations

While these results provide valuable information regarding the role of SP and TGF-β in HIV infection in the FGT, there are a few limitations to our study that must be taken into consideration. Firstly, we used a primary GEC *ex-vivo* model to study the interactions between SP, TGF-β, and the FGT epithelial lining. While there are significant advantages to using a primary cell culture system composed of a single cell type (endometrial columnar epithelium), this cell culture model does not take into account the complexities found in the tissue microenvironment. Although we have optimized the cell culture system by using primary epithelial cells isolated from hysterectomy samples under conditions where they closely mimic the polarized monolayer formation seen in *in*

vivo settings, there is an absence of other factors present in the FGT including mucus, commensal bacteria, spermatozoa, and immune cells. Nevertheless, such single-cell models are still very useful in conducting mechanistic studies and the examination of functional pathways as it allows the focused analysis of specific factors and single cell types while eliminating variability when multiple factors and complex tissues are present. Additionally, findings from primary cell cultures are more physiologically relevant than those reported from cell lines, which are commonly used in similar studies.

A second limitation that warrants consideration is the definition of the acute stage of HIV infection in the studies described in this thesis. While many studies use similar definitions for identifying acute versus chronic infection, there is a widely used biological definition of acute infection which is the detection of HIV RNA or p24 antigen in the blood before HIV antibodies have formed (Cohen, et al., 2010; Keele, et al., 2008). Some studies, including ours, draw a metaphorical "line in the sand" and define AI men as being within 6 months of their first HIV-positive test who were either negative or indeterminate by immunoblot (Bassett, et al., 2012). It is well acknowledged that it is very difficult to identify men who are within 1-2 weeks of HIV infection since the virus is in what is known as the eclipse phase. During this period of between 7-14 days following transmission, viral infection is established locally in the mucosa, submucosa, and draining lymphoreticular tissues, but viral RNA is not yet detectable in plasma (Cohen, Shaw, et al., 2011; McMichael, et al., 2010). Given the inordinate difficulty in identifying and obtaining samples from men who are within 1-2 weeks of early HIV infection, it is likely that the men we defined as AI men were past the actual acute phase of HIV infection and

had established a stable viral set point and CD4 count. Nevertheless, even though these men may not be in the classical acute stage, it is clear that the immune system is still responding to HIV infection, since we were still able to measure differences in proinflammatory cytokine levels between SP samples from AI men and CI men. We also considered men who have been infected with HIV for longer than 6 months to be in the chronic stage of infection. Although one may argue that an individual may not really be in the chronic stages of HIV infection just after 6 months post-infection, we used a previously published study from our collaborators at the University of Toronto as a benchmark for defining chronic infection in our SP samples (Sheth, et al., 2006).

The SP samples used in these studies were obtained through collaborations with colleagues at the University of Toronto where the cohort of HIV-infected, ART-naive men was formed. Due to the difficulty in obtaining ART-naive samples, particularly from AI men, the studies conducted in this thesis were carried out with a small number of SP samples (n=7 for AI men; n=12 for CI men). Although we added more SP samples for the second study, which further confirmed our initial observation of high active TGF-β levels in SP from CI men, it was difficult to achieve the statistical power needed for significance in the Spearman rank correlation test. For example, our analysis of the correlation between sCD14 and active TGF-β in SP was trending towards significance with a p value of 0.06; inclusion of a larger number of samples could improve the statistical power for this analysis and possibly provide statistical significance. Nevertheless, we were still able to observe a significant negative correlation between active TGF-β and semen viral load in SP in our second study.

It is important to note that our SP samples were diluted to 10% prior to addition to GECs to reduce any cytotoxic effects of the SP. The cytotoxic effects of SP are well known by groups who work in this area with many researchers using diluted semen samples in their studies (Lourenco, et al., 2014; Martellini, et al., 2011; D. J. Sharkey, Macpherson, et al., 2012; D. J. Sharkey, et al., 2007). However, it is unclear whether during normal intercourse when semen is deposited into the FGT whether there is a natural dilution that occurs because of the presence of cervical mucus and other secretions, or whether the mixing of male and female secretions reduces any cytotoxic effects of semen which are seen *in vitro*.

Finally, the dilution of SP from infected men for our studies may have also diluted out any cell-free HIV. We know from the previous study in our lab that GECs have a threshold at which they will respond to known concentrations of HIV (Nazli, et al., 2010). Due to the highly diluted nature of our SP samples, it is unlikely that cell-free HIV was present in sufficient concentrations to warrant a response from GECs. To the best of our knowledge, any cell free virus present in infected semen was diluted beyond any direct effect, but one cannot rule out that there may be additive effects when exogenous virus and cytokines were added in our *in vitro* cultures.

5.4 Future Directions

During the time I completed work for my thesis, I demonstrated that both SP and TGF-β modulated the epithelial barrier in the presence of HIV-infection. This was shown through measuring TER in confluent EC monolayers, measuring TNF-α production by GECs, and examining ZO-1 tight junction proteins via immunofluorescent staining. My

results showed that short term exposure to SP and TGF-β protected the EC barrier in the presence of HIV by decreasing TNF-α production and preventing disruption of ZO-1. These responses appear to be time-dependent as long-term exposure to TGF-β lead to a more proinflammatory cytokine response in GECs characterized by increased levels of TNF-α, and exposure to SP from AI men with gp120 increased TNF-α production in GECs. Overall, our studies suggest a protective role for short-term exposure of TGF-β and SP, but long-term exposure may yield a more inflammatory cytokine response in GECs. The next plausible direction in examining the role of TGF-β on barrier function would be to investigate the mechanism by which TGF-β may be modulating the mucosal barrier in the presence of HIV. Since HIV has been shown to increase TNF-α production in GECs, I anticipate that TGF-β is affecting the TNF-α signalling pathway possibly through disruption of the TLR pathway. This has been previously suggested by Naiki et al, where they determined that TGF-β interfered with NF-κB and subsequent proinflammatory cytokine signalling in endothelial cells stimulated with TLR 2, 4, and 5 ligands in a MyD88-dependent manner (Naiki, et al., 2005). In addition to modulating the TNF-α pathway, TGF-β may also prevent barrier disruption by increasing expression of tight junction proteins in the presence of HIV similar to what has been observed in Howe et al, in response to enteropathogenic *E. coli* (Howe, et al., 2005).

We focused on cell-free HIV in this thesis; however several studies have shown that SP from HIV-infected men also contains a significant amount of cell-associated virus which may play an important role in HIV transmission (D. J. Anderson & Le Grand, 2014; Bernard-Stoecklin, et al., 2013; Houzet, Matusali, & Dejucq-Rainsford, 2014;

Politch, et al., 2014). Cell-associated HIV and its role in mucosal transmission is an important, yet often overlooked area of investigation. Cell-to-cell HIV transmission has been described as being more efficient, rapid, and resistant to host immune responses compared to cell-free virus (Bernard-Stoecklin, Gommet, Cavarelli, & Le Grand, 2014; Rudnicka, et al., 2009). Dr. Deborah Anderson, a prominent researcher in the area of cell-associated HIV transmission, recently stated in a review of this area that failure of several current microbial and vaccine candidates may be due in part to the different molecular events which occur during cell-free and cell-associated HIV transmission (D. J. Anderson & Le Grand, 2014). Therefore, further studies should be conducted to examine the role of SP and cell-associated virus on modulation of GEC cytokine responses and barrier function.

Furthermore, subsequent studies should examine how other immune modulating factors in semen may influence TGF-β effects on GECs since it has been shown that TGF-β works synergistically with PGE2 in semen to induce an immunoregulatory microenvironment in the FGT (Baratelli, et al., 2005; M. O. Li, et al., 2006; Robertson, et al., 2013), but can also enhance inflammation by contributing to the differentiation of naive T cells into Th17 cells in tandem with IL-6 (Korn, et al., 2009; Mangan, et al., 2006). Due to the biphasic nature of TGF-β and our observation of both proinflammatory and immunoregulatory responses from this multifunctional cytokine, determining under which environment TGF-β becomes pro- or anti- inflammatory, especially in the context of HIV infection, would be beneficial for the development of future therapeutic strategies.

5.5 Conclusion

To put the whole role of semen in HIV transmission in context, there are a variety of factors present in the FGT microenvironment which can influence HIV transmission and infection. While we have a better understanding of how some factors, such as co-infecting STIs and imbalance of the local microbiota can either directly or indirectly contribute to inflammation and enhanced HIV infection, the interactions between FGT and semen are more complex as our studies have demonstrated that semen contains both HIV-enhancing and inhibiting components. Semen from AI men contain high concentrations of proinflammatory cytokines which, coupled with high semen viral loads, can further promote proinflammatory responses from GECs and barrier disruption which can subsequently enhance HIV infection in the FGT. In contrast, SP from CI men had reduced proinflammatory cytokines but high levels of active TGF-β which can lead to a dampened down inflammatory response from GECs and maintenance of the epithelial barrier in the presence of HIV. ART-treated men have reduced TGF-β levels suggesting a potential role of TGF-β in regulating local immune activation, possibly due to the existence of other pathologies or co-infecting infections in the MGT.

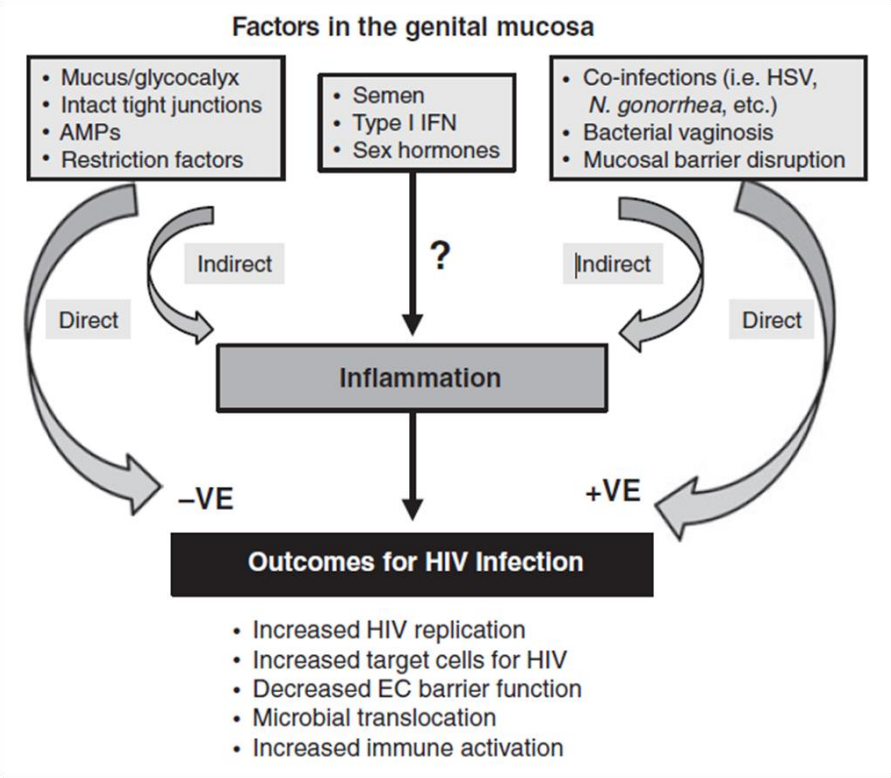


Figure 1: Factors in the FGT microenvironment and their influence on HIV infection. Physical barriers (such as, intact tight junctions and mucus/glycocalyx) and chemical/biological barriers, such as, AMPs and restriction factors, can prevent HIV acquisition in the FGT by directly inhibiting infection and/or replication. In contrast, co-infection with HSV and *N. gonorrhea*, bacterial vaginosis, or mucosal barrier disruption can either directly enhance HIV infection/replication or indirectly via the induction of inflammation. Mucosal co-factors such as these may also contribute to increasing the availability of HIV target cells, increasing immune activation, decreasing barrier function, and microbial translocation, all of which contribute to enhanced infection and disease pathogenesis. It is not clear whether factors such as semen, type I IFN, or sex hormones contribute to regulating HIV infection/ replication in the FGT as the studies suggest they can both inhibit or enhance HIV infection/replication depending on the FGT microenvironment. Figure used with permission from John Wiley & Sons (Ferreira, et al., 2014).

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--- APPENDIX ---

Co-Authored Manuscripts and Reviews

The Journal of Immunology

HIV-1 gp120 Induces TLR2- and TLR4-Mediated Innate Immune Activation in Human Female Genital Epithelium

Aisha Nazli,[*,†] Jessica K. Kafka,[*,†,1] Victor H. Ferreira,[*,†,1] Varun Anipindi,[*,†] Kristen Mueller,[*,†] Brendan J. Osborne,[‡] Sara Dizzell,[*,†] Sarah Chauvin,[*,†] M. Firoz Mian,[*,†] Michel Ouellet,[§] Michel J. Tremblay,[§] Karen L. Mossman,[*,†] Ali A. Ashkar,[*,†] Colin Kovacs,[¶] Dawn M. E. Bowdish,[*,†] Denis P. Snider,[*,†] Rupert Kaul,[‡] and Charu Kaushic[*,†]

Although women constitute half of all HIV-1–infected people worldwide (UNAIDS World AIDS Day Report, 2011), the earliest events in the female reproductive tract (FRT) during heterosexual HIV-1 transmission are poorly understood. Recently, we demonstrated that HIV-1 could directly impair the mucosal epithelial barrier in the FRT. This suggested that the HIV-1 envelope glycoprotein gp120 was being recognized by a membrane receptor on genital epithelial cells, leading to innate immune activation. In this study, we report that pattern-recognition receptors TLR2 and -4 bind to HIV-1 gp120 and trigger proinflammatory cytokine production via activation of NF-κB. The gp120–TLR interaction also required the presence of heparan sulfate (HS). Bead-binding assays showed that gp120 can bind to HS, TLR2, and TLR4, and studies in transfected HEK293 cells demonstrated that HS and TLR2 and -4 were necessary to mediate downstream signaling. Exposure to seminal plasma from HIV-1–infected and uninfected men with gp120 added to it induced a significant proinflammatory cytokine response from genital epithelial cells and disruption of tight junctions, indicating a role for gp120 in mucosal barrier disruption during HIV-1 heterosexual transmission. These studies provide, for the first time to our knowledge, a possible mechanism by which HIV-1 gp120 could directly initiate innate immune activation in the FRT during heterosexual transmission. *The Journal of Immunology*, **2013, 191: 4246–4258.**

The sexually transmitted virus HIV-1 is primarily transmitted via mucosal routes. Despite a low transmission probability per exposure event, the female reproductive tract (FRT) accounts for ~40% of all new HIV infections each year (1). Early events following HIV-1 exposure in the FRT have not been completely elucidated (2). Cumulative results from in vivo SIV studies, ex vivo explants models and in vitro culture studies suggest that HIV-1 transmission can occur in either the upper or lower genital tract and that infection can be initiated by one to three transmitter/founder viruses in isolated foci within 48–72 h postexposure (3). Local mucosal replication in T cells or, possibly, some dendritic cell (DC) subsets, precede the viremic phase of the infection, characterized by systemic viral dissemination that is initiated in draining lymph nodes (3, 4).

Epithelial cells that line the gastrointestinal and genital mucosa are the first cells that HIV-1 encounters during sexual transmission. In addition to providing a physical barrier, epithelial cells are specialized to recognize and respond to incoming pathogens, resulting in initiation of the early and induced innate immune responses (5, 6). Most studies that have examined the early steps of HIV infection focus on the ability of the virus to cross or infect mucosal epithelial cells, rather than the innate responses of the epithelium. Results from our previous study demonstrated that HIV-1 gp120 may initiate early inflammation on the mucosal surface that could facilitate barrier disruption and generation of immune activation (7). We examined the early interactions between HIV-1 and the mucosal epithelium and showed that both intestinal and primary genital epithelial cells (GECs) recognize and respond to HIV-1 gp120 by producing proinflammatory cytokines, including TNF-α, that in turn impair the tight junction barrier between epithelial cells, thereby increasing mucosal permeability and allowing microbial translocation (5, 7).

The implications of this study were two-fold. First, it indicated that HIV-1 may subvert and take advantage of the host innate immune response to enhance its chances of crossing the mucosal barrier and initiate an infection in the underlying target cells. Secondly, it indicated a possible mechanism for the phenomenon of immune activation seen in HIV-1–infected individuals. Immune activation during HIV infection is thought to be mediated by

[*]McMaster Immunology Research Centre, Michael G. DeGroote Centre for Learning and Discovery, McMaster University, Hamilton, Ontario L8S 4K1, Canada; [†]Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario L8S 4K1, Canada; [‡]Department of Medicine and Immunology, University of Toronto, Toronto, Ontario M5S 1A1, Canada; [§]Department of Medical Biology, Laval University, Quebec City, Quebec G1V 0A6, Canada; and [¶]Canadian Immunodeficiency Collaborative, Toronto, Ontario M5B 1L6, Canada

[1]J.K.K. and V.H.F. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Charu Kaushic, Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, Michael DeGroote Centre for Learning and Discovery, Room 4014, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada. E-mail address: kaushic@mcmaster.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: AI, acutely infected; CS, chondroitin sulfate; DC, dendritic cell; FRT, female reproductive tract; β-gal, β-galactosidase; GEC, genital epithelial cell; HS, heparan sulfate; PDTC, pyrrolidine dithiocarbamate; poly (I:C), polyinosinic-polycytidylic acid; SP, seminal plasma; TER, transepithelial resistance; ZO-1, zona occludens-1.

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microbial translocation across the intestinal and genital mucosa and is concomitant with chronic systemic inflammation, as detected by measurable levels of serum LPS and soluble CD14 (8, 9). Presence of these factors in the blood has been directly correlated with disease progression (10, 11).

The exact mechanism by which HIV participates in the generation of immune activation is not known, but our previous studies implicate the viral glycoprotein gp120 as a primary mediator of this response. Cells infected with HIV-1 in vitro spontaneously shed the gp120 envelope protein in quantities greater than other viral proteins (12, 13), and detection of plasma gp120 levels have been associated with higher plasma concentrations of IL-6 and TNF-α in acutely infected (AI) individuals (14). Overall, studies suggest a paradoxical role, with some evidence for gp120-mediated suppression of T cell and DC function (15–18), whereas others show activation of $CD4^+$ T lymphocytes and myeloid cells, in the absence of direct infection (19, 20).

In the current study, we report for the first time, to our knowledge, the mechanism by which HIV-1 gp120 activates proinflammatory pathways in GECs. Our results show that gp120 signals through TLR2 and -4 in the presence of heparan sulfate (HS). We propose that gp120 in seminal plasma (SP) can initiate TLR-mediated activation of proinflammatory pathways that could lead to the disruption of the mucosal barrier and the initiation of immune activation by HIV-1.

Materials and Methods

Patient participation

Endometrial and endocervical tissues were obtained from women aged 30–59 y (mean age 42.9 ± 7.2 y) undergoing hysterectomies for benign gynecological reasons at Hamilton Health Sciences Hospital. The most common reasons for surgery were uterine fibroids and menorrhagia (heavy bleeding). Informed written consent was obtained in accordance with the approval of the Hamilton Health Sciences Research Ethics Board. HIV-infected, antiretroviral therapy–naive men were recruited through the Canadian Immunodeficiency Research Collaborative; all were men who have sex with men. Uninfected SP was obtained from either heterosexual male volunteers or uninfected men who have sex with men recruited through the Canadian Immunodeficiency Research Collaborative. Informed written consent was obtained in accordance with the approval of the Research Ethics Boards at the Mount Sinai Hospital, the University Health Network, and the University of Toronto.

Primary GEC cultures

Detailed protocols for isolation and culture of primary GECs have been described previously (21, 22). Briefly, endometrial and endocervical tissues were minced into small pieces and digested in an enzyme mixture at 37°C with shaking. GECs were isolated by a series of separations through nylon mesh filters (Small Parts, Miramar, FL), resuspended in DMEM/F12 primary growth medium (Invitrogen, Burlington, ON, Canada), and seeded onto Matrigel-coated (BD Biosciences, Mississauga, ON, Canada) tissue-culture inserts (BD Biosciences). GEC cultures were grown for 5–7 d until confluent monolayers were formed. A total of 300 μl fresh primary growth media was added to the apical and 500 μl to the basolateral compartment every 48 h. The confluency was monitored by transepithelial resistance (TER) measured by a volt ohm meter (EVOM; World Precision Instruments, Sarasota, FL). Confluent monolayers showing TER values >1000 Ω/cm were used for further experiments. The purity of GEC monolayers was between 95 and 98%, with no trace of any hematopoietic cells. TER was measured before exposing the cells to gp120 (pretreatment TER) and 24 h after and expressed as a percent of pretreatment TER.

Virus strains and GEC exposure

HIV-1 virus strain ADA (M-tropic) was prepared by infection of adherent monocytes from human PBMCs, followed by virus concentration using the Amicon Ultra-15 filtration system (Millipore, Billerica, MA). Viral stocks were titered for infectious viral units per milliliter using the TZM-bl indicator cell assay as previously described (23). To prepare UV-inactivated HIV-ADA, 10^5 infectious units/ml virus was subjected to 25–100 μJ/cm^2 UV light using a UV cross-linker (Fisher Scientific, Ottawa, ON, Canada). UV inactivation of virus was confirmed by titration on TZM-bl cells. Env-deleted HIV-1 (env^-), prepared on an NL4-3 backbone, was kindly provided by Dr. D. Johnson (National Cancer Institute). Recombinant HIV-1 gp120 protein (strain ADA) was initially obtained through the National Institutes of Health AIDS Research and Reference Reagent program and compared with commercial endotoxin-free recombinant gp120 expressed in a baculovirus expression system (Immunodiagnotics, Woburn, MA), with similar results. Although the primary GECs used in this study express TLR4, they are unable to respond to LPS due to the absence of MD2 and CD14 (24). Nevertheless, each batch of recombinant gp120 from both sources was checked for endotoxin contamination using a commercial endotoxin detection assay (Lonza, Allendale, NJ), and no trace of endotoxin was measured.

For viral exposure, primary GECs were grown to confluency on transwell inserts and exposed to HIV-1 ADA (10^5 infectious viral units in a volume of 100 μl, corresponding to a multiplicity of infection of 1; p24 concentration of 280 ng/ml), UV-inactivated HIV-1 ADA (100 μl/well, equivalent dose of 10^5 infectious viral units), HIV env^- (at a p24 concentration of 79 ng/ml), or recombinant gp120 (0.1 μg/ml) for various time points. Mock infection controls included exposure to the same volume of media without HIV-1.

To explore the role of cell-surface receptors on various aspects of HIV/GEC interactions, epithelial monolayers were treated with neutralizing Abs against TLR2 (eBioscience; clone TL2.1), TLR4 (eBioscience; clone HTA125), TLR5 (Invivogen; polyclonal rat anti-human) (all at 10 μg/ml), CD4 (DakoCytomation; clone MT310, 20 μg/ml) (25), CCR5 (BD Biosciences; clone 2D7/CCR5, 20 μg/ml) (26), CXCR4 (BD Biosciences; clone 12G5, 20 μg/ml) (27), or their respective isotype controls at similar concentrations during the course of viral exposure. In some experiments, degrading enzymes were added to monolayers 1 h prior to viral exposure to remove cell-surface receptors. These included the heparinase III from *Flavobacterium heparinum* (Sigma-Aldrich, Oakville, ON, Canada; 6 mIU/ml), chondroitinase ABC from *Proteus vulgaris* (Sigma-Aldrich; 10 U/ml), and phospholipase C from *Clostridium perfringens* (Sigma-Aldrich; 10 U/ml) for digesting HS, chondroitin sulfate (CS), and phospholipid, respectively. Exogenous HS (Sigma-Aldrich, 40 μg/ml) was used in some experiments to replace cell-surface receptors. The concentrations of these Abs or inhibitors were based on previous optimization experiments.

To block the effect of gp120, a specific neutralizing Ab (Polymun Diagnostic, Klosterneuburh, Austria; clone 2G12, 25 μg/ml) was added to primary GEC cultures for the duration of viral exposure. As positive controls for TLR2, TLR4, and TLR5 signaling pathways, primary GECs were exposed to Pam3CSK4 (InvivoGen, San Diego, CA), FimH (24), and flagellin from *Salmonella typhimurium* (Alpha Diagnostic, San Antonio, TX), respectively, at a concentration of 10 μg/ml.

Immunofluorescent staining

GECs were exposed to HIV-1, gp120, or polyinosinic-polycytidylic acid [poly (I:C)] (Sigma Aldrich; 25 μg/ml; positive control) (28) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Primary rabbit anti–NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human zona occludens-1 (ZO-1; Life Technologies, Burlington, ON, Canada) Ab was incubated for 1 h at 37°C followed by secondary Alexa Fluor 488–conjugated (Life Technologies) Ab for 1 h. Cells were counterstained with propidium iodide (nuclear stain; Life Technologies). Membranes were excised from inserts and mounted on slides prior to being imaged on an inverted confocal laser-scanning LSM 510 microscope (Carl Zeiss Canada, Toronto, ON, Canada) using standard operating conditions. For each experiment, confocal settings for image acquisition and processing were identical between control and treated monolayers, and three separate, random images were acquired and analyzed for each experimental condition. Images are presented as en face or as a composite Z-stack reconstruction, which shows the monolayer in transverse profile. Images were analyzed using Image J software (National Institutes of Health) for measuring levels of nuclear colocalization of NF-κB.

Cytokine analysis

GEC apical and basolateral supernatants were analyzed for cytokines and chemokines at several time points post–HIV-1 ADA, UV-inactivated HIV-1, HIV env^-, or recombinant gp120 exposure, using Luminex multianalyte technology (Luminex, Austin, TX), as previously described (7). Multiplex bead-based sandwich immunoassay kits (Millipore) were used to measure levels of IL-1β, IL-6, IL-8, MCP-1, and TNF-α. In some experiments, TNF-α and IL-8 in cell-culture supernatants were measured by ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Baseline cytokine levels were found to vary between different tissue samples, but in all tissues, significant induction of cytokines was observed reproducibly after treatment with HIV-1 or recombinant gp120. To confirm

that proinflammatory cytokine induction in primary GECs was primarily mediated by NF-κB, cells were pretreated with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich; 10 μM) for 1 h prior to exposure with either gp120 or HIV-1. Cell-culture supernatants were subsequently collected and measured by bead-based immunoassay.

Blue dextran leakage assay

Blue dextran dye (Sigma Aldrich) was dissolved in primary medium to a working concentration of 2.3 mg/ml (7) and added to the apical surface of confluent epithelial cell monolayers grown to confluency on transwell inserts. At 24 h post–gp120 exposure, 100 μl basolateral supernatant was collected, loaded into 96-well plates, and measured in a Tecan Safire microplate reader (Tecan, Männedorf, Switzerland) at 610 nm. Blue dextran leakage into the basolateral compartment was expressed as a percent of the dye added to the apical surface.

TLR, gp120, and HS bead-binding assay

Polystyrene microspheres (4.5 μm; Polysciences, Warrington, PA) were washed in borate buffer (0.1 M) according to the manufacturer's instructions (Tech note 238E; Polysciences). Washed microbeads (0.25 mg/ml) were coated at room temperature with 1 μg/ml recombinant gp120, human TLR2, human TLR4, 50 μg/ml HS, or 10 mg/ml BSA in borate buffer (pH 8.5) overnight with vigorous shaking. Following a buffer wash, two 30-min incubations were performed with 10 mg/ml BSA in borate buffer and 10 mg/ml BSA in PBS to block nonspecific binding on all beads. The coated beads were resuspended in FACS buffer (2 mg/ml BSA in PBS) at 0.5×10^6 beads/ml and stored at 4°C. For binding assays, coated beads were incubated with gp120 (0.5 μg/ml or 1 μg/ml) or rTLR4 (0.5 μg/ml) for 1 h at room temperature in FACS buffer, washed, and then incubated for 30 min with biotinylated anti-gp120 or anti-TLR2 or -4 Ab followed by a 20 min staining with streptavidin PE-Cy7. Beads were subsequently analyzed using a BD LSRII Flow cytometer (BD Biosciences). Single beads were selected for analysis on the scatter plots, and mean fluorescence intensity of gp120 or TLR4 Ab staining on coated beads was compared with BSA controls to analyze gp120 binding to TLR2, TLR4, or HS.

Plasmids and HEK293 transfection assays

Expression vectors for human TLR2, TLR4, CD14, MD2, β-galactosidase (β-gal), and NF-κB–luc inducible reporter plasmid were kindly provided by Dr. Cynthia Leifer (Cornell University). All plasmids were amplified and purified using Endo-free Midi Prep columns (Qiagen, Toronto, ON, Canada). Transfections for luciferase assays were carried out using the HEK293 cell line. Subconfluent HEK293 cells were transfected with 100 ng pSV β-gal (internal transfection control), 100 ng pNF-κB–luc reporter plasmid (firefly luciferase, experimental reporter), 30 ng pUNO-hTLR2 or pUNO-hTLR4 with or without 30 ng CD14, and pUNO hMD2 expression plasmids and pBABE (empty vector) for a total of 1 μg DNA/well. Transfections were completed using Gene Juice transfection reagent (EMD Millipore). At 24 h posttransfection, cells were treated with Pam3CSK4 (TLR2 ligand; 100 μg/ml), FimH (TLR4 ligand; 10 μg/ml), LPS (TLR4 ligand; 100 ng/ml), or gp120 (0.1, 1.0, and 10 μg/ml). Alternatively, transiently transfected HEK 293 cells were treated with or without heparinase III (6 mIU/ml) and/or exogenous HS (40 μg/ml) for 1 h prior to gp120 stimulation (0.1 μg/ml). At 48 h posttreatment, the cells were harvested, lysed, and luciferase activity was measured using a Stratagene luciferase assay kit (Agilent Technologies, Mississauga, ON, Canada) as per the manufacturer's instructions. Activity of pSV–β-gal luciferase was also measured by reporter assay (Luminescent β-galactosidase detection kit II; Clontech Laboratories, Mountain View, CA) according to the manufacturer's instructions. The fold increase in NF-κB luciferase activity was normalized with β-gal expression as an internal control of transfection.

SP collection and preparation

Semen from HIV-uninfected and HIV-infected men was collected, and SP was isolated as previously described (29). All study participants had to abstain from sexual intercourse or masturbation for 48 h prior to sample donation. Semen was collected directly by masturbation into 10 ml sterile RPMI 1640 (Invitrogen) containing 100 U/ml penicillin and 100 mg/ml streptomycin. SP was cryopreserved at −80°C after centrifugation to pellet semen lymphocytes and spermatozoa. SP HIV-1 RNA was measured by Versant HIV-1 RNA 3.0 assay (Bayer Diagnostics, Berkeley, CA; lower limit of detection of RNA was 50 copies/ml) at Mount Sinai Department of Microbiology-Public Health Laboratory. SP was diluted 1:6 in RPMI 1640 prior to exposure to GECs. Pooled SP samples used in the study were from three AI subjects with viral loads of 14,154, 19,908, and 29,160 RNA copies/ml.

SP and primary GEC exposure

Confluent monolayers of primary GECs were exposed for 24 h to uninfected SP spiked with 0.1, 0.2, or 0.3 μg/ml recombinant gp120 (Immunodiagnostics, Woburn, MA) or uninfected/infected SP alone. Recombinant gp120 (0.1 μg/ml) was used as a positive control for comparison. TNF-α production was measured in apical supernatants using TNF-α ELISA as per the manufacturer's instructions (R&D Systems), and cells were fixed for immunofluorescent staining of ZO-1 tight junctions. Primary GECs were also exposed for 24 h to pooled, diluted SP samples from three AI subjects (acute SP), acute SP combined with recombinant gp120 (0.1 μg/ml), acute SP combined with recombinant gp120 (0.1 μg/ml), and anti-gp120 Ab (35 μg/ml; Polymun Scientific) or acute SP with recombinant gp120 (0.1 μg/ml) and isotype control Ab (35 μg/ml; Southern Biotechnology Associates, Birmingham, AL). Mock infection or recombinant gp120 (0.1 μg/ml) were used as controls.

Statistical analysis

GraphPad Prism Version 4 (GraphPad Software, San Diego, CA) was used to compare three or more means by one-way or two-way ANOVA, depending on the experimental conditions. When an overall statistically significant difference was measured ($p < 0.05$), a Bonferroni posttest was performed to adjust the p value for multiple comparisons. The p values and the respective comparison for which they were calculated are indicated in the figure legends.

Results

Kinetics and specificity of HIV-1 gp120 for induction of proinflammatory cytokines by GECs

Previously, we demonstrated that HIV-1 could directly disrupt epithelial barrier function in cultured intestinal or primary endometrial GECs following exposure to HIV-1 or gp120 (7), but we did not measure whether similar events occurred in the endocervix. To confirm that the phenomenon of HIV-1 and gp120-mediated barrier disruption was also applicable to primary endocervical GECs, we exposed endocervical monolayers to gp120 or UV-inactivated HIV-1 ADA and measured a significant decrease in TER (results not shown), upregulation of TNF-α (Fig. 1A), and disruption of epithelial barrier integrity (Fig. 1B), relative to unexposed controls. This barrier impairment and upregulation of TNF-α was analogous to what was previously measured in endometrial GECs following exposure to HIV-1 (7). For further experiments examining the mechanism of barrier function, endometrial tissues were used because they were more abundantly available; however, endocervical epithelial cell cultures were used to confirm key findings.

In our previous study examining the barrier impairing function of HIV-1, we did not directly measure whether GEC proinflammatory cytokine induction differed with respect to whether the cells were exposed to HIV-1 or gp120. Thus, confluent, polarized monolayers of GECs were exposed to HIV-1 or recombinant gp120 protein at previously standardized doses (7), and apical and basolateral supernatants were analyzed for the proinflammatory cytokines TNF-α, IL-6, and IL-1α and chemokines IL-8 and MCP-1. Significantly enhanced production was observed for all cytokines and chemokines; in particular, TNF-α and IL-8 were upregulated in response to both HIV-1 ($p < 0.05$) and gp120 ($p < 0.01$), as early as 1 h postexposure (Fig. 2A), compared with mock-infected controls.

To determine whether HIV-1 gp120 alone was sufficient for facilitating this effect, we compared GEC responses to HIV-1, gp120, UV-inactivated HIV-1 and an env-deleted mutant, which lacks the HIV-1 viral envelope precursor gp160 (30). HIV-1 and UV-inactivated HIV-1 enhanced the levels of cytokine production by GECs, comparable with levels induced by recombinant gp120 protein exposure. In contrast, the env-deleted HIV-1 mutant failed to induce proinflammatory cytokines in GECs; cytokine levels in this treatment group were comparable to mock exposure (Fig. 2B).

To confirm the specificity of gp120 in GEC proinflammatory cytokine induction, viral preparations as well as recombinant

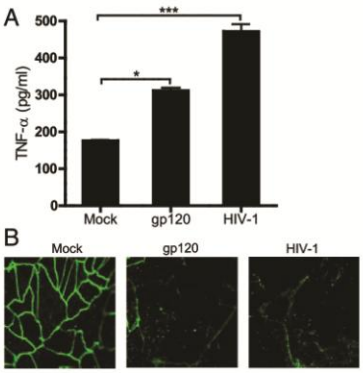

FIGURE 1. (**A**) Primary endocervical GECs were exposed to gp120, UV-inactivated HIV-1, or mock infection for 24 h. TNF-α production was measured in apical cell supernatants by ELISA. Bars represent mean ± SEM of five to six replicate inserts for each experimental condition. Data are representative of two separate experiments conducted on GECs from different tissues, with similar results. Data was analyzed by one-way ANOVA. (**B**) GEC monolayers were fixed and stained post–viral exposure for ZO-1 tight junction protein (green), as explained in *Materials and Methods*. Images were captured by a laser-scanning confocal microscope. Original magnification ×1260. $*p < 0.05$, $***p < 0.001$.

gp120 were incubated with anti-gp120–neutralizing Ab prior to exposure to GECs. gp120 neutralization reduced proinflammatory cytokine induction to baseline levels (Fig. 2C). Altogether, these results indicate that HIV-1 gp120 can directly induce a proinflammatory cytokine response in GECs.

Induction of proinflammatory cytokines by HIV-1 gp120 is mediated through NF-κB activation

Because proinflammatory cytokines can be induced in GECs by different intracellular pathways, we examined the involvement of the transcription factor NF-κB, which is strongly associated with the induction of downstream proinflammatory responses (31), following HIV-1 or gp120 exposure. GEC monolayers were exposed to HIV-1 and gp120, and the p65 subunit of NF-κB was detected. Nuclear translocation of NF-κB was observed following 2 h of exposure to HIV-1 or gp120 (Fig. 3A). Peak translocation of NF-κB was observed between 30 min to 2 h postexposure with HIV-1 or gp120 (Fig. 3B). Treatment with PDTC, an NF-κB–specific inhibitor, significantly inhibited TNF-α and IL-8 upregulation (Fig. 3C, 3D), indicating that NF-κB was necessary for the induction of proinflammatory cytokines by gp120 and HIV-1. Because the induction of cytokines and intracellular signaling pathways were identical for HIV-1 and gp120, we focused on the mechanism of gp120 for the rest of the study.

gp120-mediated cytokine induction and barrier disruption occurs via TLR2 and TLR4 signaling pathways

We next sought to determine whether TLRs played a role in gp120-mediated cytokine induction and epithelial barrier disruption, as NF-κB induction has been associated with multiple TLR signaling pathways. We examined three distinct cell-surface TLRs—TLR2, TLR4, and TLR5—because these three TLRs are primarily responsible for recognizing glycoproteins at the surface of cells and inducing intracellular signaling (32). TLR1 and -6 are also associated with cell-surface protein recognition, but act in association with TLR2 and were therefore not included in our assessment. We first measured whether TLR2, -4, or -5 were associated with gp120-mediated induction of proinflammatory cytokines. GECs were treated with neutralizing Abs against TLR2, TLR4, and TLR5 and exposed to gp120. In the presence of either TLR2 or TLR4 Ab, induction of TNF-α and IL-8 by gp120 was partially, but significantly, blocked (Fig. 4A, 4B). However, treatment with a combination of TLR2- and TLR4-neutralizing Abs reduced cytokine production to baseline. In contrast, treatment of GECs with isotype control or TLR5 neutralizing Ab did not have any effect on gp120-mediated cytokine production. The neutralizing capacity of the TLR5 Ab was confirmed by exposing primary GEC monolayers to TLR5 ligand flagellin in the presence or absence of the neutralizing Ab or isotype control (Supplemental Fig. 1). Pam3CSK4, a synthetic triacylated lipopeptide, known to act through TLR2 and FimH (fimbriae protein), a TLR4 ligand that is known to induce this pathway in GECs, were used as controls to determine the specificity of the TLR2 and -4 Abs (24, 28).

To confirm that cytokine production induced by gp120 activation of TLR2 and TLR4 was associated with barrier disruption, we directly examined barrier disruption in the presence of the TLR-neutralizing Abs (Fig. 4C–E). Monolayers treated with gp120 following preincubation with Abs against TLR2, TLR4, or both receptors did not show a significant decrease in TER (Fig. 4C), suggesting that disruption of barrier integrity involves TLR2 and -4 signaling pathways. In contrast, TLR5 or isotype control Ab did not prevent a gp120-mediated drop in TER. Immunofluorescence staining of ZO-1, an epithelial cell tight junction barrier protein that is disrupted by HIV-1 gp120 (7), was in concurrence with the TER results (Fig. 4E). Measurement of dextran blue dye leakage across the epithelial monolayer of gp120 exposed GECs, in the presence or absence of TLR Abs, also confirmed that epithelial cell permeability was completely abrogated in the presence of both TLR2- and TLR4-neutralizing Abs (Fig. 4D). In contrast, TLR5 Ab did not block dye leakage in the presence of gp120, confirming that gp120 activated proinflammatory cytokines via TLR2 and TLR4, but not TLR5.

NF-κB translocation mediated by gp120 is blocked in the presence of TLR2 and TLR4 Abs

We next sought to determine if neutralizing Abs to TLR2 and TLR4 abrogated gp120-mediated activation and nuclear translocation of NF-κB. Incubation of GEC monolayers with Abs prior to treatment with gp120 blocked NF-κB translocation in the presence of TLR2 or TLR4 Ab, but not TLR5 or an isotype control (Fig. 5). This confirmed that gp120-mediated NF-κB activation was mediated via TLR2 and TLR4 signaling pathways.

HS is required for gp120-mediated proinflammatory cytokine induction and barrier disruption

Next, we determined whether signaling through any of the known canonical HIV-1 receptors contributed to the disruption of the GEC barrier or mediated induction of proinflammatory cytokines, in addition to TLR2 and TLR4. Pretreatment of GEC monolayers with neutralizing Abs against CD4, CXCR4, or CCR5 did not have any effect on gp120-mediated decrease in TER, disruption of ZO-1 staining, or induction of proinflammatory cytokines (Fig. 6A–C), suggesting that canonical HIV-1 receptors were not involved in these GEC signaling pathways.

Cellular proteoglycans, such as syndecans, have also been previously shown to be important for HIV-1 gp120 attachment, including on epithelial cells (33–36). We therefore examined if HS or CS moieties played a role in gp120-mediated signaling in GECs. Heparinase III, chondroitinase ABC, and phospholipase C were used to remove HS, CS, or phospholipids (as control), re-

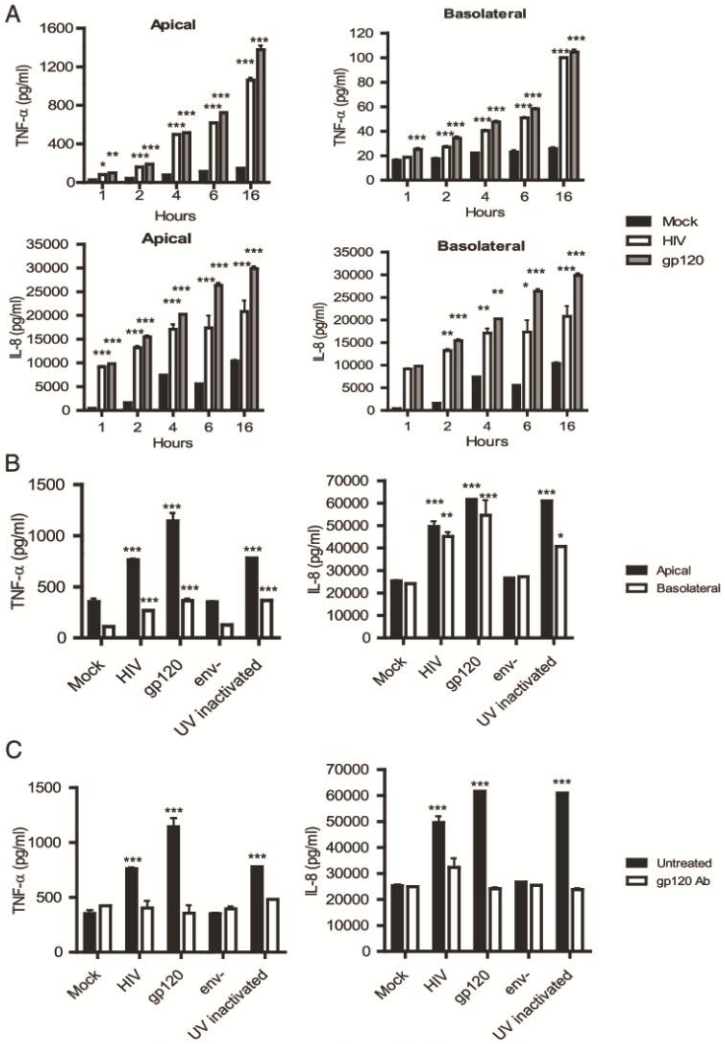

FIGURE 2. Specificity of HIV-1 gp120 for induction of proinflammatory cytokines and chemokines from primary GECs. (**A**) Primary endometrial GECs were exposed to HIV-1 or gp120, and the time kinetics of apical and basolateral TNF-α and IL-8 production were measured from 1–16 h postexposure. Cytokines were analyzed by multianalyte bead assay. (**B**) Confluent primary GECs were exposed to mock infection (media), HIV-1, gp120, env-deleted HIV-1 (env-), or UV-inactivated HIV-1 for 24 h, and supernatants were analyzed for TNF-α or IL-8. (**C**) To determine whether these were gp120-specific responses, confluent primary GECs were exposed for 24 h to mock infection, HIV-1, gp120, env-deleted HIV-1 (env-), or UV-inactivated HIV-1 with or without anti-gp120 neutralizing Ab. TNF-α and IL-8 production in apical supernatants is shown. Significance shown in each graph is relative to the respective mock-infected group for each experiment. Data shown are representative of three to five separate experiments from individual tissues with similar results and represents mean ± SEM of triplicate cultures of the representative tissue. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

spectively, from the cell surface prior to gp120 treatment. Removal of HS resulted in complete blockade of gp120-mediated TER decrease, impairment of ZO-1 barrier protein, and induction of TNF-α and IL-8 (Fig. 6A–C). Removal of CS resulted in partial blocking of gp120-mediated TNF-α induction, which resulted in partial ZO-1 delocalization, but no significant decrease on TER. Treatment with phospholipase C in the presence or absence of gp120 decreased TER and affected the viability of the epithelial monolayers (data not shown).

Further studies showed that addition of exogenous HS in the presence or absence of cellular HS enhanced the ability of gp120 to induce cytokine production in primary GECs (Supplemental Fig. 2). These results indicate that HS is an essential cofactor in gp120-mediated signaling through TLR2 and TLR4.

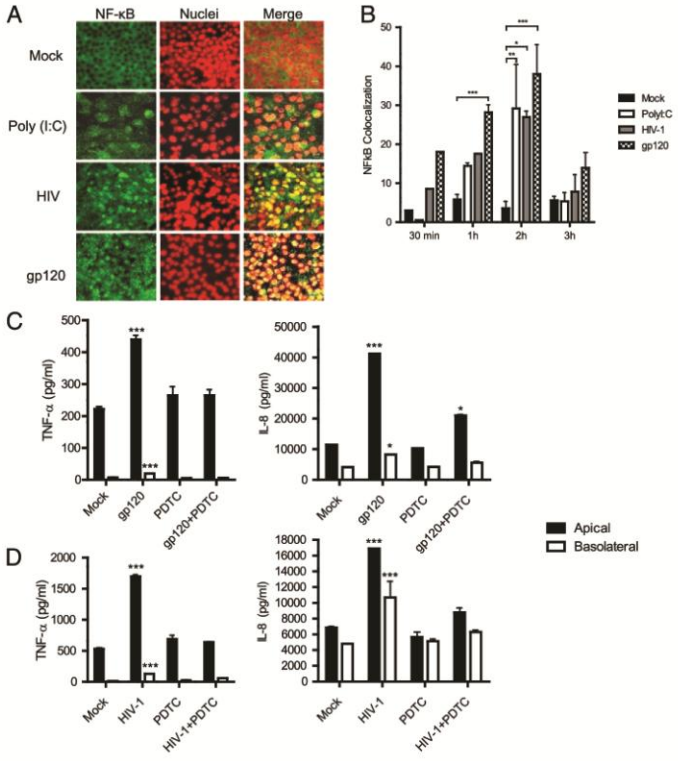

FIGURE 3. Induction of proinflammatory cytokines by HIV-1 gp120 is mediated through NF-κB. (**A**) Primary GECs were exposed to HIV-1, gp120, or poly (I:C) (as a positive control) for 2 h. Cells were fixed and stained for the NF-κB p65 subunit (green). Nuclear counterstaining, as seen in red, was achieved using propidium iodide. Images are representative of one of three separate experiments. Original magnification ×1260. (**B**) Confocal images were taken from 30 min to 3 h post–HIV-1, gp120, or poly (I:C) exposure and NF-κB translocation and nuclear colocalization were measured by Image J software. To confirm that proinflammatory cytokine induction in primary GECs was primarily mediated by NF-κB, cells were incubated with the NF-κB inhibitor PDTC prior to exposure with gp120 (**C**) or HIV-1 (**D**). Supernatants were collected after 24 h and assayed by multianalyte cytokine bead assay. Results are shown for TNF-α and IL-8. Significance shown in each graph is relative to the respective mock-infected group for each experiment. Data shown represent mean ± SEM of a representative tissue from four separate experiments done on cells isolated from four different tissues with similar results $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Soluble gp120 and HS can bind TLR2 or TLR4

Although gp120 is known to have four HS binding sites (37, 38), it is not known whether gp120 or HS can bind to TLR2 or TLR4, as suggested by our results. To further assess this, we developed bead-based binding assays. To standardize the assay, polystyrene beads were coated with soluble TLR4 or gp120, and efficient protein coating was verified by flow cytometry (Fig. 7A). Next, binding assays were conducted by coating beads with soluble TLR2, TLR4, or HS and subsequently incubating them with soluble gp120. Using a gp120 detection Ab, we observed that gp120 was specifically bound to these receptors (Fig. 7B). The binding specificity of gp120 to TLR4 was also supported by a direct enhancement in mean fluorescence intensity, as increasing gp120 concentrations were used (Fig. 7C). Furthermore, we also saw that TLR4 was specifically bound to HS (Fig. 7D). BSA-coated beads were used as controls to demonstrate the specificity of gp120 and TLR4 binding. Overall, these experiments indicate that HIV-1 gp120 can bind to TLR2, TLR4, or HS on host epithelial cells.

gp120 and HS are required and sufficient for TLR-mediated signaling

To confirm that HS and gp120 were necessary to induce cytokine signaling in epithelial cells via TLR2 and TLR4 pathways, we used an artificial signaling expression system in the kidney embryonic cell line HEK293, which has been extensively used for testing TLR function (38, 39). HEK 293 cells were transfected with an NF-κB–luciferase reporter plasmid and a TLR2 or TLR4 expression plasmid and stimulated with increasing doses of gp120 or known TLR ligands (positive control). gp120 induced significant NF-κB activation, in a dose-dependent manner, compared with mock treatment (Fig. 8A). In TLR2-transfected cells, NF-κB activation was comparable to that seen in response to Pam3CSK4 stimulation at the highest dose of gp120. HEK293 cells transfected with NF-κB–luciferase and stimulated with gp120 or Pam3CSK4 in the absence of TLR2 did not show any NF-κB activation (Supplemental Fig. 3). Transfection of HEK293 with CD14 in addition to TLR2 further enhanced NF-κB activation. A similar dose-dependent enhancement of NF-κB activation was seen when HEK293 cells were transfected with TLR4 and NF-κB reporter plasmids (Fig. 8A, *right panel*). Similar to FimH (23), we found that gp120 could also induce NF-κB activation through TLR4, even in the absence of CD14 and MD2.

To determine if HS was playing an essential role in gp120-mediated NF-κB activation via TLRs, transfected HEK293 cells were pretreated with heparinase III prior to stimulation with gp120, Pam3CSK4, or FimH (Fig. 8B). Removal of HS resulted in gp120 not being able to stimulate NF-κB activation, but did not affect NF-κB stimulation by either Pam3CSK4 or FimH, indicating HS is essential for gp120-mediated signaling. Addition of exogenous HS in the absence of gp120 did not result in NF-κB activation, indicating that induction required gp120 in the context of HS. These results confirm that HS and TLR2/TLR4 are essential for NF-κB transduction and proinflammatory cytokine induction in transfected HEK 293 cells produced in response to HIV-1 gp120.

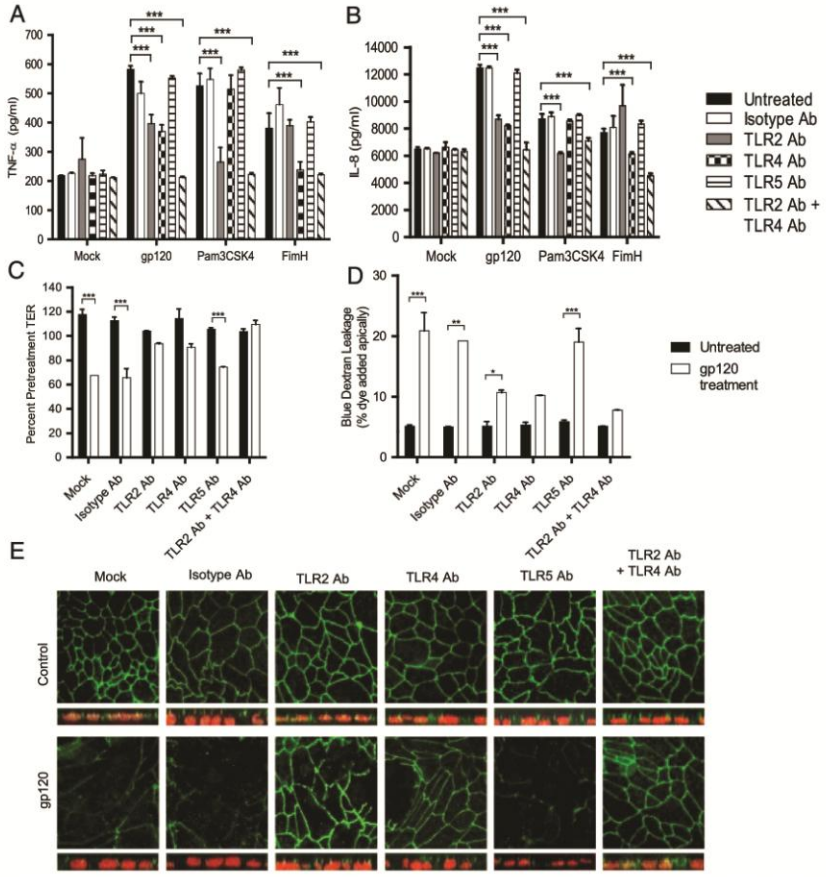

FIGURE 4. Neutralization of TLR2 and TLR4 pathways prevents the induction of proinflammatory cytokines and epithelial barrier breakdown by gp120. GEC monolayers were mock treated (media) or treated with gp120, Pam3CSK4, or FimH in the presence or absence of neutralizing Abs against TLR2, TLR4, TLR5, or isotype control Abs. Supernatants were collected and analyzed for TNF-α (**A**) and IL-8 (**B**) at 24 h postexposure, or GEC TER (**C**) was measured at pretreatment (0 h) and 24 h postexposure and expressed as a percent of pretreatment TER. To further measure barrier function, blue dextran dye leakage was measured across epithelial monolayers (**D**). Blue dextran in tandem with gp120 was added to primary GECs in the presence or absence of TLR neutralizing or isotype control Abs. At 24 h postexposure, basolateral supernatants were collected and absorbance was measured and calculated as a percent of apical blue dextran absorbance at 0 h. Significance shown in each graph above is relative to the respective mock-infected group for each experiment. Data shown represent mean ± SEM of the triplicate cultures from the representative tissue from three separate experiments. (**E**) Monolayers were fixed at 24 h post–gp120 treatment in the presence or absence of neutralizing Abs or isotype controls, and immunofluorescent staining for ZO-1 was performed. The corresponding Z-stack series are featured below each panel and show ZO-1 (green) and nuclei staining (red). Images were captured by a laser-scanning confocal microscope. Images are representative of one of three separate experiments with similar results. Original magnification ×1260. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

GECs induced TNF-α in response to gp120 present in semen samples

To determine the relevance of TLR activation in primary GECs following gp120 exposure to heterosexual transmission, we examined the effect of gp120 added to either SP from HIV-1–uninfected or infected individuals on primary female GECs. Confluent monolayers of primary GECs were exposed to 1:6 diluted uninfected SP or uninfected SP containing known amounts of recombinant gp120 protein. The dilution of SP was based on our previous studies, so that SP did not compromise the viability of GECs (28). Recombinant gp120 protein was used for comparison. Results indicated that GECs responded to gp120 present in SP in a dose-dependent manner, inducing significant levels of TNF-α, similar or slightly higher than the levels seen with gp120 alone (Fig. 9A). Furthermore, when GECs were exposed to HIV-1–infected SP, TNF-α was upregulated by two-fold compared to uninfected SP exposure (Fig. 9A). Because infected SP contains other proinflammatory cytokines that in turn induce inflammatory responses from GECs (28), we next determined whether addition of gp120 to AI SP, which contains the maximum amount of inflammatory cytokines, would have an additive effect on the induction of TNF-α from primary GECs. Confluent primary GECs monolayers were exposed

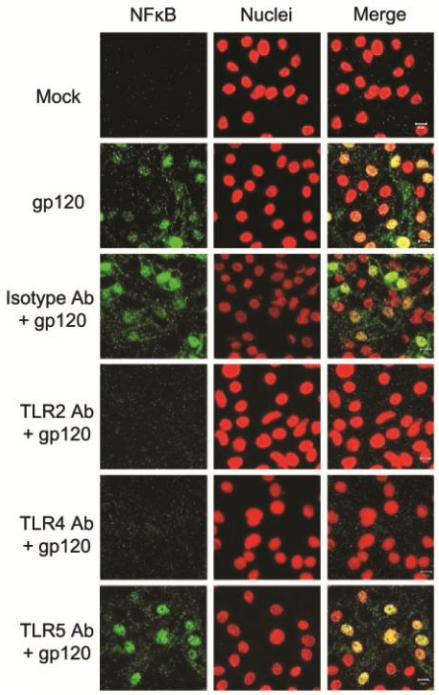

FIGURE 5. Neutralization of TLR2 and TLR4 on cell-surface blocks gp120-mediated NF-κB activation and nuclear translocation. Primary GECs were exposed to gp120 or mock treatment (media) for 1 h in the presence or absence of neutralizing Abs against TLR2, TLR4, TLR5, or isotype controls. Cells were fixed, and immunofluorescent staining for the NF-κB p65 subunit was performed. Nuclear counterstaining, as seen in red, was achieved using propidium iodide. Images were captures by a laser-scanning confocal microscope. Images are representative of one of three separate experiments with similar results. Original magnification ×1260.

to pooled SP from AI men in the presence or absence of exogenous recombinant gp120 with or without a gp120-neutralizing Ab. Mock infection, recombinant gp120 alone, or pooled SP from AI men containing exogenous recombinant gp120 and SP containing isotype control Ab were used for comparison. Although both pooled SP from AI men and gp120 alone increased TNF-α production from GECs, exposure to a combination of pooled SP with recombinant gp120 produced an additive effect on induction of TNF-α from GECs (Fig. 9B). Incubation with anti-gp120 Ab in addition to infected SP and recombinant gp120 reduced TNF-α production to comparable levels observed with pooled SP alone (Fig. 9B). The direct effect on the epithelial barrier was seen by a significant disruption of tight junctions by exposure to both uninfected SP spiked with recombinant gp120 and infected SP (Fig. 9C). These results indicate that gp120 can induce inflammatory responses in the presence of SP. Further, other proinflammatory factors present in infected SP can add to the effect of gp120 on barrier disruption.

Discussion

In the current study, we provide evidence for a novel intracellular mechanism by which HIV-1 surface gp120 can be recognized by innate pattern-recognition receptors, specifically TLR2 and TLR4, present on the female upper genital tract (endometrial and endocervical) epithelium. HS, a noncanonical attachment receptor for HIV-1, was found to be indispensable for gp120-mediated induction of TLR signaling in GECs. This interaction among HIV gp120, TLR2/4, and HS resulted in the activation of intracellular NF-κB pathway, leading to downstream upregulation of proinflammatory cytokines and chemokines, including TNF-α. Upregulation of these inflammatory factors was associated with tight junction disruption and loss of barrier function. Although binding interaction between gp120 and HS has been well described in previous studies, this is the first report, to our knowledge, that shows that gp120 can also bind directly, in a dose-dependent manner, to TLR2 and -4. Based on this, we propose that a trimolecular complex composed of gp120, HS, and TLR2 or TLR4 is required to activate the innate proinflammatory cytokine cascade in GECs in the context of an HIV infection.

Although this study is the first one, to our knowledge, to describe this mechanism in detail, induction of cytokines in epithelial cells by HIV-1 has been reported by other studies. Li and colleagues (41) showed that in response to R5 tropic HIV-1, human vaginal epithelial cell cultures produced significant amounts of chemokines MIP-3α and IL-8, whereas Fanibunda et al. (42) showed that genital epithelial cell gene signatures were changed in response to gp120, resulting in upregulated gene expression of TNF-α and the chemokines CXCL1 and CXCL8. Neither of these studies examined the mechanism by which induction could take place or whether the inflammatory factors had any effect on epithelial barrier functions.

The interaction between HIV-1 and the innate immune system is not well understood and has only recently started to garner attention (43). gp120 itself has been linked to immune activation of brain endothelial cells leading to the disruption of the blood–brain barrier and activation of LFA-1 on $CD4^+$ T cells, enhancing their susceptibility to HIV-1 (44, 45). It is interesting to speculate whether innate recognition and activation of TLR pathways in response to gp120 could occur in DCs, which are also equipped for innate sensing, like epithelial cells. So far, to the best of our knowledge, HIV-1 has not been shown to activate DCs, although a previous study suggested that recognition of HIV-1 viral ssRNA by TLR7/8 on plasmacytoid DCs and monocytes may partially contribute to immune activation seen in HIV-infected individuals (43). Other studies have shown that binding of gp120 by DC-specific ICAM-3–grabbing nonintegrin can lead to suppression of IFN-α and apoptosis signal–regulating kinase 1–dependent cell death (46, 47). A recent report by Manel and colleagues (48) found that monocyte-derived DCs were unable to respond to HIV-1 in the absence of a productive infection, but when replication could be artificially induced, type I IFN responses were produced. This response was dependent on the interaction between newly synthesized HIV-1 capsid proteins with cellular cyclophilin A and the subsequent activation of the transcription factor IRF3. Although we did not investigate type I IFN production in this study, the proinflammatory responses seen in this study were not dependent on viral replication, because UV-inactivated virus and recombinant gp120 alone could induce these responses. One reason for such distinct responses among DCs and GECs could be differences in glycosaminoglycan chain expression on the cell surface of these cells, which could account for differences in recognition of HIV-1 and subsequent induction of innate responses. For example, CS is the major glycosaminoglycan chain at the surface of mononuclear lineages (49), including human monocyte-derived DCs, whereas HS moieties outnumber CS moieties (50) in the human female genital tract. Based on our results, HS is critical for the recognition of HIV-1 gp120 by GEC and activation of TLR pathway, whereas CS does not appear to act in a similar manner. Altogether, these results

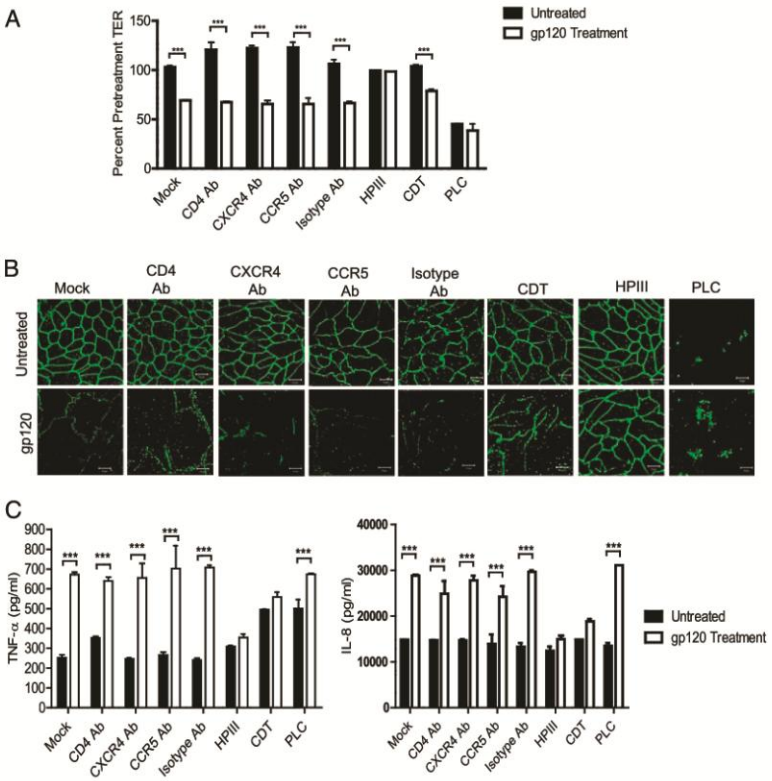

FIGURE 6. The presence of HS, but not of HIV canonical receptors, is necessary for gp120 to disrupt the epithelial barrier. Confluent GEC monolayers were pretreated with neutralizing Abs against CD4, CCR5, or CXCR4 during the entire course of gp120 exposure or were treated with chemical inhibitors including heparinase III (HPIII), chondroitinase ABC (CDT), phospholipase C (PLC), or exogenous HS for 1 h prior to exposure with gp120. At 24 h post–gp120 exposure, GEC TER was measured at pretreatment (0 h) and 24 h postexposure and expressed as a percent of pretreatment TER (**A**), or monolayers were fixed, and immunofluorescent staining for ZO-1 was performed (**B**). Images were captured by a laser-scanning confocal microscope. Images are representative of one of three separate experiments. Original magnification ×1260. Alternatively, apical and basolateral supernatants were collected at 24 h post–gp120 exposure and measured for TNF-α and IL-8 (**C**) by multianalyte cytokine bead assay. Results were compared with cells exposed to mock treatment. Significance shown in each graph above is relative to the respective mock-infected group for each experiment. Data shown represent mean ± SEM of one of three separate experiments with similar results. $***p < 0.001$.

suggest that each cell type expresses a distinct repertoire of cell-surface molecules and therefore likely engages in unique interactions with HIV-1, resulting in activation of differential signaling pathways and inflammatory responses.

The relevance of gp120-mediated TLR activation in heterosexual transmission was examined in experiments in which gp120 was found to exert the same effect in the context of semen. Despite the fact that the majority of HIV transmission takes place in the context of semen, the role of semen in HIV infection is not clearly understood. There is conflicting information whether semen inhibits or facilitates HIV infection (51–53). Our recent work showed that SP from both HIV-infected and uninfected men induced inflammatory responses in the epithelial cells of the FRT. However, higher levels of TGF-β1 in SP correlated with decreased proinflammatory cytokine production by GECs (30). Because semen can induce both inflammatory and immunoregulatory responses, we tested the effect of gp120 added to uninfected SP as well as SP from infected individuals. The results show that both SP spiked with gp120 and SP from HIV-infected individuals induced significant TNF-α induction from GECs and led to disruption of tight junctions (Fig. 9). Because SP from HIV-1–infected men contains proinflammatory cytokines that could in turn induce TNF-α from GECs, we examined the effect of combination of gp120 and infected SP and found an additive effect on the induction of inflammatory cytokines from GECs when the two are combined. This implies that physiologically, a combination of gp120 along with inflammatory cytokines present in infected semen could work together to enhance barrier disruption in FRT. In light of this, it would be interesting to determine if other sources of inflammation such as bacterial and viral coinfections and/or infected SP could exacerbate HIV-1 gp120 effects or, alternatively, effectively lower the amount of virus needed for barrier disruption.

Although the current study demonstrates that HIV-1 gp120 present in semen could directly activate inflammatory innate responses in GECs, the clinical significance of this pathway needs

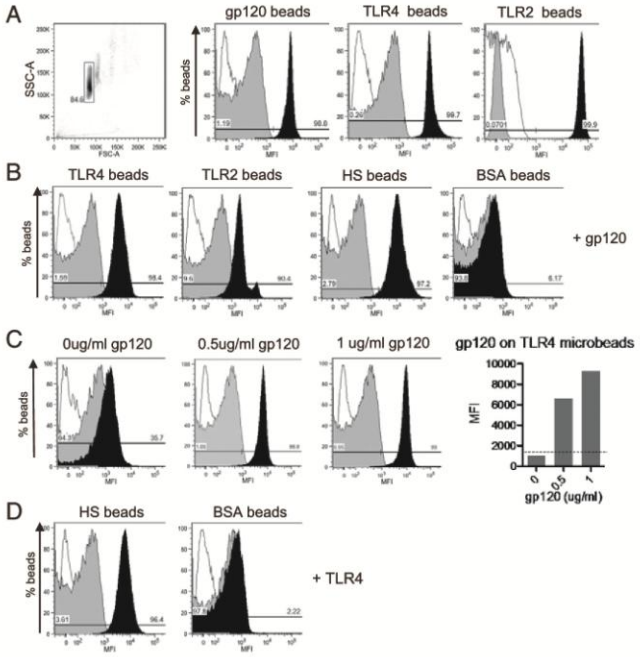

FIGURE 7. HIV-1 gp120 binds to TLR2, TLR4, and HS-coated microbeads. (**A**) The specificity of gp120, TLR4, and TLR2 coating on polystyrene microbeads was determined by flow cytometry using biotinylated anti-gp120, anti-TLR4, and anti-TLR2 Abs. Soluble TLR2, TLR4, and HS-coated beads were incubated for 1 h with gp120 (**B**), and binding was determined by biotinylated anti-gp120 Ab (**C**). Binding specificity of gp120 was confirmed by adding different doses of gp120 to TLR4 microbeads. (**D**) Soluble TLR4 was added to HS-coated beads, and TLR4 binding was determined by biotinylated anti-TLR4 Ab. In all experiments, BSA-coated beads were used as controls to establish positive staining of gp120 and TLR4 bead binding. Nonshaded peak represents unstained beads, gray-shaded peak represents background staining by streptavidin PE-Cy7 alone without primary Ab, and the black peak represents staining in the presence of primary and secondary Abs. Data shown are representative of three separate experiments with similar results.

to be further explored. Whether gp120 concentrations comparable to those used in this study are present in SP is a contentious issue (54). The source of gp120 in semen could be from infectious, intact virions or as soluble gp120 shed from viral particles or infected cells. Soluble gp120 has been associated with a variety of biological activities and has been detected (~10 ng/ml) in the circulation of acutely or chronically infected individuals (14). Santosuosso et al. (55) showed that concentrations of gp120 >300 ng/ml could be detected in secondary lymphoid tissues obtained from HIV-infected subjects, even when gp120 was undetectable in

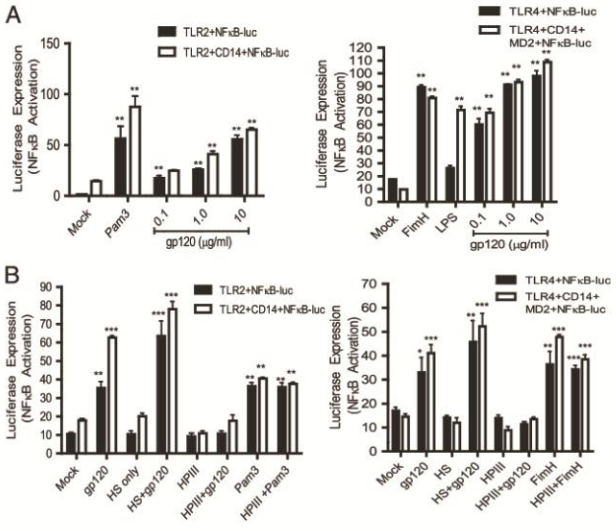

FIGURE 8. gp120 activates NF-κB through TLR2 and TLR4, facilitated by HS. (**A**) HEK 293 cells were transiently transfected with an NF-κB–luciferase reporter plasmid and a TLR2 (*left panel*) or TLR4 (*right panel*) expression plasmid, with or without a CD14 expression plasmid, and 24 h posttransfection were exposed to mock treatment (media), Pam3CSK4 (Pam3), FimH, LPS, or gp120. At 48 h posttreatment, cells were disrupted, and fold increase in luciferase activity was measured in cells as a readout for NF-κB activation using commercial luciferase kits. (**B**) Alternatively, transiently transfected HEK 293 cells were treated with, or without heparinase III (HPIII) and/or exogenous HS for 1 h prior to gp120 stimulation. At 48 h posttreatment, cells were disrupted, and fold increase in luciferase activity was measured in cells as readout for NF-κB activation. Bars represent mean ± SEM of triplicates. Significance shown in each graph above is relative to the respective mock treated group for each experiment. Data shown are representative of three (A) or two (B) separate experiments with similar results. $^{**}p < 0.01$, $^{***}p < 0.001$.

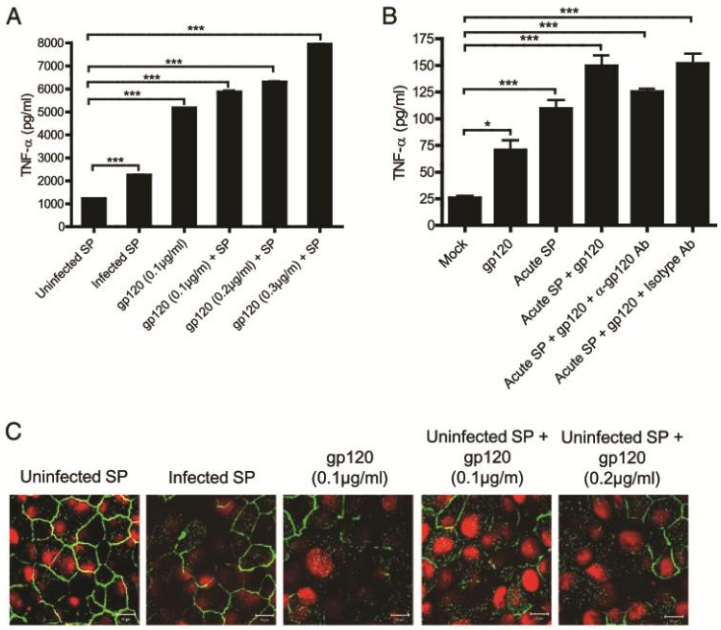

FIGURE 9. TNF-α production and epithelial barrier disruption in response to gp120 present in SP samples. (**A**) Primary GECs were exposed for 24 h to SP collected from uninfected or HIV-1–infected individuals or to uninfected SP spiked with known concentrations of gp120. TNF-α production was measured in supernatants using TNF-α ELISA. Bars represent mean ± SEM of triplicate cultures run for each condition from the same tissue sample. Data shown are representative of two separate experiments with similar results. (**B**) Primary GECs were exposed for 24 h to media alone (mock infection), gp120, pooled SP samples collected from three separate AI men, pooled SP from AI men with gp120, pooled SP from AI men with gp120 and anti-gp120 Ab, or pooled SP from AI men with gp120 and an isotype Ab. TNF-α production was measured in apical supernatants by ELISA. Data shown represent mean ± SEM. (**C**) GEC monolayers were fixed following treatment with SP samples and stained for ZO-1 tight junction protein via confocal microscopy. Original magnification ×1260. $*p < 0.05$, $***p < 0.001$.

their blood plasma. Such high local concentrations of gp120 could be relevant in light of studies showing compartmentalized viral shedding between blood and semen seen in HIV-infected men, which could indicate a local reservoir of HIV-1 in the male genital tract (56–58). Our attempts to directly measure gp120 in SP of HIV-1–infected men have given varied results and proven to be technically challenging. Nevertheless, the mechanism shown in the current study provides a possible physiological scenario in which exposure to HIV-1 or soluble gp120 from semen from infected men could directly initiate immune activation.

The recognition of HIV-1 gp120 by GECs has important implications for HIV-1 susceptibility and pathogenesis. The innate recognition and inflammatory response of GECs could inadvertently provide an advantage to HIV-1. The direct disruption of tight junctions could allow paracellular leaking of HIV due to loss of mucosal barrier and be the source of HIV infection in the female upper genital tract. Although the exact mechanism is unclear, experimental studies in nonhuman primate models provide strong evidence that the female upper genital tract, particularly the endocervix, is a susceptible site for HIV-1 infection (3). Furthermore, our studies provide an explanation for the etiology of mucosal barrier disruption during the acute phase of HIV-1 infection, which has been associated with chronic immune activation, a hallmark of HIV disease progression (10, 11). Microbial translocation across the intestinal and possibly genital mucosa is considered to be the most likely reason for increased immune activation and inflammation during HIV-1 infection (9, 59). Activated T cells in the intestinal mucosa have been cited as the source of proinflammatory cytokines that breach the mucosal barrier in the gut (9, 60, 61). Our results show that HIV-1 gp120 directly induces an inflammatory response in GECs and provides an alternative explanation of a direct mechanism of barrier disruption and microbial translocation early after exposure to HIV (7).

In conclusion, this study provides evidence for the first time, to our knowledge, that gp120 can act as a TLR ligand on GECs in the presence of HS. This signaling complex leads to activation of TLR2 and TLR4 pathways, NF-κB activation, and subsequent induction of proinflammatory cytokines. Proinflammatory cytokines, specifically TNF-α, induce destabilization and disruption of tight junction proteins, loss in barrier function, and increased barrier permeability. Subsequently, both viral and bacterial translocation can take place across the epithelial monolayers. Altogether, these studies suggest that the initial innate recognition of HIV-1 could result in barrier loss and initiation of immune activation that could be the first step in chronic immune activation, a hallmark of HIV-1 pathogenesis. Strategies to prevent barrier loss following exposure to HIV-1/gp120 could provide the basis for prophylactic treatments for prevention of immune activation during HIV-1 infection.

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Disclosures

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REVIEW ARTICLE

Influence of Common Mucosal Co-Factors on HIV Infection in the Female Genital Tract

Victor H. Ferreira, Jessica K. Kafka, Charu Kaushic

Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, Michael G. DeGroote Institute of Infectious Diseases Research, McMaster University, Hamilton, ON, Canada

Keywords
Epithelial barrier function, female genital tract, HIV susceptibility, immune activation, seminal plasma, sexually transmitted infections

Correspondence
Charu Kaushic, Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, Michael G. DeGroote Institute of Infectious Diseases Research, McMaster University, 1200 Main Street West, Hamilton, ON, Canada.
E-mail: kaushic@mcmaster.ca

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Women constitute almost half of HIV-infected population globally, and the female genital tract (FGT) accounts for approximately 40% of all new HIV infections worldwide. The FGT is composed of upper and lower parts, distinct in their morphological and functional characteristics. Co-factors in the genital microenvironment, such as presence of hormones, semen, and other sexually transmitted infections, can facilitate or deter HIV infection and play a critical role in determining susceptibility to HIV. In this review, we examine some of these co-factors and their potential influence. Presence of physical and chemical barriers such as epithelial tight junctions, mucus, and anti-microbial peptides can actively block and inhibit viral replication, presenting a significant deterrent to HIV. Upon exposure, HIV and other pathogens first encounter the genital epithelium: cells that express a wide repertoire of pattern recognition receptors that can recognize and directly initiate innate immune responses. These and other interactions in the genital tract can lead to direct and indirect inflammation and enhance the number of local target cells, immune activation, and microbial translocation, all of which promote HIV infection and replication. Better understanding of the dynamics of HIV transmission in the female genital tract would be invaluable for improving the design of prophylactic strategies against HIV.

Introduction

The global demographics of HIV and AIDS have changed dramatically over the course of the past 30 years. It was first discovered as a disease that primarily affected men who have sex with men (MSM) and intravenous drug users but has evolved into an epidemic where currently approximately half of 34 million adults living with HIV globally are women.[1] Although vaginal intercourse carries a lower HIV transmission probability per exposure event than anal intercourse or parenteral inoculation,[2] the female genital tract (FGT) has been estimated to be the predominant site of HIV acquisition globally, with approximately 40% of all new infections originating at this mucosal site.[2,3] Thus, it has become increasingly clear that a better understanding of the microenvironment in the FGT is critical for developing strategies for the prevention of HIV transmission globally.

Transmission of HIV in the female genital tract

The FGT can be divided into two major compartments: the lower genital tract (LGT), consisting of the vagina and ectocervix, lined by stratified squamous epithelium; and the upper genital tract (UGT) consisting of the endocervix, endometrium, and fallopian tubes, lined by a single layer of columnar epithelium.[4] Tight junction proteins between the

columnar epithelium of the UGT form a mechanical barrier, preventing pathogens from breaching the protective lining. In the stratified epithelium of the LGT, continuous sloughing of dead superficial genital epithelial cells (GECs) prevents many pathogens from colonizing or establishing infections. 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,[2] particularly when breaches occur in the epithelium, such as during sexual intercourse.[5,6]

Despite much debate, there is no clear consensus regarding the primary site of transmission of HIV-1 in the FGT. Although tiny microabrasions in the LGT are a common occurrence during normal sexual intercourse and could provide an easy portal for viral transmission,[7] other studies, particularly those that examined acute simian immunodeficiency virus (SIV) infection in non-human primates[8], indicate that HIV may preferentially invade through the UGT and local viral amplification likely precedes systemic dissemination. Adding further credence to this concept is the fact that a large number of activated $CD4^+$ T-cells populate the cervical transformation zone, providing a rich source of target cells for HIV-1.[9] Furthermore, a recent study using human tissue found that HIV-1 could penetrate both intact human cervical columnar and squamous epithelial barriers to depths where the virus could encounter potential target cells.[10]

Natural barriers to HIV infection in the female genital tract

The FGT contains a number of endogenous barriers that provide protection against HIV acquisition. 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.[11] 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 1000 times more slowly than it would in water.[12] GECs, as well as resident immune cells, such as macrophage and dendritic cells (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.[13] More recently, antiproteases, such as serpins and cystatins expressed by GECs, have also been shown to inhibit HIV binding and replication and reduce local inflammation.[14]

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),[15,16] tripartite motif 5α (TRIM5α),[17] bone marrow stromal antigen 2 (BST2; also known as tetherin)[18] SAM domain and HD domain 1 (SAMHD1)[19,20], and myxovirus resistance 2 (MX2 also known as MxB).[21] Interestingly, type I IFN has also been implicated as a contributor to HIV pathogenesis[22]; increased type I IFN is a component of the signature associated with chronic immune activation.[23] The benefit/harm of IFN responses may likely depend on the net outcome of a number of factors, including the stage of infection. Evidence from our laboratory suggests that in response to HIV-1 gp120, GECs significantly upregulated IFN-β and neutralization of this IFN-β resulted in enhanced induction of the HIV long terminal repeat (LTR) promoter in transfected Jurkat T-cells (A. Nazli, V.H. Ferreira & C. Kaushic, unpublished results). These results suggest that early type I IFN responses originating at the site of transmission may play a role in reducing HIV replication in the FGT, in contrast to the effects of IFN that take place during the chronic stages of infection which may contribute to immune activation.

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.[24] IFN-ε is the only type I IFN family member to be expressed by HeLa cells.[25] Moreover, seminal plasma was also found to upregulate expression of IFN-ε in cervicovaginal tissues,[26] 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, increased HIV-specific CD8 T-cell responses were observed in the spleen, genito-rectal draining lymph nodes,

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and Peyer's patches.[27] 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*[28,29] by inhibiting HIV-1 integration and post-transcriptional events.[30] Interestingly, 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.[4] In addition, primary human uterine GECs 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.[31] PRR recognition of pathogens typically initiates an intracellular signaling cascade resulting in the activation of transcription factors such as NFκB and the production of a variety of cytokines and chemokines.[32] Many of these cytokines and chemokines also have the ability to directly interfere with HIV infection. The chemokine stromal-derived factor-1 (SDF-1 or CXCL12), which is found within the subepithelial layer of the cervix, is able to inhibit X4 strains of HIV competitively.[33] Similarly, the β-chemokines macrophage inflammatory protein 1-α (MIP-1α), MIP-1β, and regulated on activation, normal, T-cell expressed, and secreted (RANTES) are all secreted by cells of the upper and lower genital tracts constitutively and under infectious conditions,[34–36] and, as natural ligands for the CCR5 receptor, may also play a role in blocking R5-tropic viruses from establishing an infection.

Epithelial barrier in FGT during HIV infection

The mucosal barrier formed by the epithelial cells of the FGT forms the first line of defense against the entry of pathogens. Sexually transmitted organisms, including HIV-1, have to breach this barrier to establish infection. There is evidence that during HIV infection, both the intestinal and genital mucosal barriers are disrupted and memory $CD4^+$ T-cells at these mucosal surfaces are severely depleted.[37–39] HIV-1 infection is characterized by chronic systemic inflammation, seen by increased levels of serum lipopolysaccharide (LPS) and soluble CD14 (sCD14) in HIV-1-infected individuals.[37,40–44] HIV-1 disease progression has been correlated with increased circulating levels of LPS, an indicator of microbial translocation, which is associated with mucosal barrier disruption.[43] The chronic immune activation associated with HIV disease is considered to be one of the main driving forces leading to immunodeficiency. The etiology of microbial translocation associated with HIV infection is not clearly understood and has been linked to impairment in the mucosal epithelial barrier. Our recent work supports this premise by demonstrating that primary human GECs directly interact with HIV-1 surface glycoprotein gp120 leading to production of an array of proinflammatory cytokines.[45] Among these cytokines, TNF-α production induced a rapid decrease in transepithelial 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 could result directly from early interactions between HIV-1 and the genital epithelium leading to initiation of microbial translocation and initiating immune activation. Further studies have since revealed that gp120-mediated activation of proinflammatory cytokine pathways in GECs utilizes TLR2 and TLR4 in addition to cell surface heparan sulfate moieties.[46] Further ongoing studies are examining the *in vivo* relevance of gp120-mediated increased permeability.

More recently, a role for local immune factors such as IL-22 in the maintenance of barrier function in the intestinal mucosa in both human and SIV models has emerged. IL-22 is a member of the IL-10 family of cytokines with epithelial reparative and regenerative properties.[47] Recent studies suggest that the absence of IL-22 contributes to HIV-1 and SIV pathogenesis.[48,49] Kim *et al*[48] recently demonstrated that during chronic HIV-1 infection, IL-22-producing Th22 cells were severely depleted, and this was accompanied by compromised epithelial integrity in the intestinal mucosa. Further, *in vitro* IL-22 treatment protected intestinal epithelial cells against HIV-1- or TNF-α-induced barrier dysfunction. Similar IL-22-mediated

protection is also observed in GECs (A. Nazli & C. Kaushic, unpublished results). These studies indicate that local immune factors in the mucosa could play an important regulatory role in epithelial barrier maintenance related to HIV-1 pathogenesis.

HIV interactions with cells of the female genital tract

If the intrinsic barriers in the FGT, described above, are overcome, HIV-1 is capable of crossing the genital epithelium and establishing an infection. HIV virions have been suggested to traverse the epithelium through several pathways, including direct infection of GECs[50]; transcytosis of viral particles across the epithelium[51–53]; and penetration of the virus through epithelial breaches.[5,6] While there is evidence that HIV-1 can infect GECs from the LGT[54,55] or UGT,[50,53] these findings are contentious, particularly as to whether these epithelial cells are productively infected. In agreement with some of the earlier work done on epithelial cell 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.[56] Consequently, the de novo virus was transmitted to target CD4 T-cells in co-culture in a contact-dependent manner. In recent studies performed in our laboratory, exposure of primary human endometrial and endocervical GECs to R5 or X4 tropic strains of HIV-1 did not result in the detection of HIV-1 pro-viral DNA integration, RNA splicing or reverse transcription products, although virus was seen to be taken up by endocytosis into GECs, suggesting that primary human GEC could be non-productively infected (V.H. Ferreira & C. Kaushic, unpublished results).

The nature of viral entry into GECs is likely distinct from the canonical HIV-1 entry pathways as GECs demonstrate inconsistent expression of CD4 and the chemokine co-receptors CCR5 and CXCR4.[54,57,58] In lieu of these molecules, GECs may facilitate HIV transmission using cell surface glycosphingolipids, sulfated lactosylceramide expressed by vaginal GECs[59], and galactosylceramide expressed by ectocervical GECs,[58] which have been found to bind HIV-1 gp120 and foster transcytosis. Interactions of HIV-1 gp120 with transmembrane heparan sulfate proteoglycans, such as syndecans, expressed by GECs, may also contribute to HIV-1 attachment and entry.[52,55] A variant of salivary agglutinin named gp340, which is expressed on cervical and vaginal GECs, has also been implicated in the passage of HIV through the epithelium.[60,61] The relative contribution of these receptors to HIV entry and infection in GECs is unclear.

In addition to GECs, there are a number of resident immune cells in the FGT that may contribute to HIV acquisition or transmission, most notably DCs and T-cells. DCs appear to play a major role in HIV transmission and dissemination, as well as driving the early inflammatory response to infection.[62] However, the relative contribution of different types of DCs is not completely understood. Some studies purport that resident Langergans cells (LCs) and $CD4^+$ T-cells are the primary target of HIV-1 in the genital tract,[3] whereas others have shown that the presence of vaginal LCs does not alter HIV transmission.[63] Furthermore, the transformation zone where the ectocervix changes into the endocervix has an enriched population of $CD4^+$ T-cells and APCs and may therefore be a particularly susceptible site for HIV acquisition.[9]

Female sex hormones, hormonal contraception, and altered susceptibility to HIV infection

A number of studies in the last two decades indicate that endogenous female sex hormones and exogenous hormonal contraceptives affect HIV-1 infection or disease progression (reviewed in[64]). Non-human primate studies have consistently found that the administration of depot medroxyprogesterone acetate (DMPA) to rhesus macaques enhances the risk of SIV acquisition,[65–67] whereas estradiol (E2) and its derivatives have been shown to be protective against SIV infection.[68] DMPA is a progesterone (P4)-based synthetic contraceptive, used by more than 100 million women around the world, and it is particularly popular in resource-poor settings in the developing world, where HIV rates remain high.[69] Human epidemiological studies have shown that DMPA use may lead to increased risk of HIV-1 infection, disease progression, and mortality compared to women who did not use the injectable contraceptive formulation DMPA.[65,70–72] More recently, a prospective cohort study of nearly 3800 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

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hormonal contraceptives and that HIV-infected women who used injectable hormonal contraceptive were twice as likely to transmit HIV to an uninfected male partner.[73] Subsequently, UNAIDS had urgent consultations with an expert panel, and although guidelines for hormonal contraceptive were not subsequently altered, there was a renewed call for more research on hormonal contraceptives and HIV infection risk.[74,75]

Although the pathways involved in these outcomes are not clear, P4 is known to regulate a number of immunological pathways,[76] including the inhibition of CTLs[77] and natural killer cells.[78] It also decreases the production and alters glycosylation of IgG and IgA antibodies, modulates cytokine production, and upregulates HIV-1 receptor expression on $CD4^+$ T-cells.[79,80] With respect to DMPA, a recent study found that DMPA use inhibited the production of IFN-γ, IL-2, IL-4, IL-6, IL-12, TNF-α, MIP-1α as well as IFN-α in peripheral blood cells and activated T-cells following stimulation with TLR ligands.[81] Others have found that in vaginal biopsies from women receiving DMPA, the numbers of HIV target cells were significantly increased compared to vaginal tissues taken during the follicular and/or luteal phases of untreated cycles.[82] In a recent study, cervical tissue explants from 22 HIV-1 seronegative women were exposed to R5 HIV-1 *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.[83] 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.

To gain a better understanding of the mechanism by which hormones regulate susceptibility in the FGT, we have extensively studied a mouse model of genital herpes infection. Using this mouse model, we demonstrated that DMPA increased susceptibility to genital herpes simplex virus type 2 (HSV-2) infection by 100-fold.[84] Further studies indicated that prolonged DMPA treatment regimes resulted in poor mucosal immune responses and increased susceptibility.[85] In other studies, mice were ovariectomized and treated with exogenous E2 and P4 prior to primary infection with genital herpes or immunization with attenuated strain of herpes virus. The results from these studies indicate that E2 treatment regulates susceptibility while progesterone treatment leads to increased chronic inflammation and pathology.[86,87] Subsequent studies demonstrated the protective effect of E2 in intranasal immunized mice that had significantly decreased pathology compared to P4-treated mice.[88] These findings were confirmed by other studies, using an HSV-2 vaccine formulation.[89]

Recent studies in our laboratory have examined whether endogenous female sex hormones or hormonal contraceptives regulate GEC susceptibility to HIV-1. Our results indicate that HIV-1 is internalized within GECs via endocytosis. However, no early or late reverse transcription products, integrated HIV-1 pro-viral DNA, or spliced HIV RNA transcripts were measured, regardless of the presence or absence of hormones. The results suggest that female sex hormones, particularly DMPA, may regulate HIV entry and transcytosis, but not replication in GECs (V.H. Ferreira & C. Kaushic, unpublished results). Ongoing studies are investigating the significance of DMPA-enhanced HIV entry and transcytosis in the absence of productive infection.

Co-infections in the female genital tract

STIs have been associated with increased HIV genital shedding, transmission, and susceptibility.[90,91] Such infections may include gonorrhea, syphilis, bacterial vaginosis, candidiasis, and genital herpes. Bacterial STIs, such as *Chlamydia trachomatis* and *Neisseria gonorrhea*, have been epidemiologically associated with increased subsequent HIV acquisition, and, by extension, with increased sexual transmission of HIV.[92,93] The increased HIV acquisition may relate to local micro-ulcerations due to the pathologies associated with the infection, or to the local recruitment of activated immune cells, which may act as targets for HIV.[94] Bacterial vaginosis (BV) is a common disorder characterized by changes in vaginal flora in which normally predominant *Lactobacillus* species are replaced by potential pathogens including *Gardnerella vaginalis*, genital *Mycoplasma*, and fastidious anaerobic bacteria.[95,96] BV has been associated with a 60% increased risk of HIV-1 acquisition in women,[97] and, among women with HIV-1, with higher HIV-1 concentrations in cervicovaginal fluids.[98] Bacteria associated with BV can induce viral replication and shedding in the genital tract,[99] which may lead to increased HIV-1 infectiousness for women with BV.[100]

Herpes simplex virus type 2 is one of the most prevalent STIs, infecting 20–30% of sexually active adults in North America and up to 80% of adults in sub-Saharan Africa.[101] A previous meta-analysis indicated HSV-2 infection to be associated with a threefold increase in susceptibility to HIV by both men and women from the general population.[102] Part of this increased susceptibility is likely due to HSV-2-induced ulcerations, which create a breach in the physical barrier of the genital epithelium.[103] Genital HIV-1 shedding is also markedly increased during clinical HSV-2 reactivations, accompanied by an increase in HIV-1 plasma viral load.[104] Herpetic lesions and possibly asymptomatic HSV-2 mucosal shedding generate an influx of activated $CD4^+$ T-cells that persist for months after healing, which may facilitate the transmission of HIV.[105]

In response to sexually transmitted co-infections, cells in the genital tract, including GECs, upregulate inflammatory responses. 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 increases HIV replication, mediated through the HIV-LTR.[106–108] Previously, we showed that in response to common co-infecting STIs, specifically HSV-1, HSV-2, and *N. gonorrhea*, primary GECs upregulated proinflammatory cytokines including TNF-α, IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1), which contributed to indirect induction of the HIV-LTR promoter in T-cells, a process synonymous with HIV replication. Furthermore, by blocking inflammatory signaling pathways, either with the broad anti-inflammatory compound curcumin or with specific NFκB and AP-1 inhibitors, this indirect induction could be blocked.[109] Inflammation may therefore play a major role in the acquisition or spread of HIV-1 infection.[110] Interestingly, it has been previously observed that lower levels of IL-1β, IL-6, and TNF-α were measured in unstimulated PBMCs of highly exposed persistently seronegative (HESN) women, suggesting an *immunoquiescent* phenotype among this resistant cohort.[111] In another study, HIV genital tract shedding was significantly associated with higher cervico-vaginal lavage (CVL) concentrations of IL-6, IL-1β, MIP-1α, and RANTES.[112] Thus, a future exploration of using anti-inflammatory compounds for the purpose of protecting the epithelial barrier-disrupting function of HIV-1 gp120 as well as the inflammation associated with co-infecting microbes may therefore present a novel and valuable new modality for preventing HIV infection in the FGT or curbing the spread of the infection.

Influence of semen on HIV transmission

Semen is the main vector of HIV dissemination as transmission in FGT occurs primarily following exposure to virus-containing seminal fluid during sexual intercourse.[113–116] Semen contains a plethora of factors, which serve to enhance or inhibit HIV infection. For example, spermatozoa can capture virus through heparan sulfate and transmit it to DCs, thus increasing their infection.[117] In contrast, clusterin and mucin-6 in seminal plasma (SP) can compete with HIV as DC-SIGN ligands.[118–120] Several *in vitro* studies reported the existence of amyloid fibrils, formed by amyloidogenic fragments of prostatic acid phosphatase and semenogelins found abundantly in semen, which may increase the infectivity of HIV by several orders of magnitude by facilitating virion attachment to target cells.[121–125] *In vivo*, SP and cationic SEVI may facilitate the spread of physiologically lower doses of HIV-1 found during sexual transmission,[126]; however, further *in vivo* studies are needed to test this hypothesis. In contrast, other cationic polypeptides such as semenogelin contribute to anti-HIV activity in SP.[127] Furthermore, deposition of semen into the acidic vaginal environment can raise the pH, which may decrease[116,128] or increase[129] the infectivity of the virus.

The influence of semen on the FGT microenvironment can have a profound effect on HIV transmission. When semen from HIV-uninfected men is deposited into the FGT, it elicits a transient inflammatory response characterized by proinflammatory cytokine and chemokine production and immune cell recruitment, which serves to prime the FGT for the conceptus.[26,130–133] However, this response may also create an environment favorable for HIV infection as SP has also been shown to induce the overexpression of COX-2 in mare and porcine endometrium[134,135] and enhance COX-2 expression in human vaginal ECs,[136] thus promoting an inflammatory environment. Additionally, vaginal GECs have been shown to upregulate the chemokine CCL20 in response to SP, which promoted the migration of LCs into the vaginal mucosa.[137] Interestingly, when it comes to transmigration of cell-associated virus across FGT epithelium, SP decreased epithelial crossing of immune cells by

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increasing their adherence to the epithelial monolayer, and increasing TER.[138]

The presence of HIV infection in the male genital tract (MGT) can alter semen composition, which can have a profound effect on FGT responses to semen. This includes changing the cytokine network and further enriching SP with cytokines which can modulate HIV replication[139] and promote HIV shedding and local target cell activation.[140] The immunoregulatory factor transforming growth factor beta-1 (TGF-β1) is found in very high concentrations in human SP and functions to induce FGT tolerance to the allogeneic fetus.[130] Because of its abundance in semen and its immunoregulatory role, we examined the role of seminal TGF-β on FGT responses and how these responses may differ during acute and chronic stages of HIV infection. Our results showed that seminal plasma from HIV-uninfected and HIV-infected antiretroviral therapy (ART)-naive men in acute stages of infection contained higher levels of proinflammatory cytokines and lower levels of TGF-β1 compared to ART-naive men in chronic stages of infection, which leads to an increase in proinflammatory cytokine production from endometrial GECs.[141] GEC responses to SP regardless of the presence or stage of HIV infection increased HIV-LTR expression, suggesting that SP can promote HIV replication in infected target cells in the FGT.[141] More recently, we found that TGF-β1 was compartmentalized between blood and semen, and latent TGF-β1 was positively correlated with the immune activation marker sCD14 in SP, suggesting that activated monocytes and macrophages in the MGT may co-express TGF-β1 and sCD14 in response to HIV infection. Interestingly, sCD14 was negatively correlated with high levels of active TGF-β1 seen in SP of chronic ART-naive men. This suggests that high levels of active TGF-β1 could play a role in regulating chronic immune activation, thus furthering our understanding of the role of TGF-β1 in HIV transmission (J.K. Kafka & C. Kaushic, unpublished results).

Conclusion

The FGT is a key target for HIV-1 transmission in women, and the outcome of exposure to HIV is likely determined by a number of co-factors that influence this mucosal microenvironment (Fig. 1). The combination of innate physical barriers such as tight junctions and mucus in the mucosal epithelial lining as well as chemical barriers including the

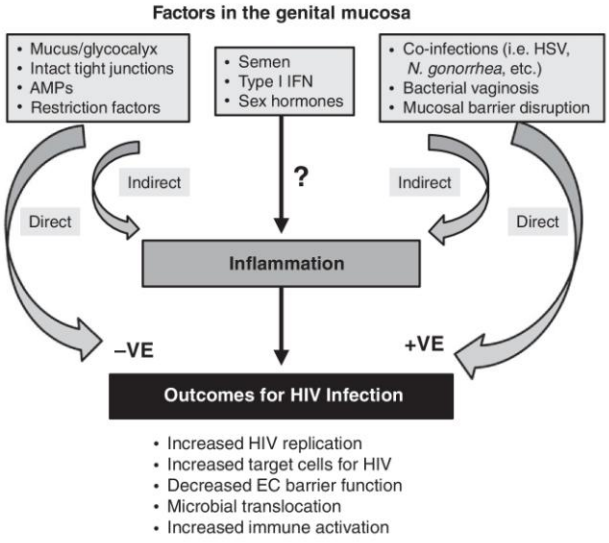

Fig. 1 Factors in the FGT microenvironment and their influence on HIV infection. Physical barriers (such as, intact tight junctions and mucus/glycocalyx) and chemical/biological barriers, such as, AMPs and restriction factors, can prevent HIV acquisition in the FGT by directly inhibiting infection and/or replication. In contrast, co-infection with HSV and *N. gonorrhea*, bacterial vaginosis, or mucosal barrier disruption can either directly enhance HIV infection/replication or indirectly via the induction of inflammation. Mucosal co-factors such as these may also contribute to increasing the availability of HIV target cells, increasing immune activation, decreasing barrier function, and microbial translocation, all of which contribute to enhanced infection and disease pathogenesis. It is not clear whether factors such as semen, type I IFN, or sex hormones contribute to regulating HIV infection/replication in the FGT as the studies suggest they can both inhibit or enhance HIV infection/replication depending on the FGT microenvironment.

anti-microbial peptides and HIV-1 restriction factors can have a direct anti-viral effect. They can also indirectly decrease HIV-1 infection and replication by inducing an anti-inflammatory microenvironment that is unfavorable for HIV. Other co-factors that may be present in the FGT, such as viral and bacterial co-infections, bacterial vaginosis, and direct interactions with HIV, disrupt the mucosal barrier, can directly and indirectly facilitate HIV-1 infection and replication. They can also create a microenvironment favorable for HIV infection and replication by attracting target T-cells into the FGT, increasing immune activation, barrier disruption, and microbial translocation. Other factors in the FGT milieu including semen, Type I IFN, and sex hormones have been shown to have both types of effects, and more work needs to be done to determine which effects are predominant *in vivo*. Further complexity is likely conferred in the genital tract because of the interactions between the co-factors present simultaneously in the FGT. Further *in vivo* studies examining the co-factors will provide insights that can assist in the development of future prophylactic strategies against HIV.

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Review Article

Primary human epithelial cell culture system for studying interactions between female upper genital tract and sexually transmitted viruses, HSV-2 and HIV-1

Charu Kaushic*, Aisha Nazli, Victor H. Ferreira, Jessica K. Kafka

McMaster Immunology Research Center, McMaster University, Department of Pathology and Molecular Medicine, 1280 Main Street West, Hamilton, Ontario, Canada L8S4K1

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HIV-1

ABSTRACT

Evidence from clinical and epidemiological studies indicates that women are disproportionately susceptible to sexually transmitted viral infections. To understand the underlying biological basis for this increased susceptibility, more studies are needed to examine the acute events in the female reproductive tract following exposure to viruses during sexual transmission. The epithelial lining of the female reproductive tract is the primary barrier that sexually transmitted viruses, such as HIV-1 and HSV-2 need to infect or traverse, in order to initiate and establish productive infection. We have established an ex-vivo primary culture system to grow genital epithelial cells from upper reproductive tract tissues of women. Using these cultures, we have extensively examined the interactions between epithelial cells of the female genital tract and HSV-2 and HIV-1. In this review, we describe in detail the experimental protocol to grow these cultures, monitor their differentiation and inoculate with HSV-2 and HIV-1. Prospective use of these cultures to re-create the microenvironment in the reproductive tract is discussed.

1. Introduction

Sexually transmitted infections (STIs) are among the most prevalent infectious diseases worldwide and are a major cause of morbidity and mortality. While bacterial STIs such as chlamydia and gonorrhea are curable, viral STIs such as genital herpes, HIV and HPV cause incurable lifelong infections. In 2008, a bulletin from the World Health Organization estimated that 536 million people aged 15–49 are infected with HSV-2, the virus that causes genital herpes [1]. In addition, 23.6 million people in this age group become newly infected with HSV-2 every year. The prevalence rates of HSV-2 infection are higher in women than men, with the lowest prevalence rates being 13% among West European men and the highest prevalence rates being 70% among sub-Saharan African women [1]. Similar to HSV-2 infections, women are also more likely to become infected with HIV. Since the 1980s, HIV has shifted from a disease caused predominantly by use of shared hypodermic needles and male–male contact to a disease caused by heterosexual transmission. In fact, recent estimates have found that 30–40% of annual worldwide HIV infections occur through heterosexual transmission in the female reproductive tract [2,3]. Currently, in sub-Saharan Africa, 57% of all people infected with HIV are women and girls, and 76% of young people (aged 15–24) living with HIV are female [4]. These and other epidemiological data from bacterial STIs, such as chlamydia and gonorrhea, have consistently found that women have higher prevalence rates compared to men [5].

2. Discrete morphological characteristics of female genital epithelial cells

One of the important considerations for understanding the heterosexual transmission of viruses in female genital tract is the morphological and functional distinctions between the epithelial cells that line the different compartments of the female reproductive tract. These epithelial cells are the first cells that sexually transmitted viruses encounter, therefore their physical and functional characteristics are important determinants in the outcome. The lower reproductive tract in women is composed of ecto-cervix and the vaginal tract. The mucosal lining in these compartments consists of stratified squamous epithelium that can be more than 25 cell layers thick [6]. In contrast, the upper reproductive tract, made up of the endocervix and endometrium, is composed of a single layer of columnar epithelium that rest on a continuous, thin basement membrane [7]. The columnar epithelium is characterized by the presence of tight junctions between cells that makes it impermeable to entry of any large molecules and particulate matter,

* Corresponding author. Address: McMaster Immunology Research Center, McMaster University, Department of Pathology and Molecular Medicine, Michael DeGroote Centre for Learning and Discovery, Room 4014, 1280 Main Street West, Hamilton, Ontario, Canada L8S4K1. Fax: +1 905 522 6750.
E-mail address: kaushic@mcmaster.ca (C. Kaushic).

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including pathogens. In comparison, the upper layers of the stratified squamous epithelium, that from the lining of the lower genital tract, have been shown to lack tight junctions and are relatively permeable to large molecular weight soluble mediators [8]. While the multiple layers in the lower genital epithelium may provide a better mechanical protection against viral invasion than the single layer columnar epithelium that lines the upper reproductive tract, at the same time, the greater surface area of the vaginal wall and ectocervix, arguably allows greater access for virus entry, particularly when breaches occur in the epithelium, such as during sexual intercourse [3,9,10].

3. Interactions of the genital epithelium with sexually transmitted viruses

While all sexually transmitted viruses have to cross the obstacle of the female genital epithelium to cause a productive infection in the host, their specific interactions with the epithelial lining in the genital tract are quite different. Herpes simplex virus type 2 (HSV-2) directly infects genital epithelium and undergoes replication within the cells [11]. It then infects adjacent epithelial cells and other cell types located under the epithelium, subsequently infecting peripheral nerves where it can become latent. The latent virus re-activates from time to time to replicate, the lining is shed in the genital tract secretions leading to further transmission. The consequence of direct infection and replication in the genital epithelium is evident in the efficiency of transmission of HSV-2. In N. America, roughly one in every 4 or 5 sexually active adults is infected by HSV-2 [12], while in Sub-Saharan Africa roughly 50–70% of the population is infected. Compared to HSV-2, the interaction of HIV-1 with the genital epithelium is still not completely understood [7,13]. Unlike HSV-2, it has a comparatively poor rate of transmission (1:200 to 1:1000 for each exposure) [14]. This makes the likelihood of productive infection in genital epithelium unlikely. However, studies done in this area have been far from conclusive. Early *in vitro* studies indicated that genital tract epithelial cell lines could be infected by HIV [15]. X4-tropic strain of HIV (T-tropic) was shown to replicate in cultured human primary uterine cells, however, R5-tropic strain (macrophage-tropic) was taken up and released from the cells, unmodified [16]. Over the years, the demonstration of alternative cellular receptors, such as GalCer, C-type lectins such as DC-SIGN, mannose receptors, proteoglycans such as heparin sulfate and syndecan that bind to HIV have raised the possibility that virus could get into mucosal epithelial cells using these alternative receptors [15,17–20]. More recently, the organ culture models of intestine, tonsil and cervix have been able to add relevant information regarding HIV transmission across epithelium [21–23]. These studies indicate that HIV-1 could possibly bind to epithelial cells via β-1 integrin and penetrate the ectocervical epithelial cell surface. Additionally, the main target of HIV-1 replication appears to be primarily the Langerhans and T cells underlying the epithelium. The overall view regarding HIV-1 infection is that it does not infect the epithelium *per se*, but is able to cross the mucosal epithelium to infect immune cells, including CD4 + DCs and T cells in the lamina propria of the mucosa. However, the debate regarding early events in HIV-1 transmission in the genital tract seems to be far from over. A recent study reported inability to detect translocation of HIV-1 across a reconstructed human vaginal mucosa. Furthermore, presence of Langerhans cells in this model did not increase HIV-1 transmission [24].

4. Immune functions of genital epithelial cells

One of the important reasons for understanding interactions of sexually transmitted viruses with genital epithelial cell is that these cells are dynamically active. As the primary cells that form the barrier between the external environment and the female genital tract, they play a critical role as the first responders to any incoming pathogen. They perform the dual function of responding directly to the pathogen as well as relaying signals to activate other innate and adaptive cells of the immune system. Genital epithelial cells (GECs) perform both these tasks very efficiently. They secrete a variety of anti-microbial factors constitutively and upon induction. Both upper and lower genital tract epithelial cells express a discrete range of TLRs [25–28]which allows them to recognize a wide array of pathogens and respond by production of cytokines and chemokines [29–31], which in turn attract other immune cells, including neutrophils and dendritic cells (DCs) to the genital tract. There is also evidence that GECs condition DCs to initiate adaptive immune responses [32].

Among the anti-microbial peptides secreted by GECs, many are produced constitutively and others are induced or upregulated upon exposure to stimuli. Studies done in *in vitro* culture systems indicate that some of these, including secretory leukocyte protease inhibitor (SLPI), lactoferrin, beta defensin and trappin-2/elafin have anti-HIV properties. The anti-leukoprotease SLPI has been shown to be secreted by ECs both in the human cervix and endometrium and suggested to have anti-HIV activity [33–35]. Epithelial cells of the genital mucosa also produce β-defensins, a family of small cationic peptides, shown to have significant antimicrobial effects [36]. More recently, the serine protease inhibitor, trappin-2/elafin has been shown to be secreted by GECs and implicated in anti-HIV activity at mucosal surfaces [37,38].

GECs also express a rich array of pattern recognition receptors. In particular, expression of, and activation by Toll-like receptors (**TLRs**) in GECs has been well described. Vaginal and cervical epithelial cells and cell lines express TLRs 1, 2, 3, 5, and 6, while primary endocervical ECs express TLRs 1, 2, 3 and 6 allowing them to sense both bacterial and viral pathogenic motifs and rapidly relay messages to other innate and adaptive cells should a pathogenic breach occur (reviewed in [30]). Primary human endometrial ECs express an even broader array of TLRs 1–9, indicating the potential of upper reproductive tract to respond to a wide range of pathogens. TLR mediated activation leads to production of chemokines and cytokines, including interleukin (IL)-6, IL-8, stromal cell derived factor (SDF)-1, the β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated upon activation, normal T-cell expressed and secreted (RANTES) [25,27,39–41].

In addition to the pro-inflammatory cytokines and chemokines, GECs are also capable of producing Type I Interferon (IFN), mainly IFN-β. Type I interferons (IFN-α, -β) impede the HIV replication cycle through numerous mechanisms, including induction of the antiviral molecule apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (**APOBEC3G**) [42,43] and 2′,5′-oligoadenylate synthetase (2′,5′-OAS), which activates a latent endo-ribonuclease, RNase L that inhibits the replication of HIV-1 [44,45]. Protein kinase R (**PKR**), inducible nitric oxide synthase (**iNOS**), myxovirus (**Mx**)-family proteins and the 9–27 protein are other interferon-inducible proteins shown to have anti-HIV properties [46]. Schaefer et al. reported the upregulation of the message levels for IFN-β, MyxA and OAS in cultures of primary endometrial ECs following exposure to TLR 3 ligand Poly I:C [47]. Since then, other studies have measured production of IFN-β in cervical and cervicovaginal cells following activation by TLR ligands or virus exposure [29,48].

5. Overview of the ex-vivo primary genital epithelial cell system

We have used an ex-vivo endometrial epithelial cell model to examine the interaction of sexually transmitted viruses, HSV-2

and HIV-1, with the female reproductive tract epithelium. These genital epithelial cells are derived from enzymatic separation from endometrial and endocervical tissues obtained from hysterectomies and grown on cell culture inserts. The culture conditions allow them to grow and differentiate into confluent, polarized, columnar epithelial monolayers [49]. We and other +laboratories have shown that the epithelial cells in these cultures reflect in vivo morphology and functional characteristics of upper genital tract epithelial cells, including tight junction formation, preferential polarized secretion of cytokines and other cellular factors [50]. The cultures are >98% pure epithelial cells with no trace of CD45 + hematopoietic cells. The purity of the culture was confirmed by flow cytometric analysis of epithelial, stromal and hematopoietic cell markers [49]. Once the monolayers are formed, they remain viable in culture for approximately another two weeks. The viability of the cultures is monitored by maintenance of transepithelial resistance values [49]. This facilitates studies where multiple time points may be examined following treatment. Using this model system, we have extensively examined interactions with both HSV-2 and HIV-1, summarized below.

5.1. Interactions between genital epithelial cells and HSV-2

In our studies, both endometrial and endocervical primary epithelial cultures were found to be susceptible to HSV-2 infection [49]. Exposure of either apical or basolateral surface of the epithelial cells resulted in successful viral infection, although virus was shed preferentially from the apical membrane. In another study, we demonstrated the innate anti-viral potential of genital epithelial cells [28]. Primary endometrial epithelial cultures were activated with eight discrete TLR ligands. When the TLR activated GEC cultures were exposed to HSV-2, differential degrees of protection were seen in response to specific TLR ligands that correlated with production of IFN-β and nitric acid production. Poly I:C, a TLR 3 ligand, induced best protection against HSV-2 infection and activated both NF-kB and IRF-3 pathways in GECs. More recently, we have observed that genital epithelial cells have the ability to sense and respond to variants of HSV-2 depending on their ability to replicate intracellularly (Ferreira et al. unpublished observations).

5.2. Interactions between genital epithelial cells and HIV-1

We recently described novel interactions between HIV and GECs, independent of HIV infection and replication, that could potentially play an important role in initiating HIV infection and immune activation [51]. This study demonstrated that exposure of GECs to HIV-1 or virus surface glycoprotein gp120 induced a remarkable upregulation of a number of different chemokines and inflammatory cytokines, including IL-1, IL-6, IL-8 and Tumor necrosis factor (TNF)-α. The increased production of TNF-α led to a significant downregulation of tight junction proteins and caused impairment of epithelial barrier function and leakage. This allowed viral and bacterial translocation across the epithelial monolayer. In addition to direct viral-GEC interactions, we have used this ex-vivo epithelial cell model to understand factors in the microenvironment of the female genital tract that could profoundly influence HIV transmission and replication. We recently reported the cellular mechanism that may drive HIV replication in the presence of viral or bacterial co-infections in the female genital tract [52]. We found that 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. In another study, we found that exposure of GECs to seminal plasma leads to NF-kB activation and pro-inflammatory cytokine production by GECs (Kafka et al., unpublished observations). High TGF-β in seminal plasma of men infected chronically with HIV suppressed pro-inflammatory cytokine production by GECs. Importantly, GEC responses to seminal plasma could activate HIV-LTR in infected CD4 + T cells.

6. Detailed methodology

The following section describes stepwise the detailed methodology of isolating genital epithelial cells, monitoring the cultures and inoculation with HSV-2 and HIV-1. The methods described here are for endometrial and endocervical primary epithelial cells. Primary ectocervical cells can also be isolated using a similar procedure and successfully cultured in keratinocyte growth media. However, due to infrequent availability of lower genital tract tissues, our experience with the ectocervical epithelial cultures is limited and therefore the methodology sections focus on upper reproductive tract cultures.

6.1. Tissue procurement

Endometrial and endocervical tissues were obtained from women aged 30–59 years (mean age 42.93 ± 7.2) undergoing hysterectomy for benign gynecological reasons at Hamilton Health Sciences Hospital. Informed consent was received in accordance with the approval of the Hamilton Health Sciences Research Ethics Board. The most common reasons for surgery were uterine fibroids or heavy menstrual bleeding (menorrhagia). Uterine tissues were only used if they were deemed free of malignancy or any other clinically observed pathology by the clinical pathologists. The best viability and yield of epithelial cells was obtained from tissues from pre-menopausal women, as epithelial yield and thickness tends to diminish in women over the age of 60. Endometrial tissues were on average 2–4 cm in length, 1–2 cm in width and roughly the thickness of a coin (Fig. 1A). Rather than the size of the overall tissue, the optimum yield of epithelial sheets was obtained when the tissue piece was sliced from the superficial layer of the endometrium and cervix on the luminal side of the tissue, where the epithelial layers are situated. The tissue piece was trimmed to minimize the amount of myometrium, since it does not contribute to epithelial cell yield.

Endometrial and endocervical tissues obtained from hysterectomy patients were received and processed within 24 h of surgery. The viability of the tissue was maintained as long as the tissue was placed in a 50 mL polypropylene conical Falcon tube (Becton Dickinson and Co. Franklin Lakes, NJ, USA; Cat No. 4-2098-11) containing 1× Hanks buffered salt solution (HBSS) (Invitrogen, Burlington, ON, Canada) and 100 U/mL of penicillin/streptomycin (Sigma–Aldrich, Oakville, ON, Canada; Cat No. P4333) within 2–3 h of surgery. The tube was stored at 4 °C until tissue processing commenced.

6.2. Tissue processing

Isolation of epithelial cells was initiated within 24 h of surgery. The enzyme digestion mixture was comprised of 1.5 mg/mL collagenase D from *Clostridium histolyticum* (Roche Diagnostics, Mannheim, Germany; Cat No. 11-088-882-001), 3.45 mg/mL pancreatin from porcine pancreas (Sigma–Aldrich, Oakville, ON, Canada; Cat No. P3292), 0.1 mg/mL hyaluronidase from sheep testes (Roche Diagnostics, Mannheim, Germany; Cat No. H6254) and 2.0 mg/mL D-glucose (EMD Chemicals Inc. Gibbstown, NJ, USA; Cat No. DX0145-1). Typically, an average sized tissue as described above was digested with 20 mL of the enzymatic digestion mixture prepared in 1× HBSS.

To prepare the tissue, it was removed from the Falcon tube using a scalpel and placed into a small, sterile 100 × 15 mm polystyrene Petri dish (Fisher Scientific, Ottawa, ON, Canada; Cat No. 0875712) (Fig. 1A). The tube and media that the tissue was stored in was set aside to be rinsed later, as it may contain some sheets of viable epithelial cells. A small volume (approximately 1 mL) of the HBSS was layered overtop of the tissue to insure it does not dry out while being processed. The tissue was minced into <2 mm^3-sized pieces using a scalpel and blade (Fig. 1B) and evenly divided in a total of 20 mL of the enzymatic mixture placed into the wells of a 6-well flat bottom tissue culture plate (Becton Dickinson and Co. Franklin Lakes, NJ, USA; Cat No. 35-3046) (Fig. 1C). The enzyme mixture with the tissue was incubated at 37 °C for 30 min to an hour on a plate shaker (Lab Line Instruments Inc., Mumbai, Maharashtra, India; Model No. 4625) with continuous shaking at medium speed. The tissue was monitored under an inverted light microscope every 5 min following the half hour time point to determine if the epithelial sheets could be seen separating from the tissue pieces (Fig. 1D). The enzymatic digestion was continued until there was no obvious increase in number of epithelial cell sheets under the microscope, usually between 35 and 45 min of incubation. At this point the tissues were removed from the shaker and transferred into a 50 mL Falcon tube and briefly pulse vortexed.

The sheets of genital epithelial cells were next separated from the digested tissue by passing through a series of nylon mesh filters of two different pore sizes: 250 and 20 μm (Component Supply Company, Fort Meade, Fl, USA; Cat. Nos. U-CMN-20 for 20 μm pore size and U-CMN-250 for 250 μm pore size). The mesh filters are placed in holders so that the nylon fabric is spread tight, forming a secure containment that allows for manipulation of digestion mixture without leakage. The 250 μm filter separates the epithelial sheets from digested tissue as the tissue debris is retained on top of the filter while the sheets go through the large pores into the filtrate (Fig. 1E). The second filter (20 μm pore size) separates out epithelial sheets from single cell populations, typically hematopoietic cells and fibroblasts, with the sheets retained on the top and single cells in the filtrate. The filtration assembly was placed with 250 μm-sized filter in place, in a large, sterile 150 × 15 mm Petri dish (Fisher Scientific, Ottawa, ON, Canada; Cat No. 0875714) to collect the filtrate. The contents of the 50 mL Falcon tube containing the digestion mixture were passed through the filter. An additional 50 mL of fresh HBSS solution was used to rinse out any remaining digested tissue and passed through the filter assembly. The 50 mL HBSS was dispensed slowly onto the filter, using a 10 mL serological pipette and using the pipette to scrape and wash the tissue pieces. Once the filtration and rinsing was completed, the filtrate was examined under the microscope. The filtrate in the petri dish should contain numerous sheets and clumps of columnar epithelial cells, indicating a good yield (Fig. 1F). Next, the HBSS solution that was used for storing the tissue was passed through the filter to collect any epithelial cell sheets that may be present in the storage tube. The washing of the tissue in the filters was repeated till no new clumps/sheets of epithelial cells were being released from the tissue. All filtrates were pooled. Typically we collect ~120–150 mL of filtrate and washes containing epithelial sheets.

The filtrate from the first set of filtration was then passed through a second set of filter assembly with a finer 20 μm pore size nylon mesh. The filtrate collected in the previous step was added to the second filter, 10 mL at a time. Once all the washes were passed through the filter, the epithelial sheets that remain on top of the filter were carefully harvested. The filtrate collected in the Petri dish contained all the single cell populations including single stromal fibroblasts and hematopoietic cells. A few doublets of epithelial cells also go through the 20 μm, but these are infrequent and majority of sheets are retained on the filter. To collect the epithelial sheets, the top surface of the filter was washed gently with 50 ml of 1× HBSS by gently gliding the pipette along the surface of the filter while applying gentle suction to retrieve the epithelial sheets. The sheets were collected into a new, sterile 50 mL Falcon tube.

The vast majority of the cells collected following the filtration steps described above were epithelial cells. However, in order to insure that there was no significant stromal fibroblast contamination that would hinder the formation of polarized monolayers, additional steps were taken to remove stromal cells. To achieve this, the enriched epithelial cell preparation was gently spread onto a 100 × 20 mm tissue culture dish (BD Falcon, Becton Dickinson and Co. Franklin Lakes, NJ, USA) covered and incubated for 30–45 min at 37 °C and 5% CO_2. Fibroblasts typically adhere rapidly to the plastic on bottom of the plate whereas epithelial cells do not. The adhered fibroblasts can be easily visualized under the microscope, especially after the removal of media containing epithelial cells. Following the incubation, the media in the petri dish (with epithelial cells) was carefully pipetted into a new, sterile 50 mL Falcon tube and centrifuged at 700*g* for 15 min at 20 °C with no brakes. The supernatant was decanted and the cell pellet was gently dispersed. The epithelial cells were then resuspend in primary epithelial culture medium (phenol red free Dulbecco's modified Eagle medium [DMEM/F12]; Invitrogen, Burlington, ON, Canada; Cat No. 11039-021) supplemented with 10 mM HEPES (Invitrogen, Burlington, ON, Canada; Cat No. 15630-080), 2 mM L-glutamine, (Invitrogen, Burlington, ON, Canada; Cat No. 21051-024), 100 U/mL pen/strep (Invitrogen, Burlington, ON, Canada), 2.5% Nu Serum culture supplement (Becton Dickinson and Co. Franklin Lakes, NJ, USA; Cat No. 355104), 2.5% Hyclone defined fetal bovine serum (FBS; Thermo Scientific, Fisher Scientific, Ottawa, ON, Canada) and 500 μL of 250 μM/ml fungizone (Invitrogen, Burlington, ON, Canada; Cat No.15290-018). The viability of epithelial cells was tested by Trypan blue dye exclusion. The volume of primary epithelial culture medium added to cells for final suspension depends on the cell yield. An approximate count of epithelial cells may be obtained by taking a sample volume (approximately 100 μL) and passing it gently through a 1 mL syringe a few times to disperse the epithelial sheets into single cells and then obtaining a cell count on a haemocytometer.

6.3. Seeding cultures

Epithelial cells were seeded onto and grown on Matrigel-coated (Becton Dickinson and Co. Franklin Lakes, NJ, USA; Cat No. 356235), 0.4-μm pore-size polycarbonate membrane tissue culture transwell inserts (Becton Dickinson and Co. Franklin Lakes, NJ, USA; Cat No. 353095) (Figs. 1F and 2). The transwell inserts were unwrapped and placed into a 24-well tissue culture plate (BD Falcon, Becton Dickinson and Co. Franklin Lakes, NJ, USA; Cat No. 353047) using sterile forceps. Matrigel was diluted 1:3 using ice-cold primary epithelial cell media and 60 μL of the diluted Matrigel was gently added to the top of the membrane at the bottom of transwell insert. To insure that the Matrigel coated the entire membrane evenly the plate was gently rocked back and forth a few times. After one minute of incubation at room temperature, the excess matrigel was removed using a suction vacuum, leaving a thin transparent layer of Matrigel on the transwell inserts. It is critical to work rapidly with Matrigel as it starts to thicken and gel at room temperature. We recommend all steps with Matrigel be carried out and completed on ice. Following coating, the plate containing the coated inserts was set aside for 15–20 min to allow Matrigel to cure. The epithelial cells were seeded into the apical chamber of the transwell at a density of approximately 1×10^5 cells per insert in 300 μL of primary medium. 500 μL of primary epithelial cell medium was added to the basolateral side of the well.

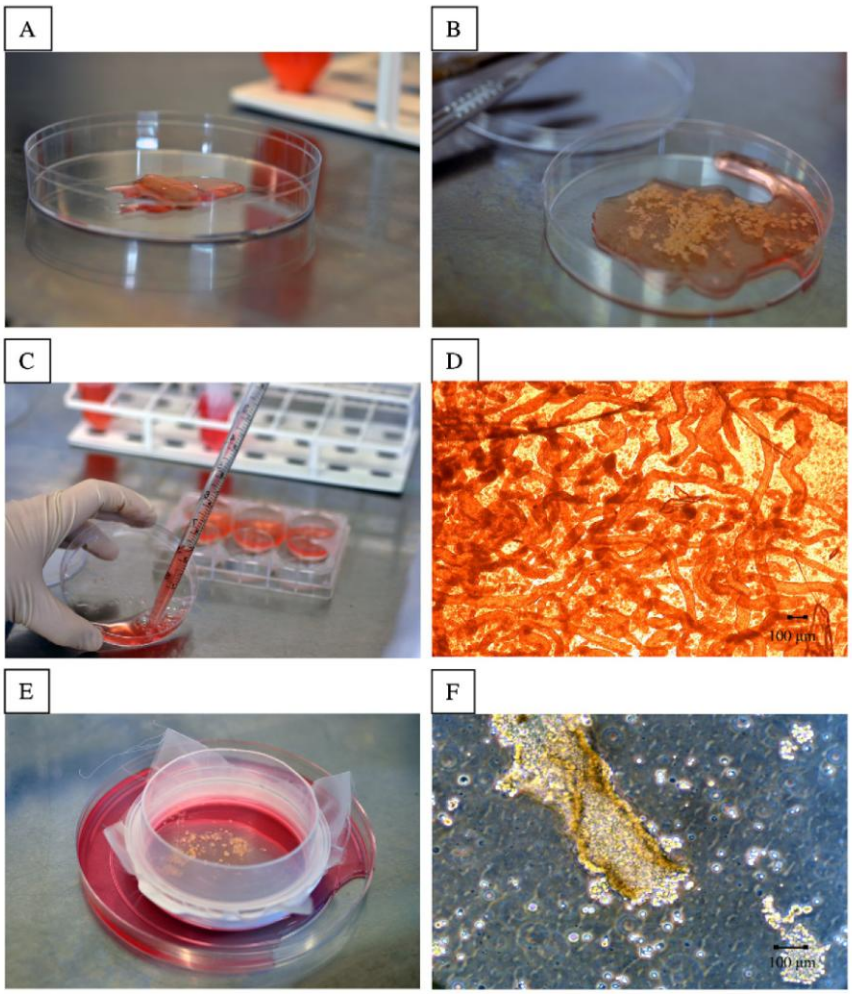

Fig. 1. Processing of endometrial tissue for isolation of genital epithelial cells. (A) Tissue pieces obtained from hysterectomy samples were transferred to a culture dish in a small volume of HBSS to keep the tissue moist. (B) Tissue was minced into smaller pieces and (C) transferred to a six well tissue culture dish with enzyme mixture and digested at 37 °C for 30–45 min. (D) The digestion mixture observed under an inverted microscope can be seen to contain abundant epithelial sheets indicating a successful digestion. Magnification 40×. (E) The epithelial sheets were separated from the tissue by passing through a 250 µm pore size nylon mesh filter followed by a second filtration through a 20 µm pore size nylon mesh filter. (F) The isolated epithelial sheets were then seeded onto a Matrigel-coated transwell insert. Magnification 100×.

6.4. Monitoring epithelial cell cultures

The first replacement of medium in the cultures is critical and the timing is determined by examining the cells microscopically. Typically within 24 h after seeding, the epithelial cells adhere to the Matrigel-coated basement membrane. The adhered cells can be observed by phase contrast microscopy and have the appearance of flattened cobble-stone pattern because of the columnar morphology. However, if the cells do not appear to have adhered to the bottom of the inserts, the addition of fresh medium may be delayed by 24 h. The spent medium was gently removed from the cultures and replaced with fresh primary culture medium (300 µL apical and 500 µL basolateral). Subsequently, medium was changed every 48 h for the duration of cultures. Polarized, confluent monolayers were typically formed within 5–10 days (Fig. 4). Confluency of epithelial cell monolayers was monitored by measurement of trans-epithelial resistance (TER) across polarized monolayers measured by a Volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA; Model EVOM, Serial No. 56734 G07E) with the STX2 chopstick electrode (World Precision Instruments, Sarasota, FL, USA). By comparing visual appearance of monolayers (by staining) with TER measurements, we established that

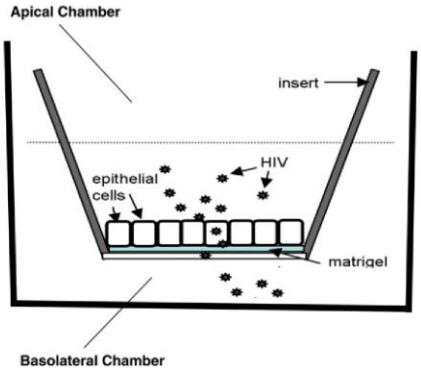

Fig. 2. Schematic diagram depicting the primary genital epithelial cell transwell insert culture system. Primary genital epithelial cells form a confluent monolayer of epithelium on a Matrigel-coated porous 0.4 μm pore size transwell filter. The monolayers become polarized giving rise to apical (upper) and basolateral (lower) compartments.

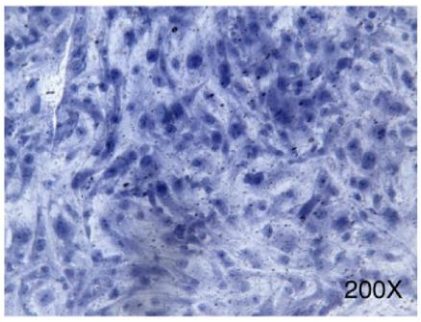

Fig. 3. Visualization of an epithelial monolayer. Primary endometrial monolayers can be visualized by staining with hematoxylin. Magnification 200×.

under these culture conditions, TER values higher than 1 kΩ/cm^2 correlated consistently with polarized, confluent monolayers and this criteria was subsequently used for determining when monolayers could be used for further experiments.

Achievement of polarized state and confluency by epithelial monolayers is a critical step and may be affected by a number of factors. The two most frequent reasons for failure to attain confluent monolayers are (1) low seeding numbers of epithelial cells and (2) significant contamination by stromal fibroblasts. Since the epithelial cells typically do not demonstrate significant proliferation under these culture conditions, seeding cells too thinly may increase the amount of time it takes for cultures to become confluent, or if the cell number is too low, it may prevent culture from becoming confluent. This can be determined by staining the monolayers and visually examining them microscopically. Typically, incomplete monolayers may be observed as patches of epithelial cells, interspersed by sparse areas on the membrane of the insert. Presence of significant number of stromal fibroblasts may also prevent the formation of a confluent, polarized monolayer. This can be identified by an almost continuous coverage of the surface of the insert membrane by epithelial cells interspersed by fibroblast patches. Fibroblast contamination can also be identified with cell-specific markers, such as vimentin, either by flow cytometry or immunohistochemistry [49].

The viability of the cultures as well as their polarization can be easily monitored by daily measurements of TERs. TERs can also be measured prior to, during and following any experimental protocol without compromising the monolayers, as long as the cultures are kept at room temperature for short periods of time. When TER values begin to decrease, the cultures will have limited survival time. The primary genital epithelial cell cultures in our culture conditions maintain high TERs for ~3 weeks before the viability of cells start to decline.

In addition to monitoring TERs, epithelial cells may be monitored by staining and visualization of the monolayers (Fig. 3). Tissue culture inserts with epithelial monolayers were stained with Harris' hematoxylin (Sigma–Aldrich, Oakville, ON, Canada; Cat No. H9627) for 1 min, followed by extensive washing with 1× PBS. Monolayers were fixed with 100% ethanol for 10 min, followed by more washing with 1× PBS. Inserts were dried for a few hours at room temperature and plastic membranes were excised from the transwell inserts and mounted onto glass slides with glass cover slips in Permount Mounting media (eBioscience, San Diego, CA, USA; Cat No. 00-4960-56). Staining for epithelial and fibroblast specific markers can also be done as described previously [49].

6.5. Immunofluorescent staining for tight junction proteins

The epithelial monolayers may also be analyzed for differentiation and confluency by immunofluorescent staining for different tight junction proteins, as described before [51] (Fig. 5). Epithelial monolayers were fixed with 4% Paraformaldehyde (Electron Microscopy Sciences Company, Hatfield, PA. US. Cat. No. 15710), permeabilized with 0.1% Triton X-100 (Sigma–Aldrich, Oakville, Canada), and blocked for 30 min in blocking solution which is composed of 5% bovine serum albumin, (Sigma–Aldrich, ON, Canada, Cat. No. A8022-500G) and 5% goat serum (Cedarlane, Burlington, ON, Canada; Cat. No. CL1200-100) in 0.1% Triton X-100 (Sigma–Aldrich, Oakville, Canada). Primary antibodies (rabbit anti-human claudin-2, rabbit anti-human occludin, or rabbit anti-human ZO-1 from Zymed Laboratories, CA, USA) were diluted (2 μg/ml) in blocking solution and incubated with monolayers for 1 h at room temperature. Normal rabbit serum was used as a negative control to check the specificity of primary antibodies. Following incubation with primary antibodies, the monolayers were washed with PBS and secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (1.5 μg/ml, Molecular Probes, Eugene, OR) was added for 1 h at room temperature. Nuclear counterstaining was done with Propidium Iodide (500 nM, Molecular Probes, Eugene, OR, USA). After extensive washing, filters were excised from the polystyrene inserts and mounted on glass slides in mounting medium (Cat. No. H-1400, Hard set Vectashield mounting medium, Vector Lab, CA, USA). All samples were imaged on an inverted confocal laser-scanning microscope (LSM 510, Zeiss, Germany) using standard operating conditions (63 × objective, optical laser thickness of 1 μm, image dimension of 512 × 512, lasers: argon (450 nm) and HeNe (543 nm) for ZO-1 and nuclear staining, respectively. Images were captured en face to illustrate the distribution of tight junction protein immunoreactivity.

6.6. Viral inoculation – HSV-2

Confluent monolayers of endometrial and cervical epithelial cells with TERs above 1 kΩ/cms^2 were used for all HSV-2 infections. Cells were infected via the apical surface with a standard inoculation dose of 10^4 plaque-forming units (pfu) of HSV-2 in a

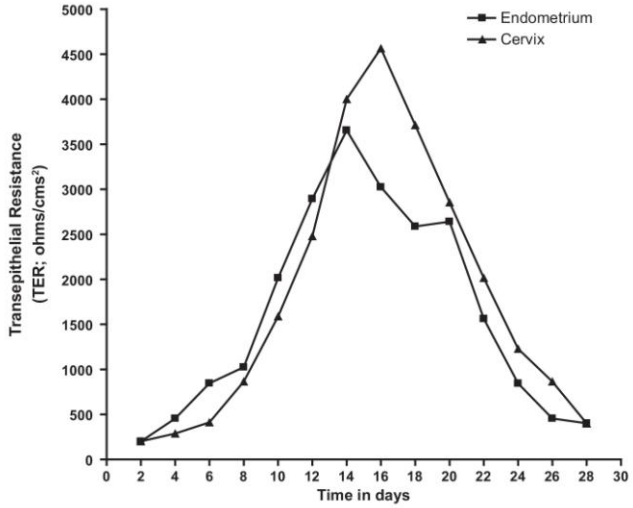

Fig. 4. Monitoring the growth and differentiation of primary genital epithelial cultures. Transepithelial resistance (TER) measurement values from Day 2 to 28 of a representative endometrial and endocervical epithelial cell culture grown on transwell inserts.

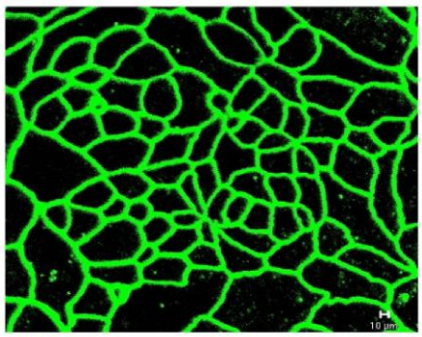

Fig. 5. Visualization of primary genital epithelial cells using immunofluorescent staining of tight junction proteins. Epithelial monolayers were fixed and permeabilized prior to incubation with antibody against tight junction protein ZO-1. After extensive washing, filters were excised, mounted on glass slides and imaged on confocal laser-scanning microscope (LSM 510). Image was captures en face to illustrate distribution of tight junction protein immunoreactivity. Magnification 200×.

volume of 100 μL of serum-free primary media (MOI of about 0.1). Following addition of the virus, the plates were gently tipped side to side to ensure that the inoculum was evenly distributed. The virus was incubated with cells for 2 h at 37 °C in 5% $C0_2$. Following this period, the inoculum was removed and the apical and basolateral chambers were washed 5 times with 500 μL of 1× PBS. Fresh primary medium was added back to the apical and basolateral chambers (300 and 500 μL, respectively). The details of HSV-2 infection kinetics and consistency of viral replication among epithelial monolayers obtained from different tissues has been previously examined [49].

Apical and basolateral supernatants were collected to measure viral shedding at 24- and 48-h post-infection and stored at −80 °C for viral titering using a standard plaque-forming Vero cell assay [53]. Intracellular replication was measured by detaching infected epithelial cells using 1× Trypsin–EDTA (Gibco, Canada; Cat. No. 15400) from the transwell inserts, 24-h post-infection, and pelleting the cells. The cell pellet underwent 2 cycles of freeze–thaw at −80 °C to lyse the cells and release the virus. The cell lysate was resuspended in 500 μL of serum free, primary media and titered using a vero plaque assay.

6.7. Viral inoculation – HIV-1

Confluent primary GECs were exposed to a standard dose of 10^5 infectious viral units (IVUs), of HIV-1 virus (R5 strain: ADA, X4 strain: IIIB), as determined by TZM-bl indicator cell assay, apically in a volume of 100 μL of serum-free primary media. The p24 values (Zeptometrix Corp., Buffalo, NY, USA) corresponding to this standard concentration were variable and depended on the viral preparation and the strain of the virus [51]. The inoculum was left on for 4 h at 37 °C in 5% Co_2. Following this period, the inoculum was removed and the monolayers were washed in the apical and basolateral chambers 5 times with 500 μL of 1× PBS. Fresh primary medium was added back to the apical and basolateral chambers (300 and 500 μL, respectively).

Our previous studies have demonstrated the effect of HIV-1 exposure on upregulation of pro-inflammatory cytokine production by primary GEC cultures. Therefore, in our studies, we use cytokine upregulation as a key readout for measuring the effect of HIV-1 on GECs. Apical and basolateral supernatants at various time points (6–48 h) were collected to measure epithelial pro-inflammatory cytokine and chemokines. Multiple cytokines were simultaneously analyzed using the Luminex 100 (Luminex Corporation, Austin, TX, USA) as previously described [28,54]. Custom made Multiplex bead-based sandwich immunoassay kits (Millipore, Billerica, MD, USA) were used to measure concentrations in

pg/ml (Ferreira et al. [52]; Nazli et al. [51]) of 8 different epithelial cell cytokines and chemokines including interleukin 1α (IL-1α), IL-6, interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), granulocyte–macrophage colony stimulating factor (GM-CSF), regulated on activation normal T cell expressed and secreted (RANTES), and TNF-α in the supernatants collected from treated epithelial monolayers. The Luminex assays were run according to the manufacturer's instructions.

7. Concluding remarks

The ex-vivo polarized primary epithelial culture system described above has proved to be useful for examining direct interactions between epithelial cells and sexually transmitted viruses. This and similar culture systems are also proving to be useful for testing the biological efficacy of microbicide candidates against HIV-1 [55]. However, as with other experimental systems, there are limitations regarding the physiological relevance of this culture system. The primary nature, homogeneity and purity of the epithelial cultures are useful characteristics for mechanistic investigation, however, under these conditions responses are measured outside the context of the microenvironment of the genital tract. Other cells and conditions in the genital tract are important considerations whose incorporation could increase the relevance of this model. This culture system can be further extended such that epithelial cells can be co-cultured with other primary cells, such as syngeneic stromal fibroblasts or peripheral blood mononuclear cells, to examine the viral interactions in a progressively complex microenvironment that recreates, albeit under defined settings, the physiological conditions in the female genital tract. Further addition of factors such as semen, female sex steroids, pathogenic or commensal microbes, all of which profoundly influence the outcome of viral exposure, into this model system could provide critical insights into the early in vivo events following the exposure to these viruses. Importantly, these cultures can provide critical information regarding the acute innate responses of these sentinel cells that are the first to encounter the sexually transmitted pathogens.

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REVIEW ARTICLE

HIV Infection in the Female Genital Tract: Discrete Influence of the Local Mucosal Microenvironment

Charu Kaushic, Vitor H. Ferreira, Jessica K. Kafka, Aisha Nazli

Center For Gene Therapeutics, Michael G. DeGroote Institute of Infectious Diseases Research, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

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Correspondence
Charu Kaushic, Center for Gene Therapeutics, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L7P4M9.
E-mail: kaushic@mcmaster.ca

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Women acquire HIV infections predominantly at the genital mucosa through heterosexual transmission. Therefore, the immune milieu at female genital surfaces is a critical determinant of HIV susceptibility. In this review, we recapitulate the evidence suggesting that several distinctive innate immune mechanisms in the female genital tract (FGT) serve to significantly deter or facilitate HIV-1 infection. Epithelial cells lining the FGT play a key role in forming a primary barrier to HIV entry. These cells express Toll-like receptors and other receptors that recognize and respond directly to pathogens, including HIV-1. In addition, innate biological factors produced by epithelial and other cells in the FGT have anti-HIV activity. Female sex hormones, co-infection with other pathogens and components in semen may also exacerbate or down-modulate HIV transmission. A combination of innate and adaptive immune factors and their interactions with the local microenvironment determine the outcome of HIV transmission. Improving our understanding of the female genital microenvironment will be useful in developing treatments that augment and sustain protective immune responses in the genital mucosa, such as microbicides and vaccines, and will provide greater insight into viral pathogenesis in the FGT.

Introduction

According to recent estimates, women constitute >50% of the 40 million people currently living with HIV worldwide.[1] In fact, the fastest growing phase of the pandemic is heterosexual transmission in women. Although vaginal intercourse carries a lower HIV transmission probability per exposure event, it contributes more new HIV cases than anal intercourse or parenteral inoculation.[2] Recent estimates suggest that 30–40% of annual worldwide HIV infections occur through heterosexual transmission via the female genital tract (FGT).[1,2] Given these statistics, it is becoming increasingly clear that a better understanding of HIV interactions in the FGT is critical to developing strategies for prevention of heterosexual HIV transmission. This review will focus on selected aspects that could influence the outcome of heterosexual exposure to HIV-1 and are unique to the local mucosal microenvironment of the FGT.

Innate Barriers in the Female Genital Tract to HIV-1 Infection

For HIV-1 to establish a productive infection in the FGT, it must first evade a number of intrinsic

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mechanical, chemical and biological barriers. The structure of the FGT forms the first line of defense against HIV. The FGT can be divided into two major compartments: the lower reproductive tract, consisting of the vagina and ectocervix, lined by stratified squamous epithelium; and the upper reproductive tract consisting of the endocervix, endometrium and fallopian tubes, lined by a single layer of columnar epithelium.[3] The tight junctions between the columnar cells of the endocervix and endometrium form a mechanical barrier, preventing pathogens from breaching the epithelium. In the vagina and ectocervix, the continuous sloughing of the superficial layers of the stratified epithelium prevents many pathogens from colonizing and establishing infections, providing a better mechanical protection against HIV invasion than the single layer columnar epithelium that lines the upper reproductive tract. However, the greater surface area of the vaginal wall and ectocervix arguably allows greater access for HIV entry, particularly when breaches occur in the epithelium, such as during sexual intercourse.[2,4,5]

Epithelial cells (ECs) of the FGT produce several biological factors that create an inhospitable environment for HIV including a hydrophilic surface layer of glycoproteins and glycolipids called the glycocalyx, and thick hydrophobic glycoprotein mucus.[6] Both the glycocalyx and the mucus act as mucosal barriers and may play a variety of important physiological functions. For example, human cervicovaginal mucus obtained from donors with normal lactobacillus-dominated vaginal flora, efficiently traps HIV, causing it to diffuse more than 1000-fold more slowly than it would in water.[7] Several innate immune proteins secreted from ECs with anti-HIV activity are also present within the secretions of the FGT. Those with established anti-HIV properties include secretory leukocyte protease inhibitor (SLPI), lactoferrin, beta (β)-defensins and trappin-2/elafin. The antileukoprotease SLPI is secreted by resident ECs and infiltrating leukocytes in the FGT.[8,9] It has been suggested to play an important role in genital mucosal defenses against HIV because of its potent ability to inhibit HIV infection *in vitro*.[10–12] Similarly, lactoferrin, a protein found in breast milk and the genital tract, has also been shown to inhibit HIV at the early stages of viral infection *in vitro*, by blocking viral adsorption and uptake.[13,14] Defensins, a family of small cationic proteins produced in the genital tract, have demonstrated significant antimicrobial effects in studies.[15,16] Alpha (α)-defensins are produced by neutrophils, macrophages and γδ T cells, whereas β-defensins are mainly produced by ECs, such as those of the genital mucosa.[17] Human β-defensin-1 (HBD-1) is expressed constitutively while HBD-2 and HBD-3 are inducible and have been shown to inhibit HIV-1, particularly X4 tropic strains.[18–20] More recently, the serine protease inhibitor, trappin-2/elafin has also been implicated in anti-HIV activity at mucosal surfaces.[21,22]

In addition to antimicrobial peptides, cells of the FGT can produce interferons (IFNs), which have a wide variety of antiproliferative, immunomodulatory and antiviral effects. Type I interferons (IFN-α, IFN-β) impede the HIV replication cycle through numerous mechanisms, including induction of the antiviral molecule apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G).[23,24] Type I IFNs also induce the production of inactive 2′,5′-oligoadenylate synthetase (2′,5′-OAS), which activates a latent endoribonuclease, RNase L, that degrades viral and cellular RNAs resulting in inhibition of protein synthesis.[25,26] The 2′,5′-OAS/RNase L pathway has been shown to inhibit the replication of HIV-1.[27,28] Protein kinase R, inducible nitric oxide (NO) synthase, myxovirus (Mx)-family proteins and 9–27 proteins are other interferon-inducible proteins shown to have anti-HIV properties.[29] A number of studies have shown the inhibitory effect of type II IFN (IFN-γ) on HIV-1 replication in monocytes, monocyte-derived macrophage and lymphocytes.[30–32] However, some studies have found a stimulatory effect of IFN-γ on HIV-1 infection.[33,34] The recently described type III IFN (IFN-λ, or IL-28/29), which has similar antiviral properties to type I IFN, has been shown to block HIV-1 infection of macrophages by the upregulation of CCR5 ligands (MIP-1α, MIP-1β), as well as anti-HIV proteins like APOBEC3G/3F and type I IFNs.[35]

The expression of Toll-like receptors (TLRs) by cells of the FGT bestows on them the ability to innately sense their environment for pathogenic motifs and rapidly relay messages to other innate and adaptive cells should a pathogenic breach occur. Vaginal and cervical EC lines express TLRs 1–3, 5 and 6, while primary endocervical ECs express TLRs 1–3 and 6.[3] Primary human uterine ECs express TLRs 1–9, indicating the potential of upper reproductive tract to respond to a wide range of pathogens. TLR-mediated activation leads to production of chemokines and cytokines, including IL-6, IL-8, SDF-1 by ECs, as well as resident immune cells in

the FGT, allowing for immediate responsiveness to pathogens.[36–39] Similarly, the β-chemokines MIP-1α, MIP-1β and RANTES are all secreted by ECs of the upper and lower genital tract constitutively and following infection.[40–42] As natural ligands for the CCR5 receptor, these may play a role in blocking R5-tropic HIV-1.

The induction of antiviral responses by activation of TLR pathways in genital ECs also provides a unique potential for utilizing TLR ligands as innate microbicides. To test this possibility, we recently examined the antiviral responses induced in genital ECs following treatment with TLR ligands. The ability of eight different TLR ligands to induce antiviral responses in genital ECs against herpes simplex virus type 2 (HSV-2) infection was determined.[43] TLR3 [poly (I:C)], TLR9 (CpG A) and TLR5 ligands (flagellin) showed the greatest ability to reduce HSV-2 replication. Poly (I:C) treatment not only induced maximum interferon-β and NO, but also enhanced production of inflammatory cytokines IL-1α, IL-6 and TNF-α. Similar effects on the inhibition of human cytomegalovirus (CMV) replication in human genital tissues were also recently reported.[44] These studies clearly demonstrate that the FGT is equipped with a number of innate defenses against reproductive tract pathogens, including HIV.

HIV interactions with epithelial barrier and target cells in the genital tract

If the intrinsic barriers of the FGT, described earlier, are overcome, HIV-1 is capable of traversing the genital epithelium and establishing an infection. HIV virions have been suggested to cross the epithelium through several pathways, including direct infection of ECs,[45] transcytosis of viral particles across the epithelium[46–48] and penetration of the virus through epithelial breaches.[4,5] HIV-1 can infect both ECs from the lower[49,50] and upper FGT.[45,48] The nature of viral entry into ECs are likely distinct from the canonical HIV-1 entry pathways as genital ECs demonstrate inconsistent or no expression of CD4 and the chemokine co-receptors CCR5 and CXCR4.[49,51,52] In lieu of these molecules, ECs may facilitate HIV transmission using cell surface glycosphingolipids, sulphated lactosylceramide expressed by vaginal ECs[53] and galactosylceramide expressed by ectocervical ECs,[52] which have been found to bind HIV-1 gp120 and foster transcytosis. Interactions of HIV-1 gp120 with transmembrane heparin sulfate molecules, such as syndecans, expressed by genital ECs may also contribute to HIV-1 attachment and entry.[47,50] A variant of salivary agglutinin named gp340, which is expressed on cervical and vaginal ECs, has also been implicated in the passage of HIV through the epithelium.[54,55] The relative contribution of these receptors to HIV entry and infection in genital ECs is unclear.

In addition to ECs, there are a number of resident immune cells in the FGT that may also contribute to HIV transmission, most notably dendritic cells (DCs) and T cells. DCs appear to play a major role in HIV transmission and dissemination, as well as driving the early inflammatory response to infection.[56] However, the relative contribution of different types of DCs is not completely understood. Langerhans cells in the cervicovaginal epithelium express CD4 and CCR5, but not CXCR4 or the surface adhesion molecule DC-SIGN, which has been shown to assist in HIV transmission at mucosal surfaces. In a study using *ex vivo* human organ culture system, HIV-1 rapidly infected both intraepithelial vaginal Langerhans and $CD4^+$ T cells. HIV-1 entered $CD4^+$ T cells almost exclusively by CD4 and CCR5 receptor-mediated direct fusion, without requiring passage from Langerhans cells, resulting in productive infection. By contrast, HIV-1 entered $CD1a^+$ Langerhans cells primarily by endocytosis, and virions persisted intact within these cells for several days without active replication.[57] In contrast, a recent study was unable to detect translocation of HIV-1 in reconstructed human vaginal mucosa, and presence of Langerhans cells did not alter HIV-1 transmission.[58] These studies suggest that the main target cells for HIV-1 are $CD4^+$ DCs and T cells in the lamina propria of genital mucosa. Consequently, the enriched population of $CD4^+$ T cells and APCs present in the transformation zone, where the ectocervix transitions into the endocervix, may be a particularly susceptible site for HIV entry.[59]

In addition to the interactions described earlier, we recently reported a novel mechanism that may allow HIV to breach the epithelial barrier of the intestinal and genital mucosae, resulting in translocation of both HIV-1 and other luminal microbes.[60] In this study, we demonstrated that HIV-1 surface glycoprotein could directly reduce transepithelial resistance, a measure of epithelial monolayer integrity, by 30–60% in primary genital epithelial and intestinal cell lines cultures. The disruption in barrier functions was associated with viral and bacterial

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translocation across the epithelial monolayers and was mediated by direct response of ECs to the envelope glycoprotein of HIV-1 seen by upregulation of inflammatory cytokines that lead to impairment of barrier functions. The increased permeability could be responsible for small but significant migration across the mucosal epithelium by virus and bacteria present in the lumen. This mechanism could be particularly relevant to mucosal transmission of HIV-1.

Hormonal contraception and altered susceptibility to HIV infection

The regulatory effects exerted by the cyclic presence of sex hormones confer the female reproductive tract with a unique microenvironment. Estradiol and progesterone play a key role in regulating physiology and functions of the FGT, including immune responses (reviewed in[3]). Therefore, the use of hormonal contraception needs to be carefully studied, as long-term, or even short-term administration of these hormones may have far-reaching effects on host responses. Long-acting, progesterone-based contraceptives, such as depot medroxy-progesterone acetate, are highly effective and currently used by more than 100 million women worldwide.[61] Multiple studies in humans and rhesus macaques suggest that the use of progesterone-based formulations may predispose one to increased risk of HIV-1 or SIV infection, higher viral burden and increased viral shedding.[62–64] A recent study further suggested that women who use progesterone-based contraceptives display accelerated HIV-1 disease progression and mortality, compared to women who do not.[65] Although the pathways involved in these outcomes are not clear, progesterone is known to regulate a number of immunological pathways, including the inhibition of CTLs and natural killer cells.[66–68] It also decreases the production and alters glycosylation of IgG and IgA antibodies, modulates cytokine production and upregulates HIV-1 receptor expression on $CD4^+$ T cells.[69,70] In contrast to progesterone, estrogen and its derivatives may exert a strong protective effect against HIV-1 infection.[71] Systemic administration of estrogen in the form of subcutaneous implants protected against intravaginal challenge of ovariectomized female rhesus macaques with highly pathogenic SIV_{MAC251}.[72] While these studies suggest that hormone-based contraceptives may perturb the mucosal environment resulting in altered HIV-1 susceptibility, the mechanisms underlying these observations need to be clearly elucidated. This is because several studies have failed to observe an overall effect of hormonal contraception on the incidence of HIV-1 infection,[73,74] while other studies report increased risk of infection, but only in subgroups of subjects differing in age and HSV-2 status.[75,76] Interpretation of these studies is complicated by multiple factors (type, dose and method of administration of hormonal contraceptives) and design of studies (cross sectional, longitudinal).

To gain a better understanding of mechanism by which hormones regulate susceptibility in the FGT, we have conducted studies in a model of genital herpes infection. Using this model, we demonstrated that long-acting progestational formulation Depo-Provera increased susceptibility to genital HSV-2 infection by 100-fold.[77] Further studies indicated that longer progesterone treatment regimes resulted in poor mucosal immune responses and increased susceptibility.[78] In other studies, mice were ovariectomized and treated with exogenous estradiol and progesterone prior to primary infection with genital herpes or immunization with attenuated strain of herpes virus. The results from these studies indicate that estradiol treatment regulates susceptibility while progesterone treatment leads to increased chronic inflammation and pathology.[79,80] Further support for the role of estradiol in controlling pathology comes from a more recent study where mice immunized with attenuated HSV-2 via subcutaneous or intranasal route under the influence of estradiol, developed remarkably decreased pathology compared to progesterone-treated mice, following genital challenge.[81] These findings were recently confirmed using an HSV-2 vaccine formulation.[82]

Co-infections in the female genital tract

Sexually transmitted infections (STI) and other genital infections have been associated with increased HIV genital shedding, transmission and susceptibility.[83,84] These may include CMV, gonorrhea, syphilis, bacterial vaginosis, candidiasis and genital herpes. Bacterial STIs, such as Chlamydia and gonorrhea have been epidemiologically associated with increased subsequent HIV acquisition and, by extension, with increased sexual transmission of HIV.[85,86] The increased HIV susceptibility may relate to local micro-ulcerations because of the pathologies associated with the infection or to the local recruit-

ment of activated immune cells, which may act as targets for HIV.[87] HSV-2 is one of the most prevalent STIs, infecting 20–30% of sexually active adults in North America and over 70% in sub-Saharan Africa.[88] A recent meta-analysis demonstrated HSV-2 infection to be associated with a threefold increase in susceptibility to HIV by both men and women from the general population.[89] Part of this increased susceptibility is more likely attributed to HSV-2-induced ulcerations, which creates a breach in the physical barrier of the genital epithelium.[90] Genital HIV-1 shedding is also markedly increased during clinical HSV-2 reactivations, accompanied by an increase in HIV-1 plasma viral load.[91] Other studies have shown that, among individuals shedding both viruses, the amounts of viral shedding are closely related.[92] Herpetic lesions and possibly asymptomatic HSV-2 mucosal shedding generates an influx of activated $CD4^+$ T cells that persist for months after healing, which may facilitate the transmission of HIV.[93] HSV-2 replication is also associated with a 10-fold increase in the number of immature DCs expressing DC-SIGN and a threefold increase in CCR5 expression on $CD4^+$ T cells.[94] Therefore, it is possible that HSV-2 infection may increase the number of HIV-target cells in the FGT, facilitating the transmission of HIV. Certain immediate early proteins of HSV-1 such as infected cell protein (ICP)-0 and ICP4 have been shown to interact with the HIV-1 LTR to induce HIV replication,[95] suggesting that HSV can directly upregulate HIV replication. Recent observations in our laboratory show that genital ECs infected with HSV-2 or treated with an array of TLR ligands, representative of various bacterial and viral pathogens, are capable of inducing HIV replication, directly and indirectly (V.H. Ferreira and C. Kaushic, unpublished). Altogether, these studies demonstrate that coinfections may directly or indirectly enhance HIV acquisition and transmission.

Influence of semen on HIV transmission

Semen represents the main vector of HIV dissemination, as transmission occurs more efficiently from men to women, and men to men than from women to men.[96] It is composed of cells and secretions from the testes, epididymis, prostate, seminal vesicles and bulbourethral gland, and it has been reported to enhance HIV infection.[97] A number of components of seminal fluid, whose physiological purpose is to protect spermatozoa, also protect HIV virions. For example, basic amines such as spermine, spermidine, putrescine and cadaverine commonly protect both spermatozoa and HIV virions from the threat of acid inactivation in the vaginal tract.[97] In recent studies, fractionation of semen from healthy donors has led to the identification of a semen-derived enhancer of viral infection (SEVI). SEVI consists of amyloid fibrils composed of internal 34–40 amino acid proteolytic fragments from prostatic acid phosphatase, a protein present at a concentration of approximately 1–2 mg/mL in semen that can enhance HIV infection up to 105-fold in cell culture.[97,98]

Semen also plays an active role in transforming the molecular and cellular environment of the FGT. Studies suggest that vaginal epithelium secretes the chemokine CCL20 in response to seminal plasma, which enhances recruitment of Langerhans cells to the vaginal mucosa. This may facilitate the transport of virions across the vaginal epithelium barrier to the lymph nodes.[99] It was also found that the HT-29 human EC line was sensitive to HIV-1 in the presence of whole semen resulting in a two-fold increase in infectivity.[100] Others have shown that seminal plasma can upregulate expression of proinflammatory cytokines in human genital epithelium raising the possibility of the role of seminal plasma in enhancing STI, including HIV-1, in the FGT.[101,102] Transforming growth factor β1 (TGF-β1) concentration in human seminal plasma is one of the highest measured in biological fluids.[103] When deposited into the FGT, TGF-β in seminal plasma plays both proinflammatory and immunosuppressive roles in preparing the FGT for the conceptus.[103] We have recently observed that semen from men infected with HIV may contain different concentrations of TGF-β, depending on the stage of infection, and this in turn may induce differential responses in the FGT (J.K. Kafka and C. Kaushic, unpublished).

Conclusions

The FGT is a unique mucosal environment and a key target site for heterosexual HIV-1 transmission. The mucosal ECs are key sentinels that serve the dual function of forming the primary barrier as well as being the first responders to HIV-1 in the event of a breach. Recent studies show that these cells express TLRs and other receptors that can respond directly and rapidly to HIV-1 exposure. Several other biological factors secreted in the FGT, such as SLPI, lactoferrin, defensins and trappin/elafin, provide intrinsic protection against HIV-1 infection. Discrete

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factors such as female sex hormones, other co-infections and semen may be present differentially in the microenvironment of the FGT and play a key role in determining the susceptibility of the host. The combination of these and other protective mechanisms and factors that confer susceptibility in the FGT likely determine the net outcome of HIV-1 exposure. Understanding the FGT microenvironment and its interactions with HIV-1 can assist in the development of better strategies to enhance innate and adaptive immunity and develop novel methods to prevent HIV-1 infection.

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