DEVELOPING AND TESTING *IN* VIVO MODELS TO EXAMINE THE EFFECT OF INFLAMMATION AND BARRIER FUNCTION IN THE FEMALE GENITAL TRACT IN THE CONTEXT OF SEXUALLY TRANSMITTED VIRUSES

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Abstract

A number of factors including epithelial barrier function, mucosal inflammation and HSV-2 infection are known to affect the early events of HIV transmission and pathogenesis in the female genital tract (FGT). We previously demonstrated that curcumin, a highly pleiotropic anti-inflammatory natural compound, can inhibit cytokine and chemokine production, epithelial barrier disruption and HSV-2 replication *in vitro*. Thus, the goal of the present study was to investigate the *in vitro* to *in vivo* translatability of the aforementioned study and the ability of curcumin to prevent mucosal inflammation and HSV-2 replication in a mouse model. The second objective of this study was to further our understanding of the genital epithelial barrier *in vivo* and develop an assay system that would allow for the experimental determination of *in vivo* genital barrier function. The work summarized in this thesis has furthered our understanding of the genital microenvironment and its relevance to HIV infection.

Our results show that while crude curcumin decreased HSV-2 infection, it was most likely due to a physical barrier effect. Further examination with curcumin nanoformulations demonstrated that it was able to potently decrease TLR-induced inflammation in the FGT, particularly with direct application to the genital tissue, but not HSV-2 infection. We also established a simple, reliable and reproducible method to functionally assess changes in genital barrier function by measuring concentrations of dye in the blood following vaginal delivery. Using this system, we have demonstrated that Depo-Provera, a widely used progestin-based hormonal contraceptive that has been linked to HIV risk, severely reduces genital barrier function *in vivo*. This work

emphasizes that inflammation and barrier function are critical avenues that should be explored in the development of more effective prevention and treatment strategies for HIV infection. Understanding causes of inflammation and barrier breakdown will also prove valuable in guiding the use of safer hormonal contraceptives and vaginal healthcare products in high risk women.

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- D

Declaration of Academic Achievement

All experiments were conceived and designed by Danielle Vitali and Dr. Charu Kaushic. Danielle Vitali performed all experiments. Danielle Vitali wrote this dissertation with contributions from Dr. Charu Kaushic.

Preface

This thesis is original, unpublished, independent work by Danielle Vitali. It is prepared in the "sandwich" format outlined in the "Guide for the preparation of Master's and Doctoral Theses" available through McMaster University's School of Graduate Studies. Chapter 1 of this thesis serves as a general introduction. Chapter 2 describes the rational, hypothesis and aims. The body of this thesis consists of 2 chapters (Chapters 3-4), each of which are independent studies that have not yet been submitted for publication at the time of the thesis submission. Chapter 3 is a manuscript entitled "Examining the anti-inflammatory and anti-viral effects of intravaginal curcumin delivery," and Chapter 4 is a manuscript entitled "Depo-Provera leads to significant decrease in *in vivo* mucosal barrier functions." The appendix sections corresponding to each chapter contain additional data gathered during the duration of the study that are not included in the manuscripts. The preamble section preceding each chapter describes the contributions of other authors to the multi-authored works. Finally, the discussion (Chapter 5) and conclusion (Chapter 6) sections summarize the conclusions of this thesis and draw out the overall implications.

Table of Contents

List of Figures

CHAPTER 3: EXAMINING THE ANTI-INFLAMMATORY AND ANTI-VIRAL EFFECTS OF INTRAVAGINAL CURCUMIN DELIVERY

CHAPTER 3: APPENDIX

List of Abbreviations

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
Ab	Antibody
AIDS	Acquired immune deficiency syndrome
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
BV	Bacterial vaginosis
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CpG ODN	CpG oligodeoxynucleotides
CVL	Cervico-vaginal lavage
CXCR	CXC-Chemokine receptor
CCR	C-Chemokine receptor
CS	Cellulose sulfate
CTL	Cytotoxic T lymphocyte
COX-2	Cyclooxygenase-2
DC	Dendritic cell
DMEM	Dulbecco's modified essential media
DMAP	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
DSG1α	Desmoglein-1α
DSC1	Desmocollin-1
E2	(17)β-estradiol
EC	Epithelial cell
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FGT	Female genital tract
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GECs	Genital epithelial cells
Gp120	HIV glycoprotein 120
GRAS	Generally regarded as safe
HAART	Highly active antiretroviral therapy
H&E	Hematoxylin & Eosin
HESN	Highly exposed persistently seronegative
HPV	Human Papilloma Virus
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen – antigen D related

HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IFN	Interferon
I-FABP	Intestinal fatty acid binding protein
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IP-10	Inflammatory protein-10
IVAG	Intravaginal
LPS	Lipopolysaccharide
LRT	Long terminal repeat
MCLR	Mannose dependent C-type lectin receptor
MCP-1	Monocyte chemotactic protein-1
α-MEM	Minimum essential media
MIG	Monokine-induced by interferon
MIP	Macrophage inflammatory protein
MIP-1α	Macrophage inflammatory protein-1 Alpha
MIP-1β	Macrophage inflammatory protein-1 Beta
MMP	Matrix metalloproteinase
MPA	Medroxyprogesterone acetate
MPT	Multipurpose prevention technology
NET-EN	Norethisterone enanthate
NFκB	Nuclear factor-kappa B
NOD	Nucleotide oligomerization domain
N-9	Nonoxynol-9
NRLs	NOD-like receptors
NK	Natural killer cells
NS	Not significant
OVX	Ovariectomy
P4	Progesterone
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PIC	Pre-integration complex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLGA	Poly(lactic-co-glycolic acid)
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptors
PVP	Polyvinylpyrrolidone
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RPM	Revolutions per minute
SDF-1	Stromal cell derived factor-1
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus

SOCS3	Suppressor of cytokine 3 signaling
SREBP-1c	Sterol regulatory element-binding protein-1c
STAT3	Signal transducer and activator of transcription 3
STIs	Sexually transmitted infections
TER	Trans-epithelial resistance
Th17	T helper type 17
TGF-β	Tumor growth-factor-β
TLRs	Toll-like receptors
TNF-α	Tumor growth-factor-α
Treg	T regulatory cells
UNAIDS	United Nations join program on HIV/AIDS
WHO	World Health Organization
WT	Wildtype

CHAPTER 1: INTRODUCTION

1.1 The Human Immunodeficiency Virus (HIV)

1.1.1 Epidemiology and Life Cycle

Human Immunodeficiency Virus (HIV) infection remains one of the most serious health challenges in the world, with over 35 million people worldwide living with HIV currently (Cohen et al., 2008). The prevalence of HIV increased rapidly during the 1980s, slowed down in the mid-1990s, and began to rise again in the late 1990s because of new HIV infections and fewer deaths due to effective therapeutic options. Currently, there is an estimated 75,000 Canadians living with HIV, cumulating in a prevalence rate of 212 per 100,000 people (Canada, 2014). The burden of the HIV epidemic continues to vary considerably between countries and regions. Sub-Saharan Africa remains the most severely affected region, and there are an estimated 24.7 million people living with HIV in sub-Saharan Africa, nearly 71 percent of the global total (UNAIDS, 2014). HIV causes destruction of immune cells, which results in susceptibility to a wide range of infections and diseases (Barre-Sinoussi et al., 2013). The most advanced stage of HIV infection is Acquired Immunodeficiency Syndrome (AIDS) and can develop between 2 to 15 years following initial infection with the virus. Genetically, HIV contains single-stranded RNA that is converted into double-stranded DNA within the host cell by a virally encoded reverse transcriptase (Barre-Sinoussi et al., 2013). Viral DNA is then imported into the cell nucleus and integrated into cellular DNA by virally encoded integrase and host co-factors. Once integrated, the virus may become latent and produce new RNA genomes and viral proteins that are packaged and released from the cell to begin a new replication cycle.

1.1.2 Increased Susceptibility in Women

Interestingly, the fastest growing phase of this pandemic is currently by heterosexual transmission in women (UNAIDS, 2016). In Canada, between 1999 and 2009, the number of women living with HIV increased from 12 to 27 percent of the national total (Canada, 2014), and according to the World Health Organization and the United Nations Joint Program on HIV/AIDS, women comprise 52 percent of all people living with HIV across the world (UNAIDS, 2014). In fact, the female genital tract mucosa is a major portal for entry of HIV into the body, where 40 percent of global HIV infections are thought to initiate following exposure during heterosexual intercourse (Hladik and McElrath, 2008). The number of new HIV infections reported in women has consistently increased over the past few decades, and clinical and epidemiological studies have consistently found that women are disproportionately more susceptible to acquiring HIV upon exposure compared to men (UNAIDS, 2014; Wira and Fahey, 2008). Women also tend to acquire HIV at a much younger age than men, with women aged 15 to 24 being 8 times more likely to be HIV-positive compared to men of the same age (Dellar et al., 2015). Adolescent girls and young women are at particularly high-risk of infection. Almost 60 percent of all new HIV infections in young people occurred among adolescent girls and young women (UNAIDS, 2014), and they currently make up approximately 20 percent of the global demographic of new HIV infections (UNAIDS, 2016). The gender imbalance is even more pronounced in geographical areas with higher HIV prevalence, such as sub-Saharan Africa, where women account for almost 56 percent of the total number of people living with HIV and heterosexual sex is the predominant mode of viral transmission (UNAIDS, 2016). Infection

rates here among young women are twice as high as among young men (UNAIDS, 2014). While social, economic and behavioral factors contribute to the increased prevalence in women, less attention has been paid to biological reasons underpinning this increased incidence.

1.1.3 Women and HIV

Despite that the majority of HIV infections in women occur because of heterosexual intercourse with an infected male partner, the precise mechanisms of sexual transmission in the female genital mucosa remain elusive (Ferreira et al., 2014). Heterosexual transmission models suggest that HIV in the male ejaculate must first overcome numerous innate and adaptive immune factors, such as antibodies, mucus and anti-microbial peptides, in the vaginal lumen, transverse the mucosal barrier *via* tears or transcytosis, and ultimately infect the underlying target cells in the female genital tract (Carias et al., 2013). Here, the virus establishes a small founder population of productive infection that then expands systemically, likely *via* an influx of newly recruited target cells caused by an upregulation of inflammatory factors (Haase, 2011). Interestingly, cases of new HIV infection suggest that only one or a few virus variants are responsible for establishing productive infection in the newly infected partner (Derdeyn et al., 2004). There remains to be a clear consensus on the primary location of HIV entry in the female genital tract and the etiology of epithelial penetration by HIV. Furthermore, despite being the predominant site of HIV acquisition globally, the acute events that follow HIV exposure in the female genital tract remain unclear, and it has become increasingly apparent that more knowledge of the

microenvironment in the female genital tract is critical for developing strategies for the prevention of HIV transmission in women (Ferreira et al., 2014). Therefore, in the present study, we used mouse models to improve our understanding of the mucosal microenvironment in the female genital tract. Moreover, we addressed how factors such as inflammation and barrier function can affect susceptibility and these models can be used to examine new windows of opportunity in the development of HIV prevention strategies.

1.2 Virology

HIV is a member of the genus *Lentivirus,* part of the family *Retroviridae,* and is a single-stranded, positive-sense enveloped RNA virus surrounded by a viral envelope (Viruses, 2002a, b). HIV uses CD4, a glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages and dendritic cells, as its primary receptor to gain entry into host cells. The main co-receptors of HIV are CXC-chemokine receptor 4 (CXCR4) and CC-chemokine receptor 5 (CCR5) (Feng et al., 1996; Weiss, 2013), and binding to these chemokine receptors induces conformational changes that are necessary for the entry of X4-tropic HIV-1, which relies on CXCR4, and R5-tropic HIV-1, which relies on CCR5, into host cells (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). Upon binding to the CD4 receptor and co-receptors, the viral envelope undergoes fusion with the host cell membrane (Barre-Sinoussi et al., 2013). The viral capsid is eventually uncoated and HIV RNA and protein is released into the cytoplasm of the host cell (Barre-Sinoussi et al., 2013). A virally encoded reverse transcriptase then converts the single

stranded HIV RNA into double-stranded DNA and following formation of the pre-integration complex (PIC), the viral DNA is then imported into the cell nucleus (Barre-Sinoussi et al., 2013). Once in the nucleus, the viral DNA is integrated into the host cell DNA by virally encoded integrases and host c-factors (Barre-Sinoussi et al., 2013). Once integrated, the virus may be subsequently transcribed and translated to produce new RNA genomes and viral proteins that translocate to the cell surface to assemble into new immature virus particles at the plasma membrane (Barre-Sinoussi et al., 2013). These viruses are then released from the cell and undergo maturation. During the maturation process, protease enzymes cleave the structural polyprotein to form mature Gag proteins (Barre-Sinoussi et al., 2013). This results in the production of new infectious virions and the HIV life cycle repeats when the newly infectious viral particles find new CD4+ susceptible host cells.

1.3 The Female Genital Tract (FGT)

1.3.1 Anatomy of the Female Genital Tract

The FGT contains several endogenous barriers that provide protection against sexually transmitted infections. It can be divided into two main compartments: the lower FGT and the upper FGT (Marieb, 2012). The lower FGT consists of the vagina and the ectocervix, while the upper FGT consists of the endocervix, uterus, Fallopian tubes and ovaries (Marieb, 2012). The uterus connects to the ovaries through the Fallopian tubes, and the vagina connects to the uterus through the cervix (Marieb, 2012). The vagina also meets

the external organs of the FGT collectively referred to as the vulva, which includes the labia, clitoris and urethra (Marieb, 2012).

1.3.2 Mucosal Immunity in the Female Genital Tract

The mucosal immune system of the FGT is unique among all the mucosal sites of the human body. Similar to the intestinal and pulmonary mucosa, it is one of the first lines of defense against potential pathogens, however, the genital mucosa has also evolved to accept an immunologically distinct fetus. Female sex hormones estradiol (E2) and progesterone (P4) have a marked immunoregulatory influence on the FGT to balance pathogen defense with reproductive success, and play a role in coordinating immune cell phenotype and function with the regulation of the menstrual cycle and pregnancy (Wira et al., 2015). To support reproductive success, a pattern has evolved in which tissue specific aspects of innate, humoral and cellular immunity are either enhanced or suppressed in the upper and lower FGT in coordination with hormonal fluctuations throughout the menstrual cycle (Wira et al., 2015). For example, during the P4-high secretory phase of the cycle, uterine cytotoxic T lymphocyte (CTL) activity and natural killer (NK) cell cytotoxic activity are suppressed whereas innate components are enhanced (Kalkunte et al., 2008; White et al., 1997; Wira et al., 2015). While the resulting immune changes optimize the environment for successful implantation in the upper FGT, they may also increase the risk of acquiring sexually transmitted infections (STIs) at this point in the menstrual cycle, known as a "window of vulnerability" (Wira and Fahey, 2008; Wira et al., 2015).

1.3.3 Genital Epithelial Cells of the Female Genital Tract

Genital epithelial cells (GECs) that line the FGT form the first line of defense against pathogen entry, and sexually transmitted organisms must breach this mucosal barrier to establish productive infection (Reis Machado et al., 2014). In addition to acting as a physical barrier, they secrete mucus, anti-microbial peptides, and inflammatory factors such as cytokines and chemokines (Reis Machado et al., 2014). The upper FGT and the upper portion of the cervix are lined by a simple columnar epithelium, a monolayer which relies on the presence of tight junction proteins, such as occludin and claudin-1, to seal the intercellular space between adjacent cells and prevent pathogens from breaching the internal milieu (Blaskewicz et al., 2011). Conversely, the lower FGT and the lower portion of the cervix are lined by a stratified squamous epithelium, which relies on the presence of multiple epithelial layers to provide a protective barrier (Blaskewicz et al., 2011). The most apical layers of the lower FGT are terminally differentiated and devoid of cell to cell adhesion junctions, whereas the basal layers are metabolically active and contain the most robust adhesion junctions (Anderson et al., 2014). Despite the lack of tight junctions, continuous sloughing of dead apical layers of the lower FGT helps prevent many pathogens from colonizing or establishing infection.

1.3.4 Target Cell Distribution in the Female Genital Tract

The prevalence of CD4+ T cells, macrophages, dendritic cells (DCs) and Langerhans cells throughout the FGT has been well characterized in humans and macaques (Reis Machado et al., 2014; Shacklett and Greenblatt, 2011). Both dendritic cells and

macrophages are sentinels of the mucosal immune system, responsible for bridging the innate response with the initiation of adaptive immunity (Janeway, 2012). The endocervix is the region with the highest density of CD4+ cell populations within the superbasal layers of the squamous epithelium (Pudney et al., 2005). Langerhans cells, on the other hand, are vaginal DCs that are primarily found within the superbasal layers of the squamous epithelium and at the stromal-epithelial interface of the vaginal and ectocervix, known as the transformation zone (Miller and Shattock, 2003). Notably, the ectocervix has a slightly higher density of Langerhans cells compared to the vagina (Miller and Shattock, 2003). CD4+ T cells are most prevalent in the parabasal layers of epithelium in the cervical transformation zone and surrounding tissue, and few are contained in the vaginal tissue (Xu et al., 2013). Studying the distribution of target cell populations as well as the functional morphology of the genital epithelium can offer considerable insight into the primary location and mechanism of HIV entry into the FGT during transmission.

1.4 HIV in the Female Genital Tract

1.4.1 Site of HIV Transmission in the Female Genital Tract

HIV acquisition in women as a result of heterosexual intercourse is frequently referred to as vaginal transmission, however the actual anatomical sites where HIV crosses the mucosal barrier and establishes infection in the FGT is still widely debated (Kaushic, 2011). While the entire FGT may be vulnerable to HIV infection, the majority of infections likely occur through transcytosis of the simple columnar epithelium of the endocervix or

through tears in the stratified squamous epithelium of the ectocervix and vagina (Carias et al., 2013; Rodriguez-Garcia et al., 2013). Compared to the squamous epithelium, the columnar epithelium of the endocervix and transformation zone, between the upper and lower genital tract epithelium, has been proposed as the favored sites for HIV transmission (Carias et al., 2013). This is largely because it is composed of a single cell layer with a thickness of only 10 to 30 μm, placing the virus at closer proximity to intraepithelial and submucosal target cells (Carias et al., 2013). However, it comprises only a small portion of the total surface area exposed to virus-containing ejaculate and is coated with a protective layer of mucus that may impede the efficiency of viral interaction and epithelial penetration (Carias et al., 2013). In contrast, the squamous epithelium of the ectocervix and vagina is a thick multilayered structure that was thought to provide a substantive physical barrier against HIV invasion when healthy and intact (Shattock and Moore, 2003). Nevertheless, it lines the primary area of the FGT that comes in contact with seminal fluid containing infectious virus and comprises the majority of the FGT exposed surface area that would arguably present greater access sites for HIV entry, particularly when microtears occur in the epithelium during sexual intercourse (Hladik and McElrath, 2008). In fact, Carias *et al* (2013) eloquently showed that the lower FGT is more susceptible to HIV penetration that previously thought, demonstrating in *ex vivo* human cervical tissue that virus can penetrate a much as 10 μm into the squamous epithelium (Carias et al., 2013). In areas where epithelial integrity was compromised and cellular junctions were either absent or degraded, virus could readily interact with or penetrate the vaginal or ectocervical epithelium through diffused percolation (Carias et al., 2013). While the exact site of HIV penetration into the

underlying submucosa remains unclear, this knowledge could aid in development of more specific and therefore efficacious therapeutics that target the site of transmission within the FGT.

1.4.2 Role of Target Cells in HIV Infection in the Female Genital Tract

Regardless of the anatomical site of infection within the FGT, timed inoculation and viral inactivation studies in macaques have demonstrated that the virus crosses the mucosal barrier and establishes infection within 60 minutes of intravaginal exposure (Hu et al., 2004). 18 hours later, infected cells can be found in the draining lymph nodes (Hu et al., 2000). Possible mechanisms for transmission across the epithelium of the FGT involves viral interaction with HIV target cells, and presumably regions of higher target cell density are more susceptible to infection (Xu et al., 2013). Langerhans cells within the superbasal layers of the squamous epithelium and at the transformation zone may quickly acquire and transport virus to underlying susceptible cells in the initial minutes or hours of infection (Hu et al., 2000; Hu et al., 1998; Marx et al., 1996). While these cells do not express HIV receptors CD4 or CCR5, they do express HLA-DR (Poonia et al., 2006), CD1a and a number of mannose dependent C-type lectin receptors (MCLRs) that can facilitate viral attachment (Geijtenbeek et al., 2000; Hu et al., 2004; Turville et al., 2004). Most notably, dendritic processes from the Langerhans cells can extend through the genital epithelium to the vaginal lumen, whereby they sample luminal antigen and potentially capturing HIV virions residing in the vaginal space (Hu et al., 1998; Parr et al., 1991; Xu et al., 2013).

Once the virus has been captured, the immature DCs then migrate and present the virus to the underlying CD4+ and CCR5+ T cells in the underlying submucosa (Xu et al., 2013).

CD4+ T cells are almost exclusively infected in the acute phase of SIV infection and are among the early targets for destruction (Poonia et al., 2006; Veazey et al., 2003). Although those that reside within the vaginal mucosa are normally found in the parabasal layers of epithelium, the upper layers are devoid of tight junctions, and as a result, virions can freely penetrate and reach the CD4+ T cells among the deeper layers of epithelium (Blaskewicz et al., 2011). Whether CD4+ T cells are infected directly with HIV or following presentation by Langerhans cells in the cervicovaginal mucosa is unclear. The majority of studies extrapolate the dynamics of these interactions from tissue sections but are limited as this represents only a single 'snap shot' in time (Xu et al., 2013). It is likely that genital tract acquisition of HIV may take place at a variety of different tissues, and increasing our understanding of the mechanism associated with the early events of HIV infection in both the lower and upper FGT will provide valuable information that can be used to design effective prophylactic therapeutics.

1.5 HIV Transmission

1.5.1 The Epithelial Innate Immune Response

What is exceedingly clear is that increased mucosal inflammation enhances the rate of sexual transmission of HIV in the FGT (Haase, 2011; Kaul et al., 2011; Naranbhai et al.,

2012). Inflammatory responses at mucosal sites are largely initiated by the GECs, which produce an array of innate immune mediators, including chemokines, cytokines and anti-microbial peptides, that trigger cell recruitment and homing to the initial site of infection (Janeway, 2012; Kaushic, 2011; Wira et al., 2010). Epithelial cells express pattern recognition receptors (PRRs), specifically retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and Toll-like receptors (TLRs), which are the best characterized among all PRRs for pathogen detection (Kawai and Akira, 2007). TLRs are transmembrane or cytoplasmic proteins that recognize evolutionarily conserved patterns present in microorganisms, including bacteria, fungi, protozoa and viruses. Such patterns are classified as pathogen-associated molecular patterns (PAMPs). Thus far, 10 different TLRs have been identified in humans that comprise both extracellular and intracellular receptors. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are surface-expressed and mainly recognize extracellular microorganisms, whereas TLR3, TLR7, TLR8 and TLR9 are intracellular, localized into cytosolic endosomal compartments, and recognize microorganisms that have already crossed the cell membrane barrier (Kawai and Akira, 2007). TLR1, TLR2, and TLR4 recognize bacterial and fungal PAMPs, while TLR3 recognizes double-stranded viral RNA (Kawai and Akira, 2007). TLR7 and TLR8 recognize single-stranded viral RNA, and TLR9 binds to unmethylated CpG DNA (Janeway, 2012). With all TLRs, ligand binding induces a signaling cascade that ends in the expression of genes related to the production of pro- and anti-inflammatory factors, such as NF-κB, cell survival and proliferation, immune cell activation, interferons and anti-microbial products.

Variable TLR expression throughout the FGT supports the delicate balance between local homeostasis, fertility and inflammation in response to potential pathogens (McClure and Massari, 2014). TLR1 to TLR6 and TLR9 are expressed on vaginal epithelial cells (Fazeli et al., 2005; Pivarcsi et al., 2005). Interestingly, TLR4 expression has been shown to decline progressively along the genital tract, with the highest levels of expression in the fallopian tubes and endometrium, following by the cervix. In the vaginal tract, the MD2 accessory molecule of TLR4-signaling is notably absent, and as a result, TLR4 does not function (Nasu and Narahara, 2010). Conversely, TLR4 expression is not fully ascertained in epithelial cells of the upper FGT (Hart et al., 2009). Different patterns of cytokine and chemokine secretions characterize TLR-dependent responses. In the FGT, constitutive secretion of the following cytokines and chemokines is observed, likely due to constant exposure to commensal microbiota: granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-α (TNF-α), interleukin -1, 6, 8, leukemia inhibitory factor (LIF), stromal cell derived factor (SDF-1), transforming growth factor-β (TGF- β), macrophage inflammatory protein-1β (MIP-1β), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) (Fahey et al., 2005; Grant-Tschudy and Wira, 2005; Kayisli et al., 2002; McClure and Massari, 2014; Ochiel et al., 2008; Schaefer et al., 2005). During pathogen exposure, most cytokines and chemokines are secreted apically into the luminal compartment to attract immune cells to the mucosal surface where the pathogen is detected (Schaefer et al., 2005). Our lab has demonstrated that HIV glycoprotein 120 binds to TLR2 and TLR4, as well as heparin sulfate moieties, on the surface of primary genital epithelial

cells, resulting in the activation of NF-κB and the production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-1 and MCP-1 (Nazli et al., 2010; Nazli et al., 2013). While these early front-line defenses are designed to prevent infections in the FGT, they may also foster inflammatory conditions within the FGT microenvironment that are highly efficient for HIV transmission.

1.5.2 Genital Inflammation in HIV Transmission

Early mucosal inflammation is necessary for viral transmission in the FGT (Li et al., 2009). Recently, Masson *et al* observed a three-fold increased risk of HIV infection in South African women who had elevated levels of at least five mucosal pro-inflammatory cytokines, including IL-8, IL-1β, IL-1α and TNF-α (Masson et al., 2015). Li *et al* demonstrated that inflammatory cytokines such as MIP-1α, MIP-1β and IL-8 in the genital tract were *essential* for the establishment of productive simian immunodeficiency virus (SIV) infection following vaginal inoculation (Li et al., 2009). This is relevant as an inflammatory profile in the genital tract is necessary for the recruitment of target cells to the portal of entry, particularly T helper type 17 (Th17) CD4+ T cells and activated CD4+ T cells expressing $\alpha_4\beta_7$ or $\alpha_4\beta_1$ (Arnold et al., 2016), and the establishment of productive, systemic infection. Indeed, studies have found increases in the frequency of CD4+ T cells in the genital tract of women with pro-inflammatory cytokine profiles (Arnold et al., 2016). In addition, elevated levels of α-defensins at mucosal surfaces are also associated with increased HIV infection (Hirbod et al., 2014; Levinson et al., 2009), presumably exacerbating risk by also contributing to target cell recruitment. Conversely, reduced

inflammation in the genital tract has been associated with reduced HIV susceptibility, insight which came to light following the study of HIV exposed seronegative (HESN) female sex workers, who have decreased immune activation (Card et al., 2013) and remain uninfected despite long term and high-risk sexual activity. HESN women have reduced systemic CD4+ T cell gene expression (McLaren et al., 2010), as well as significantly lower expression of monokine-induced by interferon-γ (MIG), interferon-γ-induced protein 10 (IP-10) and IL-1α in the genital tract compared to non-HESN HIV-uninfected and infected controls (Lajoie et al., 2012).

1.5.3 Genital Barrier Function in HIV Transmission

The effects of inflammation on HIV transmission in the FGT extend beyond the recruitment of target cells. In order for successful transmission to occur, infectious virions or HIV-infected cells in the male ejaculate must breach the epithelial barrier to encounter a susceptible cell in the host for the establishment of productive infection (Carias et al., 2013). Here, the virus establishes a small founder population of productive infection that then expands systemically (Haase, 2011). However, the role of the epithelial barrier function in preventing or facilitating the transmission of HIV in the FGT remains largely understudied (Kaushic et al., 2010; Kaushic et al., 2011). Our lab has shed light on the pathophysiological mechanism by which inflammation induced upon viral exposure can facilitate viral transmission. We showed that HIV envelope protein gp120 interacts with TLR2 and TLR4 on the genital epithelium, resulting in the downstream induction of pro-inflammatory cytokines, such as TNF-α, and the rapid destruction of tight junction proteins

localized at the apical surface (Nazli et al., 2010; Nazli et al., 2013). This resulted in the impairment of barrier function and significant viral translocation across the epithelium, suggesting that the paracellular leakage of HIV across a permeable epithelium could be a major source of HIV infection in the FGT *in vivo*. Furthermore, Arnold *et al* demonstrated that a pro-inflammatory cytokines profile changes the vaginal proteome, associated with increases in factors related to loss of barrier integrity (neutrophil proteases that mediate tissue barrier breakdown) and decreases in factors related to enhanced barrier integrity (protease inhibitors) in the cervicovaginal fluid of HIV-uninfected Kenyan women (Arnold et al., 2016). Protease inhibitors, such as serpins in the genital tract have also been identified as correlates of HIV resistance in studies of HESN women (Van Raemdonck et al., 2014), and the absence of specific serpins can lead to increased levels of activated Th17 target cells are mucosal surfaces (Zhao et al., 2014). Taken together, these studies suggest that the state of inflammation and barrier function are likely key determinants of the likelihood of HIV transmission during exposure in the FGT.

1.6 Other Factors That Influence HIV Transmission in the Genital Tract

1.6.1 The Microbiota of the Female Genital Tract

There are a number of factors within the FGT that have intimate interactions with the mucosal immune system and can influence susceptibility to HIV by attracting target cells and creating a microenvironment favorable for HIV infection (Ferreira et al., 2014). The vaginal microbiota is increasingly recognized as a contributing factor in host defense

against HIV. Bacterial vaginosis (BV) is a symptomatic clinical condition diagnosed using the Nugent score or Amsel criteria, characterized by polymicrobial vaginal microbiota and overgrowth of anaerobes, and it has been consistently linked to increased risk of acquiring HIV (Atashili et al., 2008; Low et al., 2011; Martin et al., 1999; Myer et al., 2005; Taha et al., 1998). HIV-infected women with BV are also three times more likely to sexually transmit HIV to their male partner than HIV-infected women with a *Lactobacillus*-dominant vaginal microbiome (Cohen et al., 2012). Cervicovaginal bacteria modulate genital inflammation, although their role in HIV susceptibility has not yet been fully elucidated. Women with diverse genital bacteria communities often have elevated levels of pro-inflammatory cytokines and activated CD4+ T cells in their vaginal fluid compared to women with *Lactobacillus crispatus*-dominant communities (Alcaide et al., 2017; Mitchell and Marrazzo, 2014). Transcriptional analysis by Anahtar *et al* elucidated that non-*Lactobacillus* dominant communities contribute to genital inflammation by activating NFκβ through the TLR4 pathway (Anahtar et al., 2015). Subsequent recruitment of lymphocytes by chemokine production was accompanied by increased frequency of mucosal CD4+ T cells compared to those with *L. crispatus*-dominant communities (Gosmann et al., 2017). These studies indicate that microbial diversity influences mucosal factors that contribute to HIV risk during sexual transmission. Furthermore, the use of either oral and vaginal antibiotics (metronidazole) to treat BV are able to dampen cytokines in the cervicovaginal fluid of women who respond to treatment (Mitchell et al., 2009; Thurman et al., 2015; Yudin et al., 2003). Diversity of the cervicovaginal microbiome has also been shown to be associated with changes in the vaginal proteome, which may serve

to lower barrier function in the FGT and thereby facilitate viral transmission (Borgdorff et al., 2016). In fact, microbially-driven barrier disruption is a well-established phenomenon in the gut (Vindigni et al., 2016). For example, researchers have related dysbiosis of the gut microbiome in patients with fatty liver disease to increased TNF-α, IL-6 and IFN-γ and irregularly arranged and widened tight junctions in the intestinal epithelium (Jiang et al., 2015). Thus, changes in the vaginal microbiota may also drive barrier disruption in the genital tract and thereby enhance HIV risk in women.

1.6.2 Female Sex Hormones and Hormonal Contraceptives

There is also emerging evidence that commonly used hormonal contraceptives increase the risk of HIV acquisition and transmission. Injectable progestins, including depot medroxyprogesterone acetate (DMPA) and norethisterone enanthate (NET-EN), are the favored form of contraception, used by approximately 8 million women in sub-Saharan Africa (Ross and Agwanda, 2012). With continued use, circulating estradiol concentrations of DMPA users commonly fall into the postmenopausal range, ovulation is suppressed and pregnancy is prevented (Bahamondes et al., 2014; Miller et al., 2000; Mishell, 1996). Numerous observational studies have identified DMPA as a significant risk factor for the acquisition of HIV. In a recent prospective cohort study, the HIV incidence in South African women using DMPA and NET-EN was 2.93 times higher than the incidence in those not using long-term contraception, and they also had 3.92 times the frequency of cervical CCR5+ CD4+ T cells (Byrne et al., 2016a). While the biological mechanism remains unclear, DMPA has been shown to downregulate cellular adhesion molecules, such as desmoglein-1α (DSG1α) and desmocollin (DSC1) in the murine vaginal tract (Quispe

Calla et al., 2016). Enhanced genital permeability allowed for endogenous microbial translocation from the vaginal lumen into the submucosal tissue and this subsequently increased vaginal inflammation (Quispe Calla et al., 2016). Interestingly, estrogen treatment was able to reverse DMPA-mediated barrier disruption in mice (Quispe Calla et al., 2016). In SIV macaque models, it has also been shown that subcutaneous progesterone implants, which mimics a progesterone-based hormonal contraceptive, induce thinning of the vaginal epithelium and resulted in a 7-fold greater risk in SIV vaginal transmission than that observed in macaques treated with placebo implants and exposed to SIV in the follicular phase of the menstrual cycle (Marx et al., 1996). It has been consistently shown that macaques can repeatedly be infected with much lower doses of virus following the administration of progestins (Marx et al., 1996; Mascola et al., 2000; Sodora et al., 1998). However, it remains unclear whether this is entirely a result of changes in epithelial thickness and integrity, or if changes in mucus secretion and/or cyclic changes in the immunologic responses of the FGT in response to hormones could also play a role. In addition to increasing genital permeability and inflammation, the promotion of HIV replication and transcytosis, changes in the vaginal microbiome and modulating innate and adaptive immune responses are all additional proposed mechanisms through which DMPA may increase the risk of HIV transmission (Achilles and Hillier, 2013; Ferreira et al., 2015a; Hel et al., 2010; Huijbregts et al., 2013; Murphy et al., 2014).

1.6.3 Co-infections in the Female Genital Tract

A co-infection is defined as an infection that is acquired subsequently to or in conjunction with HIV and tends to share a similar route of transmission. Infections with other sexually transmitted infections can increase susceptibility to HIV by altering the mucosal microenvironment in a way that favors the establishment of infection. The most pronounced alteration is increasing the populations of CD4+ CCR5+ target cells in the genital tissue (Schust et al., 2012). For example, women infected with *Chlamydia* have markedly higher levels of CD4+, CXCR4+ and CCR5+ T cells in the endocervix compared to uninfected women (Schust et al., 2012). Other STIs, such as Neisseria gonorrhea, Chlamydia trachomatis and Trichomonas vaginalis, also induce an inflammatory microenvironment and the recruitment of HIV target cells to the FGT (Mayer and Venkatesh, 2011). STIs that induce epithelial ulceration, such as syphilis, chancroid and Herpes simplex virus type 2 (HSV-2) can also cause loss of mucosal integrity and thereby increase susceptibility to HIV.

1.6.3.1 Herpes Simplex Virus Type 2

Epidemiological studies have repeatedly demonstrated that HSV-2 infection increases the risk of HIV acquisition by several fold and therefore has contributed to the expanding HIV epidemic (Freeman et al., 2006). HSV-2 is one of the most prevalent STIs in the world. The World Health Organization estimated in 2008 that 536 million people between the ages of 15 and 49 are infected with HSV-2, and approximately 23.6 million people in this age group become newly infected each year (Looker et al., 2008). The highest

rates of HSV-2 infection are in sub-Saharan Africa, where more than 80% of women and 50% of men are positive for HSV-2 in some areas (Looker et al., 2008; Looker et al., 2015). The virus primarily infects epithelial cells, resides in the sacral ganglion at the base of the spine and recurs in the genital area (Whitley et al., 2007). Symptomology typically includes the development of small and confined genital ulcerations, but in more severe cases, such as HIV-infected or immunocompromised individuals, ulcerations can be larger, more numerous and necrotic (Whitley et al., 2007).

HSV-2 infection has received renewed attention in recent years due to our current understanding of the strong epidemiological synergy between HSV-2 and HIV. HSV-2 infection is associated with a three-fold increase in the risk of HIV acquisition, which is amplified in those with newly acquired HSV-2 infection (Freeman et al., 2006). Prospective studies have also demonstrated that individuals co-infected with HIV and HSV-2 have more frequent and persistent episodes of HSV-2 mucosal shedding (Augenbraun et al., 1995), higher plasma HIV levels (Wright et al., 2003), and five-fold higher risk of transmitting HIV on a per-contact basis during sexual transmission compared to HSV-2 seronegative individuals (Buve et al., 2001; Corey et al., 2004; Gray et al., 2001). Thus, the development and implementation of strategies to prevent both infections would result in the most advantageous outcome in controlling these epidemics. In clinical trials, long-term suppressive anti-viral therapy against HSV-2 has been shown to reduce asymptomatic HSV-2 shedding and symptomatic viral reactivation, but does not reduce the risk of HIV acquisition in HIV-uninfected individuals or the risk of transmission by HIV-infected

individuals (Celum et al., 2010). The current dogma of understanding the increased susceptibility to HIV with HSV-2 rests on two hypotheses. The first suggests that the genital ulcerations induced by HSV-2 disrupt the mucosal epithelium and the resulting loss of barrier function facilitates HIV transmission into the submucosa (Ferreira et al., 2014). The second suggests that HSV-2 infection alters the mucosal microenvironment by increasing the local production of pro-inflammatory cytokines and chemokines that recruit and activate CD4+ T cells, macrophages and dendritic cells in the submucosa (Ferreira et al., 2011; Martinelli et al., 2011; Stefanidou et al., 2013). Taken together, the likelihood that HIV will transverse the epithelium and also encounter a target cell in the submucosa are increased in the context of HSV-2 infection. We have also shown that pro-inflammatory cytokines induced in response to HSV-2 infection, including TNF-α, IL-6, IL-8 and MCP-1, contribute to the indirect induction of HIV-LTR promoter in T cells, a process synonymous with HIV replication (Ferreira et al., 2015b; Ferreira et al., 2011). Despite numerous *in vitro* studies, the complex molecular and cellular mechanisms underlying the synergistic relationship between HIV and HSV-2 infection *in vivo* still remain unclear. This is likely in part due to the absence of an *in vivo* co-infection model to study their interactions and respective pathologies.

1.6.4 Microbicides

One strategy to prevent HIV transmission is the design of topical microbicides that either create chemical, biological and/or physical barriers to infection or block or inactivate the virus at the mucosal surface where infection can occur (Weber et al., 2005). In the

formulation of vaginal microbicides, the effect of formulation ingredients on the integrity of the cervicovaginal epithelium and mucosal inflammation should be carefully evaluated. The importance of this was exemplified by the failures of two phase III clinical trials assessing the efficacy of nonoxynol (N-9) vaginal gel (COL-1492) and the 6% cellulose sulfate (CS) gel (Hillier et al., 2005; Tao et al., 2008; Van Damme et al., 2008; Van Damme et al., 2002). Both microbicides failed to demonstrate efficacy despite promising activities in pre-clinical studies, and the results of these trials emphasized the urgent need for more stringent pre-clinical protocols, with emphasis on microbicide safety.

1.6.4.1 Nonoxynol-9 and HIV Risk

N-9 is a anionic surfactant that disrupts cell membranes and was originally developed in the 1960s as a contraceptive spermicide to coat latex condoms (Weber et al., 2005). Early *in vitro* studies of N-9 yielded promising results (Bourinbaiar and Fruhstorfer, 1996; Hicks et al., 1985), demonstrating that it possessed broad-spectrum activity against several STIs, including *Chlamydia trachomatis, Neisseria gonorrhoeae,* HSV-2 and HIV (Benes and McCormack, 1985; Weber et al., 2005). As a detergent, it destroys the integrity of lipid bilayer membranes and likely acts as an anti-microbial agent by disrupting the viral envelope and bacterial membranes (Weber et al., 2005). The widespread and apparent safe use of N-9 as a contraceptive agent further supported its use as a potential topical microbicide. It proved to be safe in pre-clinical studies, and was the first microbicide to be tested in large clinical trials for the prevention of HIV transmission. Paradoxically, the

final phase 2/3 clinical trial of N-9, formulated as 3.5% N-9 in a carbomer gel, demonstrated that repetitive use of this product was associated with an almost 2-fold greater risk of HIV acquisition compared to carbomer gel alone in a high-risk population of women (Roddy et al., 1998; Van Damme et al., 2002). Increase in HIV risk associated with N-9 application has since been attributed to genital irritation, inflammation and disruption of the cervicovaginal epithelial barrier (Kreiss et al., 1992; Roddy et al., 1998; Stafford et al., 1998). In mouse models, N-9 resulted in swelling of cells in the columnar epithelium, sloughing of the superficial layers of epithelium and severe apoptosis in the cervical region within 10 minutes of application (Catalone et al., 2004; Lozenski et al., 2012). In the immediate hours post-application, very little of the superficial columnar cell layer of epithelium remained, leaving large areas of the deeper basal cell layer almost completely exposed, and the majority of the mucosa contained only one or two layers of basal cells with larger areas of complete epithelial denudation (Lozenski et al., 2012). Further investigation revealed that epithelial damage coincided with transient increases in pro-inflammatory cytokines, such as IL-1β and IL-6, and CD14+ monocyte/macrophage immune cell infiltration into the vagina (Lozenski et al., 2012).

1.6.4.2 Cellulose Sulfate and HIV Risk

Cellulose sulfate also entered into Phase III clinical trials to evaluate its effectiveness in preventing HIV transmission among sexually active women

perceived to be at considerable risk of HIV acquisition (Halpern et al., 2008; Van Damme et al., 2008). It also demonstrated potent *in vitro* activity against other sexually transmitted infections, such as *Neisseria gonorrhoeae, Chlyamdia trachomatis,* Human Papilloma Virus (HPV) and *Gardnerella vaginalis* (Anderson et al., 2002; Christensen et al., 2001; Simoes et al., 2002; Su and Caldwell, 1998). This was prompted by promising laboratory data and a good safety profile demonstrated in early-phase clinical trials (Doh et al., 2007; Malonza et al., 2005; Mauck et al., 2001a; Mauck et al., 2001b). Strikingly, the trial was stopped prematurely because of safety concerns arising after the Data Safety Monitoring Board of a parallel trial concluded that CS might be increasing the risk of HIV (Halpern et al., 2008). There was a higher rate of HIV acquisition in the CS group compared to the placebo group, although the result did not achieve statistical significance in the primary analysis. *In vitro* studies evaluated changes in the epithelial barrier upon exposure to CS using a dual-chamber culture system, and found rapid and sustained reduction in TER and a marked increase in HIV infection of T cells cultured in the lower chamber (Mesquita et al., 2009). Interestingly, CS also triggered NFκB activation in peripheral blood mononuclear cells and increased HIV replication in chronically infected U1 cells (Mesquita et al., 2009). The study of both N-9 and CS provided novel insights into the association between genital inflammation, epithelial damage and HIV susceptibility, and the illuminated the importance of safety assessments during the pre-clinical development of topical vaginal microbicides effective against HIV (Cutler and Justman, 2008). Despite

this, an effective *in vivo* model where such agents can be tested for their effect on barrier function is still absent. This type of tool will greatly improve our ability to test microbicides beyond *in vitro* safety profiles.

1.7 HIV Pathogenesis

1.7.1 Mucosal Immunity in the Gastrointestinal Tract

The gastrointestinal tract is arguably the most important mucosal site in the pathogenesis of HIV infection (Shacklett and Anton, 2010). Beneath the single layer of intestinal epithelial cells lining the gut is an abundant population of activated memory CD4+ T cells (Xu et al., 2013). Depending on the presence of foreign or dietary antigens and the intestinal microbiota, they engage in either inflammatory or regulatory immune responses. A balanced interaction between dietary antigens, the microbiota and innate immune activation is required to maintain intestinal health, while disruption of this fine-tuned regulation is believed to precipitate inflammation that can alter the mucosal integrity of the GI tract (Xu et al., 2013). Antigens within the lumen are sampled by M cells within the follicle-associated epithelium lining the Peyer's patches and presented to T and B cells, which recirculate and home preferentially to the intestinal lamina propria as activated, effector T cells or antibody secreting plasma cells (Janeway, 2012). Accordingly, many of the T cells residing within the intestinal mucosa are highly activated CD4+ CCR5+ T cells, the main targets for early HIV infection and amplification (Mattapallil et al., 2005; Veazey and Lackner, 2003; Veazey et al., 2000). Furthermore, the number of T cells that reside

within the epithelium and lamina propria of this mucosal site alone has been estimated to exceed the total number of T cells in the rest of the body (Shacklett and Anton, 2010; Xu et al., 2013). As a result, it serves as the primary tissue for HIV expansion and persistence, and depletion of these cells has dire consequences on intestinal integrity, immune function, and the clinical progression of disease.

1.7.2 Intestinal Barrier Function in HIV Pathogenesis

Impairment of barrier function in the gut is a prerequisite for microbial translocation and subsequent immune activation, two hallmarks of HIV infection that contribute to increased pathogenesis, morbidity and mortality in chronically infected individuals (Leon et al., 2015; Marchetti et al., 2011; Sandler et al., 2011; Tincati et al., 2016). A landmark study by Brenchley *et al* was first to show a significant increase in plasma lipopolysaccharide (LPS) levels, used as a measure of microbial translocation, in chronically infected patients and in rhesus macaques following inoculation with a pathogenic SIV strain (Brenchley et al., 2006). The mechanism of microbial translocation was subsequently demonstrated to be focal breaches in the epithelium that occur as a result of mucosal T cell activation in the gut, which allow for the translocation of microbial constituents from the intestinal lumen into the draining and peripheral lymph nodes and liver (Estes et al., 2010). We also revealed that HIV-mediated barrier disruption and viral translocation through genital epithelial cells is accompanied by significant amounts of bacterial translocation following 24 hours of HIV exposure *in vitro* (Nazli et al., 2010*)*. Currently, the most widely used indicator of microbial translocation is soluble CD14, which

is a biomarker of monocyte activation upon binding to LPS. Although it serves only as an *indirect* marker of microbial translocation, it is more widely used than LPS because its measurement is more easily standardized across different laboratories and it's the most reliable predictor of mortality in HIV-infected patients (Marchetti et al., 2011; Marchetti et al., 2013; Sandler et al., 2011). Macaque studies of SIV infection have also revealed that *preexisting* barrier damage before inoculation with SIV predicts disease progression to AIDS following infection (Canary et al., 2013).

An important contribution in the field was made by a study on acutely infected individuals. At the time of enrolment, which was an average of 42 days from initial infection, subjects displayed comparable levels of soluble factors in the periphery associated with intestinal barrier damage, including tight junction protein zonulin and epithelial death biomarker intestinal fatty acid binding protein (I-FABP) (Chevalier et al., 2013). Six months following enrolment, investigators found a significant increase in intestinal damage (Chevalier et al., 2013). Astoundingly, this was present regardless of their treatment option, and the initiation of antiretroviral therapy was not sufficient to halt the profound changes that occur in the intestinal mucosa throughout the course of infection (Chevalier et al., 2013). Consistent with these findings, an additional study on acutely infected patients, who were estimated to acquire the infection less than 180 days earlier, demonstrated that the immediate and timely administration or antiretroviral therapy had no effect on plasma levels of intestinal barrier damage markers (I-FABP) (Jenabian et al., 2015). Cross-sectional studies have also shown the presence of intestinal barrier damage in

HIV-infected individuals on long-term antiretroviral therapy, and aviremic individuals still have elevated levels of E-cadherin (Streeck et al., 2011). More recently, the expression of tight junction complex proteins and genes was shown to progressively decrease along the proximal-to-distal axis within the colonic epithelium of virally-suppressed patients, who started antiretroviral therapy with a poor CD4+ T count but presented favorable immune competency at the time of the study (Chung et al., 2014). These studies confirm that gut barrier disruption persists despite suppression of viral load. Currently, the major consequence of intestinal barrier disruption and microbial translocation is thought to be chronic immune activation (Gori et al., 2008; Marchesi et al., 2016).

1.7.3 Immune Activation in HIV Pathogenesis

Immune activation is one of the most unequivocally clear outcomes of HIV infection and is a more accurate predictor of disease progression, morbidity and mortality than plasma viral load or CD4+ T cell counts, independent of antiretroviral therapy (Klatt et al., 2013a; Klatt et al., 2013b). It is characterized by polyclonal B-cell activation (Lane et al., 1983), high CD4+ and CD8+ T cell turnover (Hazenberg et al., 2000) and high levels of circulating pro-inflammatory cytokines and chemokines (Valdez and Lederman, 1997), which persist independent of antiretroviral therapy. Indeed, HIV itself can induce inflammation directly by stimulating anti-viral pathways in both the innate and adaptive arms of the immune system. However, the majority of inflammation observed during chronic HIV infection persists as a result of indirect or 'bystander' factors of infection, such as microbial translocation, that be both cause and be an effect of CD4+ T cell depletion.

Some of the strongest evidence that immune activation correlates with disease progression comes from the comparison between different non-human primate models of SIV infection. SIV infection in rhesus macaques is characterized by chronically high levels of immune activation, progressive CD4+ T cell depletion and progression to AIDS with similar pathologies as observed during human infection (Appay and Sauce, 2008). In contrast, SIV-infected sooty mangabees manifest far lower levels of aberrant immune activation, maintain normal CD4+ T cell levels and ultimately resist progression to AIDS-like symptomology (Appay and Sauce, 2008). Interestingly, deposition of commensal *E. coli* protein can be found in the colonic lamina propria of infected rhesus macaques, indicative of microbial translocation, while sooty mangabees do not show similar evidences of microbial translocation (Estes et al., 2010). Following treatment with a bowel-sterilizing antibiotic regimen, plasma LPS levels were lowered in rhesus macaques, suggesting that targeting microbial translocation can help to control systemic immune activation (Brenchley et al., 2006). Furthermore, work done by our lab demonstrating that microbial translocation of the genital epithelium occurs following direct exposure to HIV in the FGT can also provide explanation for increased immune activation observed during the acute phase of HIV infection (Nazli et al., 2010).

In the past few years, research on mucosal immunology has evolved from investigation of CD4+ T cell depletion and reconstitution to the study of the homeostasis of other T cell subsets, which affect the structure and function of the GI tract (Tincati et al., 2016). The GI tract is home to IL-17 and IL-22 producing cells that were first hypothesized

to have a role in the immune pathogenesis of SIV (Ferretti et al., 2003; Klatt et al., 2012; Sugimoto et al., 2008). Mucosal Th17 cells are only slightly decreased in the earliest phases of acute HIV infection (Chevalier et al., 2013; Schuetz et al., 2014), however, depletion of both Th17 and Th22 mucosal populations is observed in chronic HIV infection (Kok et al., 2015; Page et al., 2014). Studies have also assessed the role of these cells in the pathogenesis and outcome of HIV infection. Loss of the Th17/T regulatory (Treg) cell balance in peripheral blood and rectosigmoid tissue of patients with HIV infection was associated with increased plasma concentration of microbial products as well as progressive HIV disease (Favre et al., 2010; Page et al., 2014). In untreated disease, immune-regulatory skewing of mucosal Th17 cell function characterized by an increased IL-10/TNF-α ratio was found to be independently associated with decreased systemic immune activation (Kim et al., 2013). Importantly, these defects were shown to persist throughout the course of antiretroviral treatment, particularly in patients presenting with poor CD4+ recovery. These results strengthen the hypothesis that immune activation influences clinical outcome and challenges CD4+ reconstitution on antiretroviral therapy (Gaardbo et al., 2015). Together, these studies warrant the search for therapeutic strategies that reduce microbial translocation and/or systemic immune activation in HIV-infected patients and can be taken in combination with antiretroviral therapy to address the multifactorial pathogenesis of HIV infection.

1.8 Curcumin

Curcumin *(diferuloylmethane)* with the chemical formula 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione is the primary active constituent of the spice turmeric and is responsible for its vibrant yellow color (Moghadamtousi et al., 2014). It has gained widespread recognition due to a range of anti-microbial, anti-inflammatory, anti-cancer, anti-coagulant and wound healing activities (Gupta et al., 2011; Lao et al., 2006; Prasad and Tyagi, 2015). A very early report by the Food and Agriculture Organization and the World Health Organization demonstrated that the optimal daily intake of curcumin is 0-1 mg/kg of body weight. Based on its safety, the U.S. Food and Drug Administration approved curcumin as a "generally regarded as safe" (GRAS) compound (Prasad and Tyagi, 2015). In a phase I clinical trials, curcumin was administered at a dose of 500 mg/day, following by increases to 1, 2, 4, 8 and 12 g/day for 3 months (Cheng et al., 2001). No dose-limiting toxicity was observed in any subject under any condition. Despite its safety properties in humans even at high doses (12 g per day), the inherent physicochemical characteristics, including poor water solubility, low bioavailability, chemical instability, photodegradation, rapid metabolism and short-half life, limit its pharmaceutical significance (Bansal et al., 2011; Kharat et al., 2017; Schneider et al., 2015). Thus, advanced delivery systems have been developed in recent years to overcome these pharmaceutical issues and improve the therapeutic efficacy of curcumin.

1.8.1 The Anti-inflammatory Activity of Curcumin

One of curcumin's best explored actions is its ability to interact with numerous targets of inflammation and potently modulate the inflammatory response. Acute and chronic inflammation is a major factor in the progression of Alzheimer's disease, Parkinson's disease, multiple sclerosis, cancer, allergy, asthma, colitis, rheumatoid arthritis, obesity, diabetes, cardiovascular diseases and depression (Aggarwal and Harikumar, 2009). Curcumin can downregulate the activity of cyclooxygenase-2 (COX-2), lipoxygenase and inducible nitric oxide synthase (iNOS) enzymes (Goel et al., 2008a; Goel et al., 2008b), inhibit the production of inflammatory cytokines TNF-α, IL-2, IL-6, IL-8, IL-12, MIP and MCP, matrix metalloproteinase (MMP-1, -9, -13) (Abe et al., 1999), prevent the secretion of anti-apoptotic proteins such as Bcl_2 (Park et al., 2007), activate Bax and caspase-3 (Park et al., 2007), and downregulate mitogen-activated and Janus kinases (Natarajan and Bright, 2002; Siwak et al., 2005). It mediates the majority of these effects *via* the suppression of transcription factor NFκB, involved in the regulation of inflammatory gene expression (Singh and Aggarwal, 1995). Under homeostatic conditions, it retains an inactive state in the cytoplasm as a p50-p65 heterotrimer by the inhibitory subunit Iκβα. Upon activation of the complex, Iκβα sequentially undergoes phosphorylation by kinase Iκκ, followed by ubiquitination and degradation and allows for the release of the p50-p65 heterodimer for translocation to the nucleus (Aggarwal et al., 2005; Kamat et al., 2007; Shishodia et al., 2005; Siwak et al., 2005). In the presence of curcumin, however, several *in vitro* studies have demonstrated that the Iκκ mediated phosphorylation of Iκβα is blocked, thus preventing NFκβ activation and inflammatory

gene expression (Foryst-Ludwig et al., 2004). Curcumin has also been shown to decreases inflammation through the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) (Bharti et al., 2003), as well as through down-regulation of suppressor of cytokine 3 signaling 3 (SOCS3) and sterol regulatory element-binding protein-1c (SREBP-1c) (Kuo et al., 2012). The safety and efficacy of curcumin has since been investigated in the context of a plethora of diseases. Ongoing clinical trials indicate that it may have therapeutic potential in inflammatory diseases, such as inflammatory bowel disease, pancreatitis, arthritis and chronic anterior uveitis, as well as certain types of cancer (Taylor and Leonard, 2011).

1.8.2 The Anti-Viral Activity of Curcumin

Curcumin has been evaluated as an anti-viral therapeutic in several *in vitro* experiments and in a single clinical trial. *In vitro,* curcumin has been shown to inhibit (a) viral growth (Prasad and Tyagi, 2015), (b) HIV LTR-directed gene expression (Sui et al., 1993), (c) tat-mediated transactivation of HIV LTR (Barthelemy et al., 1998), (d) HIV-1 and HIV-2 proteases (Sui et al., 1993), (e) HIV integrase (Mazumder et al., 1995), and (f) Tat protein acetylation in susceptible cells (Balasubramanyam et al., 2004). As a viral growth inhibitor, curcumin present in a polyherbal cream (Basant) has been shown to prevent the entry of HIV into HIV susceptible HeLa cell lines (Talwar et al., 2008). As a protease inhibitor, curcumin fits well into the HIV protease active site as a result of its symmetrical structure, interacting with residues Asp25, Asp29, Asp30, Gly27, Asp29 and Asp30 of HIV protease (Sui et al., 1993). Extensive hydrogen bonding promoted by the *o*-

hydroxyl and/or keto-enol structures are important for its integrase and protease inhibitory actions (Vajragupta et al., 2005). Curcumin can also interact with the integrase catalytic core of HIV integrase (Mazumder et al., 1996), and further energy minimization studies revealed that it's anti-integrase activity was associated with intramolecular stacking of two phenyl rings that bring the hydroxyl groups in closer proximity and enable tight binding to the integrase active site (Mazumder et al., 1995). Curcumin and derivatives also modulate HIV genome expression of viral regulatory protein Tat, which stimulates transcription elongation. Curcumin inhibited Tat transactivation of HIV-LTR in HeLa cells (Barthelemy et al., 1998), and other studies have demonstrated its potent and selective ability to inhibit HIV-LTR directed p24 antigen production, in cells either acutely or chronically infected with HIV (Li et al., 1993). Curcumin derivatives have also showed significant anti-HIV activity. For example, curcumin-loaded apotransferrin nanoparticles completely block the synthesis of viral cDNA in the gag region (Barthelemy et al., 1998). In addition, numerous kinases susceptible to curcumin have been reported to be involved in HIV infectivity and multiplication. Tyrosine kinase (Hong et al., 1999), mitogen-activated protein kinase (Dong et al., 2014), oncogenic kinase PAK1 (Maruta, 2014), ferritin and creatine kinase (Babiker et al., 2015), and protein kinase C (PKC) (Das et al., 2011; Reddy and Aggarwal, 1994) are involved in HIV infection and host cell dysregulation, and curcumin has the potential to potently modulate all these kinases. We have also demonstrated that curcumin blocked TLR-mediated induction of HIV replication and significantly decreased HIV-1 replication in chronically infected T cells, further implicating it in the prevention and treatment of HIV (Ferreira et al., 2015b).

Investigation of the anti-herpetic activity of curcumin is also relevant due to the epidemiological synergy between HIV and HSV-2. *In vitro,* curcumin has been shown to significantly decrease HSV infectivity and immediate early gene expression by affecting VP16-mediated recruitment of RNA polymerase II to immediate early gene promoters (Kutluay et al., 2008). However, in contrast to promising *in vitro* studies, clinical trial investigation of curcumin as an anti-HIV compound in 40 patients over an eight-week period showed no significant reduction or elevation in patients' viral load or CD4+ T cell counts, respectively (James, 1996). A major caveat to this study is that curcumin was administered orally, which results in poor bioavailability due to rapid metabolism (as described in above).

1.8.3 The Clinical Limitations of Curcumin

Despite its promising safety profile and broad-spectrum biological activity *in vitro*, the therapeutic potential of curcumin is limited because it undergoes rapid plasma clearance and conjugation. Animal studies have demonstrated that it is rapidly metabolized/glucoronidated, conjugated in the liver and excreted in the feces following oral administration (Anand et al., 2007). In a study in rats., a 500 mg/kg oral dose of curcumin resulted in a peak plasma concentration of only 1.8 ng/mL and a 40 mg/kg intravenous dose resulted in complete plasma clearance (Ireson et al., 2001). Data on the pharmacokinetics and bioavailability of curcumin in humans, mainly conducted on cancer patients, is consistent with animal studies. A phase I clinical trial conducted on 25 patients with various pre-cancerous lesions demonstrated that daily oral doses of 4, 6, and 8 g of curcumin for

three months yielded serum concentrations of only 0.51 ± 0.11, 0.63 ± 0.06 and 1.77 ± 1.87 μM, respectively (Cheng et al., 2001). Serum levels peaked between one and two hours post-dose and declined rapidly thereafter. Although data from studies conducted in healthy subjects is limited, one study using high doses (10 and 12 g in a single oral dose) in 12 healthy subjects measured serum curcumin as well as its sulfate and glucuronide metabolites at various time points up to 72 hours post-dose (Vareed et al., 2008). As in previously mentioned studies, curcumin was rapidly cleared (only one subject had detectable free curcumin in the serum) and was subsequently conjugated in the gastrointestinal tract and liver. Cumulatively, data indicate that curcumin is poorly absorbed following systemic administration. It remains to be determined if topical application of curcumin to the FGT, the primary site of virus contact during sexual transmission of HIV and HSV-2, improves its efficacy as an anti-viral compound. Furthermore, the efficacy of curcumin as a prophylactic agent for the prevention of HIV transmission or as a means to reduce chronic immune activation in HIV-infected patients has yet to be investigated.

CHAPTER 2: RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 Rationale

Although women constitute half of the estimated 35 million people living with HIV worldwide (Cohen et al., 2008), little is known about the early events of HIV infection in the FGT, where 40% of all new HIV infections are established each year (Hladik and McElrath, 2008). The early pathogenesis of HIV infection remains particularly elusive in the context of the varying endogenous and exogenous factors found in the genital microenvironment that may influence susceptibility to HIV. Mucosal inflammation, disrupted epithelial barrier function and HSV-2 infection are three main factors that have been linked to increasing susceptibility to HIV (Burgener et al., 2015; Masson et al., 2015; Passmore et al., 2016).

Several studies have reported that an inflammatory microenvironment can increase the abundance of activated HIV target cells in the genital tissue and subsequently increase the risk of the virus establishing productive infection in the submucosa during sexual intercourse (Burgener et al., 2015). Following transmission, pro-inflammatory cytokines secreted by the genital epithelium may be sufficient to directly activate HIV replication in infected target cells within the genital tract. Notably, reduced inflammation in the genital tract has been associated with reduced HIV susceptibility (Burgener et al., 2015; Lajoie et al., 2012). Our lab has also shown that the inflammation induced by genital epithelial cells upon viral exposure can result in the rapid destruction of tight junction protein within the epithelium (Nazli et al., 2010). The resulting impairment of barrier function allows for HIV

translocation across the epithelium and could be a major source of HIV infection in the FGT *in vivo* (Nazli et al., 2010). Other factors that promote barrier integrity in the FGT, such as protease inhibitors, have been identified as correlates of HIV resistance in HESN women (Van Raemdonck et al., 2014). Interestingly, HSV-2 infection can contribute to both target cell abundance and loss in barrier function in the FGT, which may explain why it is the most common HIV co-infection. The majority of these findings, however, are derived from *in vitro* studies or correlations in clinical studies, and emphasize the need to improve our understanding of the relationship between HIV susceptibility and these factors *in vivo.* It also begs the question of whether targeting inflammatory pathways to impede mucosal inflammation, barrier disruption and dampen the effects of HSV-2 on the FGT microenvironment is a plausible strategy to prevent HIV transmission in women.

We recently examined if curcumin, a highly pleiotropic anti-inflammatory compound, could be used to abrogate the production of cytokines and chemokines that (a) induce an inflammatory microenvironment capable of recruiting HIV target cells to the FGT and (b) directly impair the genital mucosal barrier that facilitates HIV acquisition in the FGT (Masson et al., 2015; Nazli et al., 2010). Results of these *in vitro* studies demonstrated that curcumin treatment abrogated the upregulation of pro-inflammatory cytokines TNF-α and IL-6, as well as chemokines IL-8, RANTES, and IP-10, and prevented barrier disruption following HIV exposure (Ferreira et al., 2015b). Interestingly, curcumin also demonstrated efficacy against HIV co-infections by inhibiting the indirect activation of the HIV-LTR promoter *via* secretion of inflammatory factors in the presence

of co-infecting STIs, and by exhibiting anti-viral activity against HSV-2 through the suppression of NF-κB (Ferreira et al., 2015b). These results raise the intriguing possibility that modulating the microenvironment in the vaginal tract with curcumin could minimize genital inflammation, strengthen the mucosal epithelial barrier and protect and/or control against HSV-2 infection *in vivo,* all with relevance to preventing HIV infection in the female genital tract.

2.2 Hypothesis

The first objective of this study was to develop an *in vivo* model to test the anti-inflammatory and anti-viral effect of curcumin. Based on previous *in vitro* studies, **we hypothesized that curcumin would have an anti-inflammatory effect in the FGT *in vivo*. Furthermore, curcumin treatment would either protect against or decrease viral replication and pathology following intravaginal HSV-2 infection *in vivo.*** The ultimate goal of these studies was to provide rationale for the future clinical testing of curcumin as a prophylactic agent for the prevention of both HSV-2 and HIV infection in women. The second objective of this project was **to develop a comprehensive assay that would allow for the experimental determination of *in vivo* genital barrier function in a preclinical murine model.** Currently there are no in vivo animal models available to assess barrier function of the genital tract. A pre-clinical model would allow us and others to test the effect of sexually transmitted viruses and anti-viral strategies on mucosal barrier in the FGT.

2.3 Aims

The following aims were outlined to address the objectives outlined above:

1. Examine the *in vivo* anti-herpetic activity of curcumin treatment in a mouse model of intravaginal HSV-2 infection
2. Examine the *in vivo* anti-inflammatory activity of curcumin treatment in a mouse model of vaginal inflammation
3. Develop and test a novel and comprehensive assay system to measure barrier function in the female genital tract *in vivo*

CHAPTER 3

EXAMINING THE ANTI-INFLAMMATORY AND ANTI-VIRAL EFFECTS OF INTRAVAGINAL CURCUMIN DELIVERY

Danielle Vitali, Puja Bagri, Jocelyn M. Wessels, Philip Nguyen, Talveer Mandur, Meenakshi Arora, Ravikumar Majeti and Charu Kaushic

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Given its potent anti-inflammatory properties, we decided to investigate whether curcumin could be used to abrogate inflammatory processes in the vaginal tract that could facilitate HIV acquisition in the FGT or contribute to HIV amplification. Our results suggest that topical delivery of curcumin-encapsulated nanoparticles to the vaginal tract can reduce the induction of inflammatory responses from the vaginal tissue *in vivo.* We did not observe the same anti-inflammatory effect locally in the vaginal tract following systemic routes of delivery, including oral and IP, likely because of poor tissue distribution to the vaginal tract. Previous studies from our lab demonstrated that curcumin can block HSV-2 viral replication *in vitro* in genital epithelial cells *via* a mechanism that involves inhibiting inflammatory transcription factor NFκB. However, this was not observed *in vivo,* as prophylactic administration of curcumin-encapsulated nanoparticles did not reduce HSV-2 acquisition or viral-load set point in our mouse model of intravaginal HSV-2 challenge, as compared with the vehicle-only control group. The lack of efficacy observed was seen across both lethal and sub-lethal doses of viral challenge. We conclude from these experiments that curcumin has potent anti-inflammatory properties when delivered locally, however, its *in vitro* anti-viral properties do not translate *in vivo* under the conditions that were tested.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments. Puja Bagri, Philip Nguyen and Dr. Jocelyn M. Wessels provided technical assistance and contributed to the generation of the data. Talveer Mandur performed the oral gavages, and Dr. Meenakshi Arora and Dr. Ravikumar Majeti provided the nanocurcumin

and PLGA formulations. I was responsible for data analysis, and Dr. Charu Kaushic and I wrote and edited the manuscript.

Examining the anti-inflammatory and anti-viral effects of intravaginal curcumin delivery

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Abstract

Objective: The fastest growing phase of the HIV pandemic is currently by heterosexual transmission in women. Sexually transmitted infections, such as Herpes Simplex Virus Type 2 (HSV-2), and an inflammatory microenvironment are two known factors that can contribute to HIV risk. We recently found that curcumin, a highly pleiotropic anti-inflammatory compound, can block HSV-2 infection and abrogate the production of cytokines and chemokines in genital epithelial cells *in vitro.* These studies raise the intriguing possibility that modulating the microenvironment in the vaginal tract with curcumin could minimize genital inflammation and/or control against HSV-2 infection *in vivo*, all with relevance to reducing susceptibility to HIV infection. In this study, we tested the anti-inflammatory and anti-viral activity of curcumin in an in vivo model.

Design and Methods: Six to eight-week-old female C57BL/6 mice were pre-treated with curcumin-encapsulated nanoparticles through various routes of delivery 2 and 4 hours prior to exposure to TLR9 ligand, CpG ODN. Inflammation was assessed based on vaginal tissue morphology following H&E staining of tissue sections and cytokine (IL-6, TNF-α and MCP-1) analysis of vaginal tissue homogenates. Distribution of nanoparticles in the vaginal tissue following different routes of delivery was imaged using PLGA particles entrapped with fluorescent dye. To examine the anti-viral activity of curcumin, mice received an intravaginal dose of curcumin-encapsulated nanoparticles prior to challenge with wildtype HSV-2, and survival, pathology and viral shedding were measured over the course of

infection.

Results: Topical delivery of curcumin into the vaginal tract abrogated inflammatory pathology and the production of pro-inflammatory cytokines IL-6, TNF-α and MCP-1. This effect was absent with systemic (intraperitoneal and oral) route of delivery because nanoparticle dissemination to the vaginal tract was poor, as seen by negligible amounts PLGA detected in the vaginal tissue. Despite potent anti-inflammatory effects, curcumin did not reduce HSV-2 acquisition or viral-load set point, as compared with the vehicle-only control group. The lack of efficacy observed was consistent across both lethal and sub-lethal doses of viral challenge in the *in vivo* mouse model.

Conclusions: Although we did not observe efficacy against HSV-2 infection in our model under the conditions tested, our results suggest that curcumin nanoparticle delivery in the vaginal tract would be useful as an anti-inflammatory formulation that could potential decrease the risk of HIV acquisition in women. which could be useful for deceasing HIV acquisition risk.

Introduction

Clinical and epidemiological studies have consistently found that women are more susceptible to acquiring sexually transmitted infections upon exposure compared to men (UNAIDS, 2016; Wira and Fahey, 2008). In fact, the fastest growing phase of the Human Immunodeficiency Virus (HIV) pandemic is currently by heterosexual transmission in women (UNAIDS, 2014). The female genital tract (FGT) mucosa is a major portal for entry of HIV into the body, responsible for initiating 40% of global infections (Hladik and McElrath, 2008). Similarly, the global incidence of Herpes Simplex Virus 2 (HSV-2) infection is higher in women than men, with the lowest prevalence rate being 13% among West European men and the highest being 70% among sub-Saharan African women (Looker et al., 2008). Interestingly, there is strong epidemiological synergy between the dual epidemics of HIV and HSV-2, whereby HSV-2 infection is associated with a 3-fold increase in the risk of HIV acquisition (Freeman et al., 2006). In addition to the social, economic and behavioral factors that contribute to the increased prevalence in women, there are biological mechanisms underpinning this increase in susceptibility and a better understanding of the microenvironment in the FGT is critical for developing strategies for the prevention of both HIV and HSV-2 transmission in women.

What is exceedingly clear is that mucosal inflammation in the FGT raises the risk of HIV infection in women (Masson et al., 2015; Passmore et al., 2016). An inflammatory profile in the genital tract is necessary for the recruitment of HIV target cells to this portal

of entry and for the establishment of productive and systemic HIV infection. T helper type 17 (Th17) CD4+ T cells and activated CD4+ T cells expressing $\alpha_4\beta_7$ or $\alpha_4\beta_1$ have been shown to be particularly important to this process, as they are the main targets for HIV infection (Arnold et al., 2016; Li et al., 2009). We have also demonstrated that the downstream induction of pro-inflammatory cytokines by genital epithelial cells results in the rapid impairment of mucosal barrier function, which could help the virus transverse the genital epithelium and ultimately infect underlying target cells in the FGT (Nazli et al., 2010; Nazli et al., 2013). Interestingly, the current dogma of understanding the increased susceptibility to HIV with HSV-2 infection rests on the hypotheses that the presence of HSV-2 increases local production of pro-inflammatory cytokines and chemokines that facilitate target cell recruitment and mucosal barrier disruption (Ferreira et al., 2014; Ferreira et al., 2011; Martinelli et al., 2011; Stefanidou et al., 2013). Certain innate inflammatory responses have also been shown to favor HSV-2 replication rather than restrict it. For example, activation of NFκB, a transcription factor involved in the regulation of inflammatory gene expression, during HSV infection increases the efficiency of HSV-2 replication (Marino-Merlo et al., 2016). Taken together, these studies suggest that targeting inflammatory pathways in the FGT should be investigated as a possible prophylactic strategy for the prevention of both HIV and HSV-2 infection in women.

Curcumin (*diferuloylmethane)* is the primary active constituent of the spice turmeric that has been extensively examined for its anti-inflammatory responses in experimental and clinical studies (Moghadamtousi et al., 2014). Some of its best-explored

anti-inflammatory actions are its ability to downregulate the activity of cyclooxygenase-2 (COX-2), lipoxygenase and inducible nitric oxide synthase (iNOS) enzymes, inhibit the production of the inflammatory cytokines TNF-α, IL-1, -2, -6, -8 and -12, MIP, and MCP and downregulate mitogen-activated and Janus kinases. Such anti-inflammatory activities are likely mediated *via* its ability to suppress NFκB (Abe et al., 1999; Goel et al., 2008b; Surh et al., 2001). We recently examined if curcumin could be used to abrogate the production of cytokines and chemokines that induce an inflammatory microenvironment capable of recruiting HIV target cells to the FGT and directly impair the genital mucosal barrier (Ferreira et al., 2015b). Results of these *in vitro* studies demonstrated that curcumin treatment abrogated the upregulation of pro-inflammatory cytokines TNF-α and IL-6, as well as chemokines IL-8, RANTES and IL-10, and prevented barrier disruption following HIV exposure (Ferreira et al., 2015b). Interestingly, curcumin also inhibited the indirect activation of the HIV-LTR promoter by inflammatory factors in the presence of co-infecting STIs, and exhibited direct anti-viral activity against HSV-2, likely through the suppression of NFκB (Ferreira et al., 2015b). These studies raise the intriguing possibility that modulating the microenvironment in the vaginal tract with curcumin could minimize genital inflammation and/or control against HSV-2 infection *in vivo*, all with relevance to preventing HIV infection in the FGT.

Thus far, the therapeutic potential of curcumin has mainly been examined following systemic administration, but efficacy was found to be limited because it undergoes rapid metabolism and conjugation in the liver (Bansal et al., 2011; Kharat et al., 2017; Schneider

et al., 2015). To address bioavailability concerns, polymeric nanoparticles are being used as oral delivery vehicles, and *Shaikh et al* has shown that encapsulating curcumin in polymer nanoparticles improves peroral bioavailability by at least 9-fold compared to crude curcumin in rodents (Shaikh et al., 2009). Their group also showed that following oral and intraperitoneal routes of delivery, nanocurcumin therapy can delay the progression of cataract development (Grama et al., 2013), protect against inflammatory markers and lipid metabolism in streptozotocin-induced diabetes and have significant beneficial effects in other disease models as well (Devadasu et al., 2011).

Based on these studies, **we hypothesized that curcumin would have an anti-inflammatory effect in the FGT. Furthermore, curcumin treatment would either protect against intravaginal HSV-2 infection or decrease viral replication and pathology *in vivo.*** To address this hypothesis, we administered the curcumin nanoparticle formulation to mice and investigated the treatment conditions favorable for anti-inflammatory and anti-viral efficacy. Anti-inflammatory and anti-viral activity were examined in the context of immunostimulatory TLR9 agonist CpG ODN and HSV-2 infection, respectively.

Methods

Mice. Inbred, sexually matured, 6-8 week old female C57BL/6 mice were obtained from Charles River laboratories (Constant, Quebec, Canada). All mice were housed and

maintained under standard temperature controlled conditions in the Central Animal Facility at McMaster University that followed a 12-hour light/dark cycle. Mice were given low-fat mouse chow and water ad libitum. All animal studies performed were approved by and were in compliance with the Animal Research Ethics Board (AREB) at McMaster University.

Depo-Provera injection and staging. Mice were subcutaneously injected with 2 mg of Depo-Provera (medroxyprogesterone acetate) as per previously published protocols (Kaushic et al., 2003). Vaginal washes were collected daily for 4 consecutive days following Depo-provera injection by pipetting 30 μL of PBS into and out of the vagina 5-6 times. Sample fluid was smeared on glass slides and was examined by light microscopy to determine the stage of the estrous cycle as described previously. The following classification was used for identifying the stage of the cycle: estrus, >90% cornified epithelial cells; diestrus, >75% polymorphonuclear cells; meta-estrus, 50% epithelial cells, 50% polymorphonuclear cells.

Histo-morphology of the vaginal tract. To study the anti-inflammatory effect of curcumin in the vaginal tract, Depo-treated mice were anesthetized and received crude curcumin or curcumin-encapsulated nanoparticles IVAG at various time points prior to IVAG delivery of 25 μg of CpG ODN or water as a control. The CpG ODN used in these studies was ODN 1826, which is 20 nucleotides in length and contains two CpG motifs (5'-TCCATGACGTTCCT-GACGTT-3'). This ODN has previously been delivered

intravaginally for immunostimulatory purposes (Ashkar, 2003; Sajic, D; Ashkar, 2004). After 24 hours, vaginal tissue was removed, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 7 μm for hematoxylin-and-eosin staining. Vaginal tissue morphology was used to assess the anti-inflammatory effects of curcumin pre-treatment. Inflammatory pathology in the vaginal tract was scored on the basis of four parameters, including 1) thickness of the epithelium and extent of inflammatory cell infiltrate within the epithelium 2) inflammatory infiltration in the submucosal tissue, 3) thickness and inflammatory infiltration in the blood vessels and 4) inflammatory infiltration in the luminal space. A four-point scale was used; 0 (-) was indicative of an absence of inflammatory pathology, evidenced by normal epithelium and minimal inflammatory cell infiltrate, and 3 (+++) was indicative of severe inflammatory pathology, evidenced by epithelial thickening and extensive inflammatory cell infiltrate. Average pathology for each experimental group was determined by added the scores of each tissue and dividing by number of animals (n).

Multiplex Cytokine Assay. Mouse vaginal tissue was collected from mice and mechanically homogenized in PBS, using metal beads and the Gold BulletBlender system (Next Advance, Averill Park, New York, USA). Homogenates were centrifuged at 8000 RPM for 5 minutes, and the supernatants were collected and stored at -80° until required. Cytokines and chemokines were quantified in duplicate in the supernatants using the Magpix technology system and the Mouse Cytokine Magnetic Kit 96-Well Plate Assay (Millipore, Billerica, MA, USA), as per the manufacturer's instructions. The following

cytokines and chemokines were measured: TNF-α, IL-6 and MCP-1, and the range of detection of this assay was between analyte concentrations 3.2 and 10,000 pg/mL

HSV-2 primary inoculation. Depo-treated mice were anaesthetized using injectable anesthetic (150 mg of Ketamine/kg with 10 mg of Xylazine/kg) given intraperitoneally at a dose of 0.1 ml/10 g of body weight. Anaesthetized mice were gently swabbed intravaginally (IVAG) with sterile, dry cotton wool. Mice were then treated with either H_2O or 0.6 mg of curcumin-encapsulated nanoparticles IVAG (kindly provided by Dr. Kumar of University of Texas). Two hours later, mice were infected IVAG with 10 μL of the lethal dose (10^4 pfu/mouse) of WT HSV-2 strain 333. After inoculation, mice were placed on their backs for approximately 30-45 minutes to allow for the inoculum to infect the vaginal tract.

Collection of vaginal washes. Vaginal washes were collected daily for 10 consecutive days following HSV-2 infection by pipetting 30 μL of PBS twice consecutively into and out of the vagina 5-6 times to give a total volume of approximately 60 μL and stored at -70°C until use.

Genital pathology. Genital pathology was monitored daily following HSV-2 infection and scored on a 5-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness; 5, severe genital ulceration extending to surrounding tissue. Animals were euthanized by

cervical dislocation when they reached stage 4/5. In order to compare groups, cumulative scores of pathology were determined by tabulating the number of mice with the highest score of pathology they achieved and the number of days that score was observed. Mice that did not survive the challenge were given the highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. The sum of these scores for all the mice was the total level of pathology for each group and then the average pathology score per mouse for each group was calculated.

Viral titers. Viral titers in vaginal washes were determined by viral plaque assay on Vero cells (ATCC, Manassas, USA) monolayers. Vero cells were grown in supplemented α-Medium Essential Medium (α-MEM) (Cat #12000063; GIBCO Laboratories, Burlington, Canada) supplemented with 5% fetal bovine serum (FBS; Cat #16000-044; GIBCO, Burlington, Canada), 1% penicillin-streptomycin (Cat #15140-122; Invitrogen, Burlington, Canada), L- glutamate (Cat #56-85-9; BioShop Canada Inc., Burlington, Canada), and 1% HEPES (Cat #15630-080; Invitrogen, Burlington, Canada). For plaque assays, Vero cells were grown to confluency in 12-well plates (Becton Dickson, Oakville, Ontario). Vaginal lavages were thawed on ice and samples were diluted (10^{-2} to 10^{-7}) in FBS-free α-MEM and added to Vero cell monolayers. Infected monolayers were incubated at 37°C and rocked to ensure even distribution of virus across monolayers every 15 minutes for 2 hours, at which point monolayers were overlaid with α-MEM supplemented with 5% FBS to stop cellular adsorption of virus. Cells were fixed and stained with crystal violet 48 hours later, and viral

plaques were counted under a light microscope. The number of PFU per millimeter was calculated using the plaque count per sample and accounting for the dilution factor.

Statistical analysis. Statistical analysis and graphical representation was performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). The Mantel-Cox log-rank test was used to calculate significant differences in survival. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test was used to calculate significant differences in cytokine levels.

Results

Anti-inflammatory efficacy in vaginal tissue is absent with systemic routes of curcumin delivery. We first tested the anti-inflammatory activity of curcumin-encapsulated nanoparticles in the vaginal tract. CpG oligodeoxynucleotides (CpG ODN) act as immunostimulants and potently activate the innate immune system, including dendritic cells, macrophages and natural killer (NK) cells. In a study by *Ashkar et al.,* local delivery of CpG ODN was used to assess the ability of TLR-mediated innate immunity to protect against HSV-2 infection in the vaginal tract (Ashkar et al., 2004; Sajic et al., 2003). Although Type I IFN responses were responsible for mediating protection, inflammatory transcription factor NFκB was also induced in response to CpG, resulting in rapid thickening of the vaginal epithelium and significant CC11b+ and NK1.1+ immune cell infiltrate (Ashkar et al., 2004). This was therefore used as an animal model of acute

inflammation in the genital tract, and these histo-morphological indicators of inflammation were used to assess and quantify the anti-inflammatory activity of curcumin.

Depo-treated WT C57BL/6 mice received a single 1.25 mg dose of curcumin-encapsulated nanoparticles (50 mg/kg body weight) or H_2O *via* oral gavage or IP injection 4 hours prior to vaginal delivery of CpG ODN (30 μg). Previous data indicate that blood levels of curcumin peak between 2 and 4 hours following oral delivery (Shaikh et al., 2009). Twenty-four hours following delivery of CpG, mice were sacrificed and vaginal tissue was collected for H&E staining (Figure 1). There was no significant difference in average inflammatory pathology between untreated positive controls (CpG only) (Figure 1A, Table 1; average score=1.67) and mice that received curcumin-encapsulated nanoparticles *via* IP (Figure 1B, Table 1; average score =1.15) or oral (Figure 1D, Table 1; average score = 1.67) routes of delivery prior to CpG. Vehicle-only nanoparticle preparations *via* IP injection (Figure 1C, Table 1; average score = 1.2) and oral gavage (Figure 1E, Table 1; average score 1.16) were also tested alongside their respective treatment groups and found to have no anti-inflammatory activity. Higher doses of curcumin-encapsulated nanoparticles, including 2.4 and 4.8 mg, were also tested with IP injection and no change in inflammation was observed (Table 1; average scores = 1.16 and 1.5, respectively). Notably, negative control mice that did not receive CpG showed complete absence of tissue inflammation (Figure 1F, Table 1; average score = 0.33). Our results indicate that curcumin did not exert anti-inflammatory effects in the vaginal tract with systemic routes of delivery, thus, alternate routes were explored in subsequent experiments.

Nanoparticles are localized within the vaginal tissue following topical application in the genital tract. Given the lack of any effect following systemic delivery, we examined the tissue distribution pattern of curcumin following IP and oral delivery to see if that could explain the lack of local anti-inflammatory activity in the vaginal tissue. To image tissue distribution of the nanoparticles, we used fluorescent poly(lactic-co-glycolic acid) (PLGA) particles. Depo-treated mice received either H_2O (Figure 2A) or PLGA particles (Figure 2B and 2C) *via* IP injection. Vaginal tissue was collected at 2 (Figure 2B) and 24 (Figure 2C) hours following PLGA delivery, and the nanoparticles were absent in the vaginal tissue at both time points. Conversely, the nanoparticles were well absorbed and retained within the vaginal epithelium following intravaginal delivery (Figure 2D). Thus, subsequent experiments tested the anti-inflammatory efficacy of curcumin-encapsulated nanoparticles following intravaginal delivery.

Curcumin reduces inflammatory pathology in the vaginal tissue following intravaginal delivery. Since oral and IP routes of curcumin administration did not dampen CpG-mediated inflammation in the vaginal tract and intravaginal administration showed promising tissue retention, we subsequently tested local delivery of curcumin. Depo-treated mice received either (A) H_2O + H_2O (negative controls), (B) 0.5 mg of curcumin-loaded nanoparticles + CpG, (C) 0.5 mg of vehicle-only nanoparticles + CpG (D) 1 mg of crude curcumin + CpG or (E) H_2O + CpG (positive controls). Mice received intravaginal curcumin treatments 2 hours prior to intravaginal inoculation with CpG. Average inflammatory pathology was measured, and mice that received the curcumin-encapsulated

nanoparticles (Figure 3B, Table 2; average score =0.5) showed lower tissue inflammation compared to mice that received vehicle-only nanoparticles (Figure 3C, Table 2; average score=1.67), crude curcumin (Figure 3D, Table 2; average score = 3) or positive controls (Figure 3E, Table 2; average score=3). In fact, tissue inflammation in animals that were treated with the curcumin-encapsulated nanoparticles prior to CpG was comparable to negative controls that did not receive CpG. Vaginal tissue was also collected for cytokine analysis and curcumin treatment resulted in significantly lower concentrations of pro-inflammatory cytokines TNF-α, MCP-1 and IL-6 in both the cervical (Figure 4A) and vaginal (Figure 4B) tissues compared to positive controls. This demonstrated that curcumin can mediate significant anti-inflammatory activity in the genital tissue following intravaginal delivery. Interestingly, treatment with vehicle-only nanoparticles also marginally decreased cytokine levels, likely due to a non-specific physical effect of the nanoparticles themselves, however the effect of curcumin-loaded nanoparticles was significantly greater.

Curcumin-encapsulated nanoparticles do not confer protection against lethal or sub-lethal primary intravaginal HSV-2 challenge. Previous *in vitro* studies revealed that curcumin treatment significantly decreases HSV-2 replication in primary genital epithelial cells through the inhibition of NFκB. Since we observed anti-inflammatory efficacy following intravaginal delivery of curcumin, we next tested if this treatment results in protection against lethal HSV-2 infection. Depo-treated mice received a single 0.5 mg dose of curcumin-encapsulated nanoparticles *via* intravaginal delivery 2 hours prior to

inoculation with a lethal dose of wildtype (WT) HSV-2 333. Survival (Figure 5A), genital pathology (Figure 5B) and viral shedding (Figure 5C) were monitored post-challenge. All mice treated with curcumin showed extensive disease pathology and a similar viral-load set point compared to curcumin untreated WT virus infected positive controls, indicating that intravaginal curcumin treatment offered no apparent anti-viral protection against HSV-2. We subsequently conducted a similar experiment using a sub-lethal dose of virus to investigate the ability of curcumin to mediate subtler anti-viral effects, such as delayed disease pathology or reduced viral replication. However, no difference in survival (Figure 6A), pathology (Figure 6B) or shedding (Figure 6C) was observed between untreated and curcumin-treated mice, despite the lower viral dose. This suggests that the anti-inflammatory effects of curcumin do not translate to anti-viral activity against HSV-2 *in vivo* regardless of the viral dose at challenge.

Discussion

We have described in this study a system of curcumin delivery that offers protection against genital inflammation *in vivo*. Our results show that topical delivery of curcumin-encapsulated nanoparticles directly into the vaginal tract abrogated CpG-induced inflammatory pathology and the production of pro-inflammatory cytokines IL-6, TNF-α and MCP-1. This effect was absent with traditional systemic routes of delivery, including oral and intraperitoneal, likely because of limited dissemination of the nanoparticles to the vaginal tract. Despite potent anti-inflammatory activity, prophylactic administration of

curcumin did not reduce HSV-2 acquisition or the viral-load set point, as compared with the vehicle-only control group. This lack of efficacy observed was consistent across both lethal and sub-lethal doses of viral challenge in our mouse model, suggesting that the *in vitro* anti-viral properties observed previously do not translate *in vivo* under the conditions tested. The reason for the lack of efficacy is unclear but it highlights the need for testing new interventions in more physiologically relevant but complex in vivo models than pure epithelial cells in culture. Furthermore, the anti-inflammatory effects of curcumin are more likely to be beneficial for the prevention HIV than HSV-2, as HIV risk is closely correlated with genital inflammation and unlike HSV-2, HIV does not directly infect epithelial cells.

It has become exceedingly clear that mucosal inflammation enhances the rate of sexual transmission of HIV in the FGT (Alcaide et al., 2017; Arnold et al., 2016; Masson et al., 2015; Passmore et al., 2016). Recently, Masson *et al* observed a three-fold increased risk of HIV infection in South African women who had elevated levels of at least five mucosal pro-inflammatory cytokines (Masson et al., 2015). Furthermore, Arnold *et al* found increased frequencies of CD4+ T cells in the endocervix of women with pro-inflammatory cytokine profiles, which is relevant as HIV preferentially infects CD4+ T cells (Arnold et al., 2016). Indeed, an innate and adaptive inflammatory cascade in response to viral exposure in the FGT is necessary for the recruitment of target cells to the portal of entry and the establishment of a productive, systemic infection (Li et al., 2009). Our lab has also shed light on the mechanisms by which inflammation can facilitate viral transmission, in that exposure to HIV envelope protein gp120 *in vitro* resulted in

impairment of barrier function and significant viral transmission across the genital epithelium (Nazli et al., 2010; Nazli et al., 2013). Given its potent anti-inflammatory properties, we previously examined if curcumin could be used to abrogate inflammatory processes *in vitro* that facilitate HIV acquisition in the FGT. Results of these *in vitro* studies demonstrated that curcumin treatment abrogated the upregulation of pro-inflammatory cytokines and chemokines, prevented barrier disruption following HIV exposure and significantly decreased HIV replication in chronically infected T cells (Ferreira et al., 2015b). However, the efficacy and practical applications of curcumin in the genital tract *in vivo* were unclear.

This study is one of few to investigate the efficacy of topical application of curcumin to the vaginal tract (Bourne et al., 1999; Lakshmi et al., 2016). Although curcumin offered impressive benefits in *in vitro* preclinical studies, the translation into *in vivo* conditions has been very poor and the doses often tested are unfeasible to administer clinically (Prasad et al., 2014). Lack of clinical success with curcumin is often linked to (a) poor solubility and (b) extensive intestinal and hepatic metabolic biotransformation resulting in poor oral bioavailability (Prasad et al., 2014). Thus, the focus of investigators has been to improve the therapeutic efficacy of curcumin *in vivo* by addressing both factors (Prasad et al., 2014). Polymeric nanoparticles have been actively explored as oral delivery vehicles for pharmaceutically challenging compounds. Shaikh *et al* has shown that encapsulating curcumin in polymer nanoparticles improved peroral bioavailability of curcumin by at least 9-fold compared to that of crude curcumin in rodents (Shaikh et al.,

2009). They have since published the ability of this nanocurcumin to delay the progression of diabetic cataracts in rats with significantly higher therapeutic efficacy than crude curcumin (Grama et al., 2013). In more recent years, the therapeutic focus of curcumin nanoformulations has been disease profiles with an inflammatory link. Wang et al. showed that curcumin-solid lipid nanoparticles, administered by IP injection, effectively suppressed airway hyperresponsiveness, inflammatory cell infiltration and expression of IL-4 and IL-13 in bronchoalveolar lavage fluid in the animal model of asthma (Wang et al., 2012). The administration route of choice by the majority of other studies investigating similar therapeutic potentials is systemic, either by oral or intraperitoneal delivery (Suzuki et al., 2009; Tamaddonfard et al., 2012; Ung et al., 2010; Young et al., 2014; Yu et al., 2011; Zunino et al., 2013). Our study is one of few to investigate local administration with topical application of curcumin to the genital tissue, and we conclude that this method of delivery results in significant anti-inflammatory benefits to the target area, which were absent following systemic delivery. Our results suggest that the curcumin nanoparticles are unable to reach the genital tissue in sufficient quantities by oral and IP delivery, which likely explains the poor anti-inflammatory efficacy observed in vaginal tissue.

The use of crude curcumin as a therapeutic in patients with HIV has been investigated in the past. A clinical trial in 40 patients over an eight-week period showed no significant reduction or elevation in patients' viral load or CD4+ T cell counts, respectively, following oral administration of curcumin (James, 1996). However, we suspect that a cream formulation containing curcumin nanoparticles that will circumvent absorption issues and

allow for retention of curcumin within the genital tract will offer the most desirable therapeutic outcome in clinical trials. There has also been renewed interest in the use of intravaginal rings to deliver pharmaceutical products into the genital tract (Thurman et al., 2013). These rings are designed to deliver sustained drug doses for extended periods of time while bypassing first pass metabolism in the gut (Thurman et al., 2013). They have already been established as an effective system to delivery hormonal contraceptives, but could be combined with microbicidal compounds like curcumin as a multipurpose prevention technology (MPT), offering protection against both unintended pregnancy and sexually transmitted infections like HIV (Thurman et al., 2013). However, as with any intravaginal formulation, appropriate tissue concentrations of drugs that are pharmacodynamically active will have to be delivered, such that there is no mucosal alterations or inflammation, and no change in the resident microbiota. These are all factors that should be investigated in a clinical setting before the implementation of either curcumin nanoparticles or curcumin-containing intravaginal rings into clinical trials for the prevention of HIV transmission in women.

Curcumin may also play a significant role in preventing or reducing chronic immune activation when delivered systemically. The gut mucosa is where the most abundant populations of CD4+ immune cells are found and loss in intestinal barrier function and massive CD4+ T cell depletion occur early in the course of infection. Translocation of the intestinal microbiota occurs as a result and is strongly believed to be one of the main contributors to chronic immune activation, characterized by increased

inflammatory markers and immune cell activation that persists even in HAART-treated individuals (Brenchley et al., 2006; Tincati et al., 2016). Paradoxically, this immune activation is a cause of and also one of the main driving forces of CD4+ T-cell depletion and HIV replication in the gut (Appay and Sauce, 2008; Klatt et al., 2013b). Repeated exposure to HIV gp120 in mucosal tissues from shed virus or from unbound gp120 may provide a continuous source for generating inflammation in mucosal tissues that facilitates barrier breakdown and microbial translocation (Nazli et al., 2010; Passmore et al., 2016). Since microbial translocation and immune activation tend to occur in the chronic stages of infection, the window of opportunity to intervene and limit these processes is likely in the immediate stages following exposure and during acute infection (Tincati et al., 2016). Using curcumin to block inflammatory pathways in the gut that lead to intestinal barrier disruption, microbial translocation and immune activation could reduce the dissemination of infection and the chronic inflammatory state that contributes to HIV pathology.

Conclusion

In conclusion, our results indicate the promising potential of a topical curcumin formulation as a potent and local anti-inflammatory in the genital tract. Genital inflammation can increase HIV risk in women, as well as contribute to the sequelae of chronic HIV infection. Thus, this curcumin nanoparticle formulation could work in tandem with current prophylactic or treatment anti-retroviral strategies and have a significant impact on HIV infection and disease progression.

Figure 1

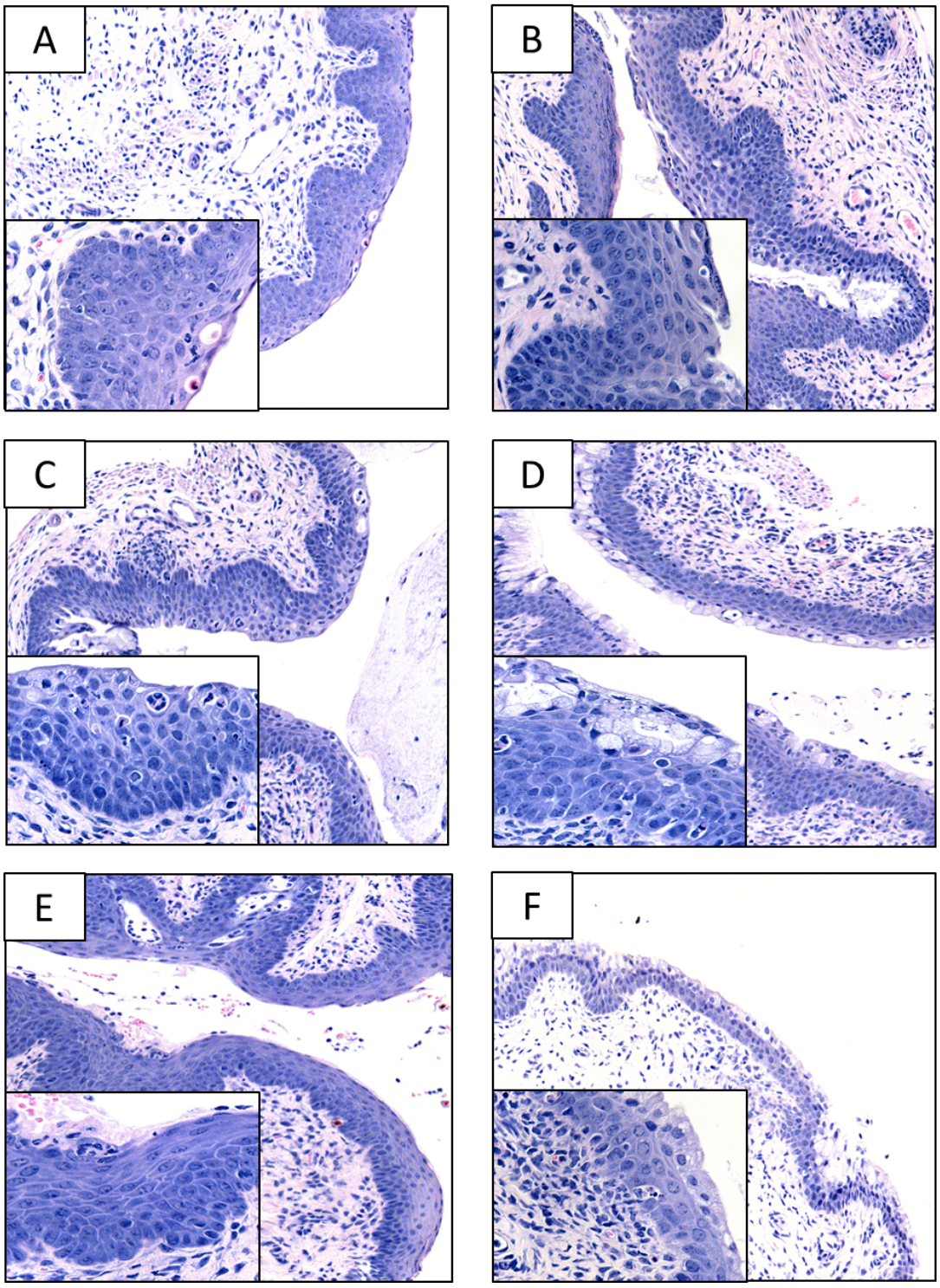


Figure 1. Anti-inflammatory efficacy of curcumin-encapsulated nanoparticles following IP and oral delivery. Depo-treated mice received either **(A)** H_2O + CpG (positive controls), **(B)** 1.25 mg of curcumin-loaded nanoparticles intraperitoneally (IP) + CpG, **(C)** 1.25 mg of vehicle-only nanoparticles IP + CpG, **(D)** 1.25 mg of curcumin-loaded nanoparticles orally + CpG or **(E)** 1.25 mg of vehicle-only nanoparticles orally + CpG. Mice received treatments 4 hours prior to intravaginal inoculation with CpG ODN (30 μg) and were compared to negative control mice that did not receive CpG **(F)**. Vaginal tissues were fixed, sectioned and stained with H&E. Representative images from multiple experiments with three to four animals per condition are shown (Magnification 10x and 40x).

Figure 2

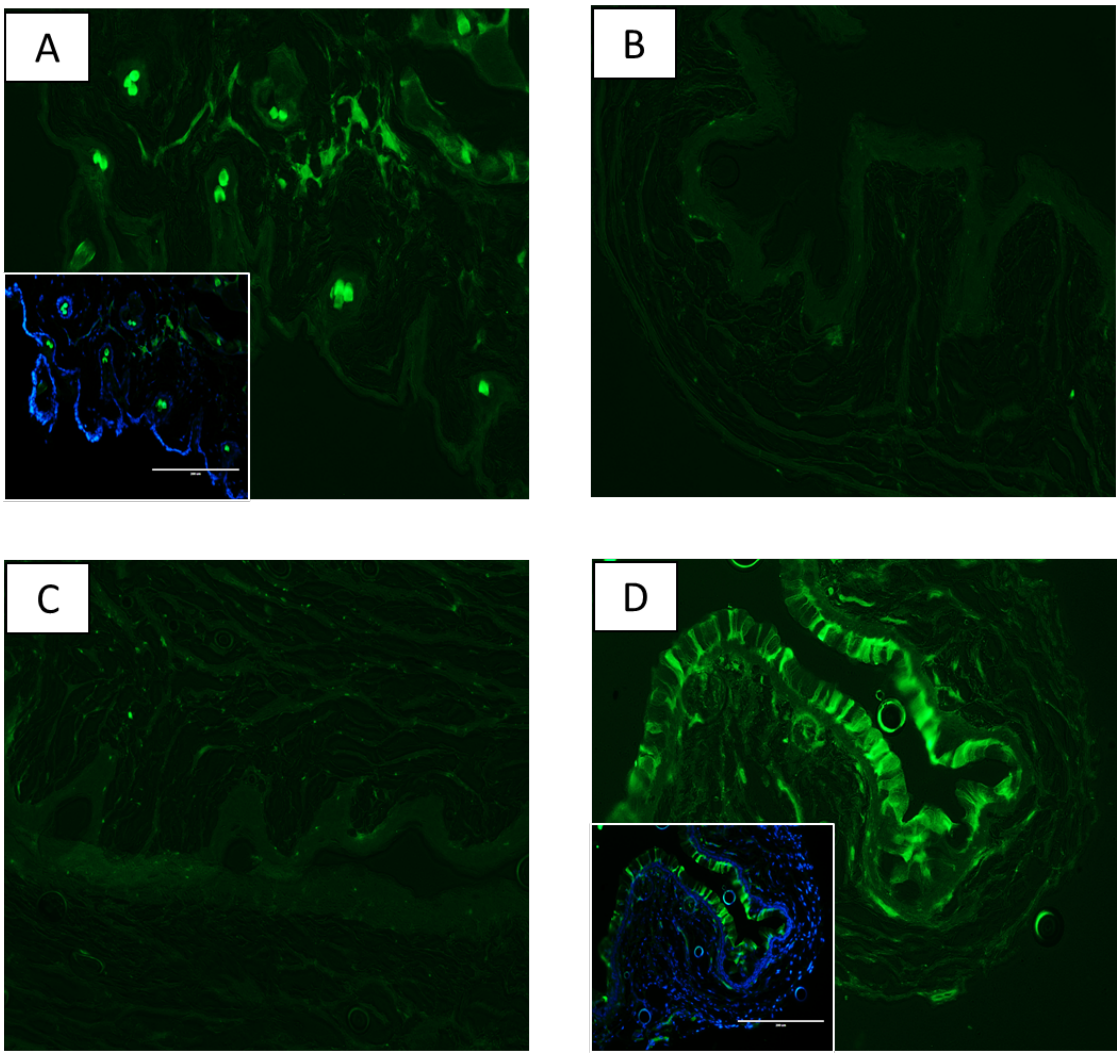


Figure 2. Vaginal tissue distribution of nanoparticle preparations following IP and intravaginal delivery. Mice received either H_2O (A) or 1.25 mg of PLGA-Fluorescein nanoparticles IP and vaginal tissue was excised after 2 (B) or 24 hours (C) following delivery. Positive control mice received 0.5 mg of PLGA-Fluorescein nanoparticles intravaginally (D) and vaginal tissue was excised 2 hours following delivery. Vaginal tissues were sectioned and observed under an EVOS fluorescent microscope. Representative images from a single experiment with three animals per condition are shown (Magnification 20x).

Figure 3

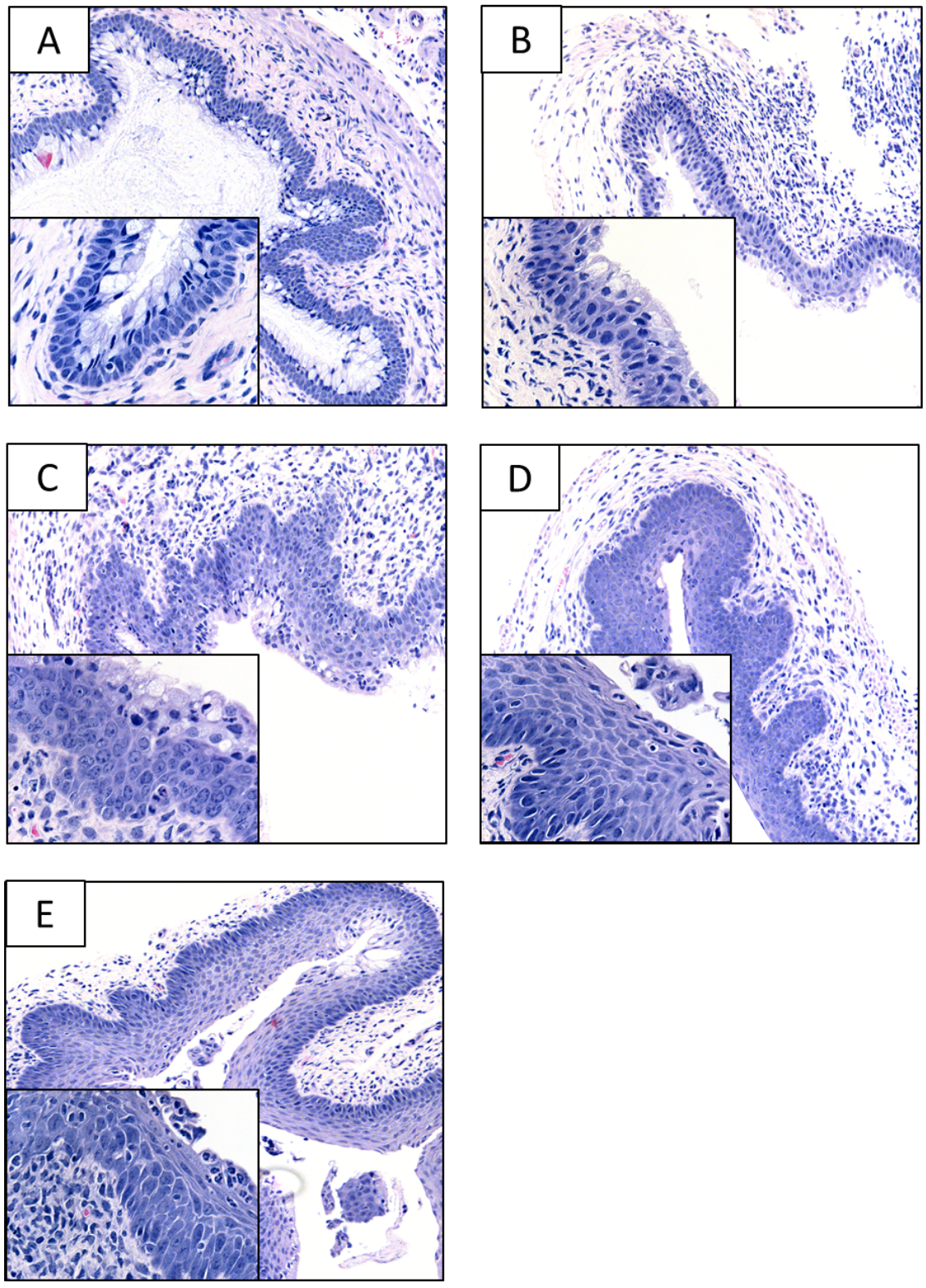


Figure 3. Anti-inflammatory effects of curcumin-encapsulated nanoparticles on tissue pathology following intravaginal delivery. Depo-treated mice received either (A) H_2O + H_2O (negative controls), (B) 0.5 mg of curcumin-loaded nanoparticles + CpG, (C) 0.5 mg of vehicle-only nanoparticles + CpG (D) 1 mg of crude curcumin + CpG or (E) H_2O + CpG (positive controls). Mice received treatments 2 hours prior to intravaginal inoculation with CpG ODN (30 μg). Vaginal tissues were sectioned and stained with H&E. Representative images from multiple experiments with four animals per condition are shown (Magnification 10x and 40x).

Figure 4

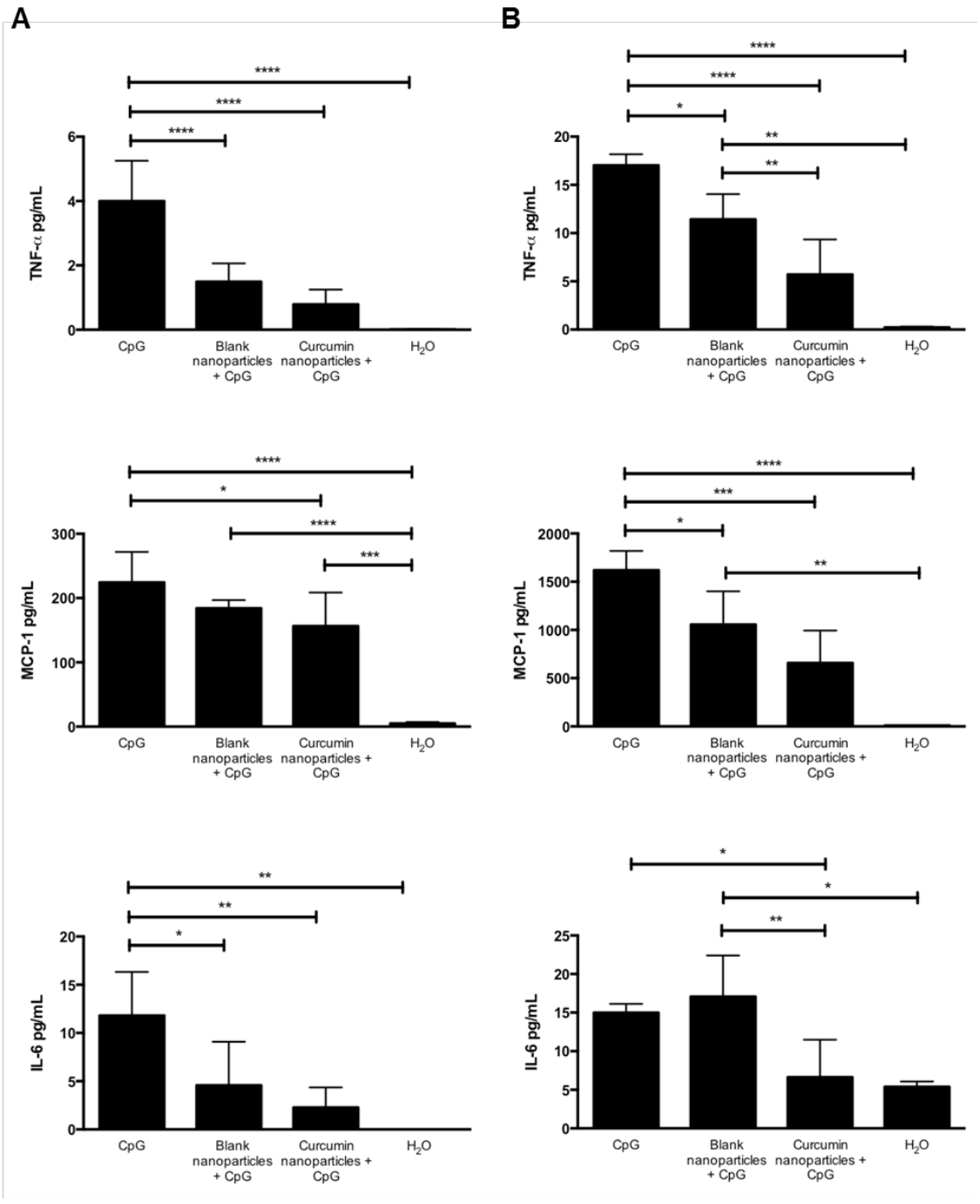
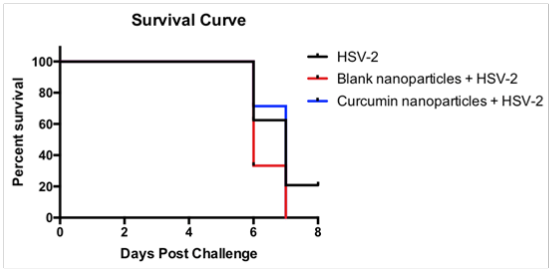

A
B
TNF-α pg/mL
MCP-1 pg/mL
IL-6 pg/mL
CpG
Blank nanoparticles + CpG
Curcumin nanoparticles + CpG
H2O

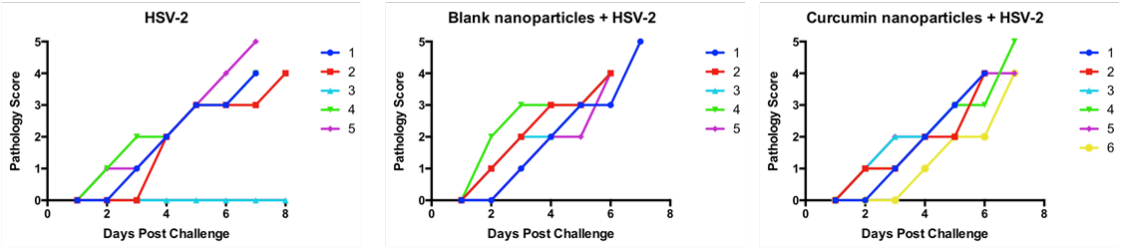
Figure 4. Anti-inflammatory effects of curcumin-encapsulated nanoparticles on inflammatory cytokine production following intravaginal delivery. Depo-treated mice received either H_2O, vehicle-only nanoparticles or curcumin-loaded nanoparticles 2 hours prior to inoculation with 30 μg of CpG ODN. Negative control mice received H_2O in the absence of CpG. Vaginal tissues were excised and homogenized using the Bullet Blender (Next Advance, New York). Magpix technology system was used to quantify the following cytokine and chemokines in duplicate in the homogenate supernatants: TNF-α, MCP-1 and IL-6, and are expressed as pg/mL of cytokine/chemokine production. Data was analyzed by one-way ANOVA * $p<0.05$, ** $p<0.01$, *** $p<0.001$, $p<0.0001$. Data includes mean pg/mL of three to four animals per experimental condition. Panel A shows cytokine levels in cervical tissue and panel B shows cytokine levels in vaginal tissue.

Figure 5

A


Survival Curve
HSV-2
Blank nanoparticles + HSV-2
Curcumin nanoparticles + HSV-2
Percent survival
Days Post Challenge

B


HSV-2
Blank nanoparticles + HSV-2
Curcumin nanoparticles + HSV-2
Pathology Score
Days Post Challenge

C

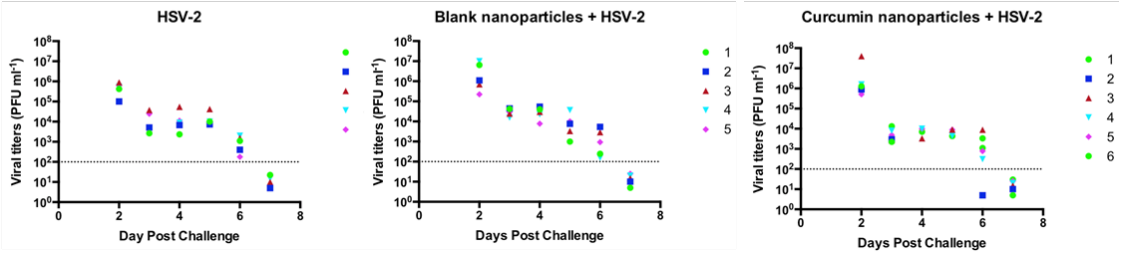

HSV-2
Blank nanoparticles + HSV-2
Curcumin nanoparticles + HSV-2
Viral titers (PFU ml-1)
Day Post Challenge
Days Post Challenge

Figure 5. Anti-viral efficacy of curcumin-encapsulated nanoparticles following lethal primary intravaginal HSV-2 challenge. Depo-treated mice received either H_2O, 0.6 mg vehicle-only nanoparticles or 0.6 mg curcumin-loaded nanoparticles 2 hours prior to inoculation with WT HSV-2 (10^4 pfu/mouse, n=5 per group). (A) Survival was monitored and (B) pathology scores were recorded for 7 days following challenge and plotted. (C) Vaginal washes were collected for 7 days following exposure from all groups and viral plaque assays were conducted, as described in Material and Methods. Plaques were counted and viral titers were expressed as pfu/mL. Each symbol represents a single animal. The dashed line in (C) shows the lower detection limit of the assay, and data points below this line indicate undetectable viral shedding.

Figure 6

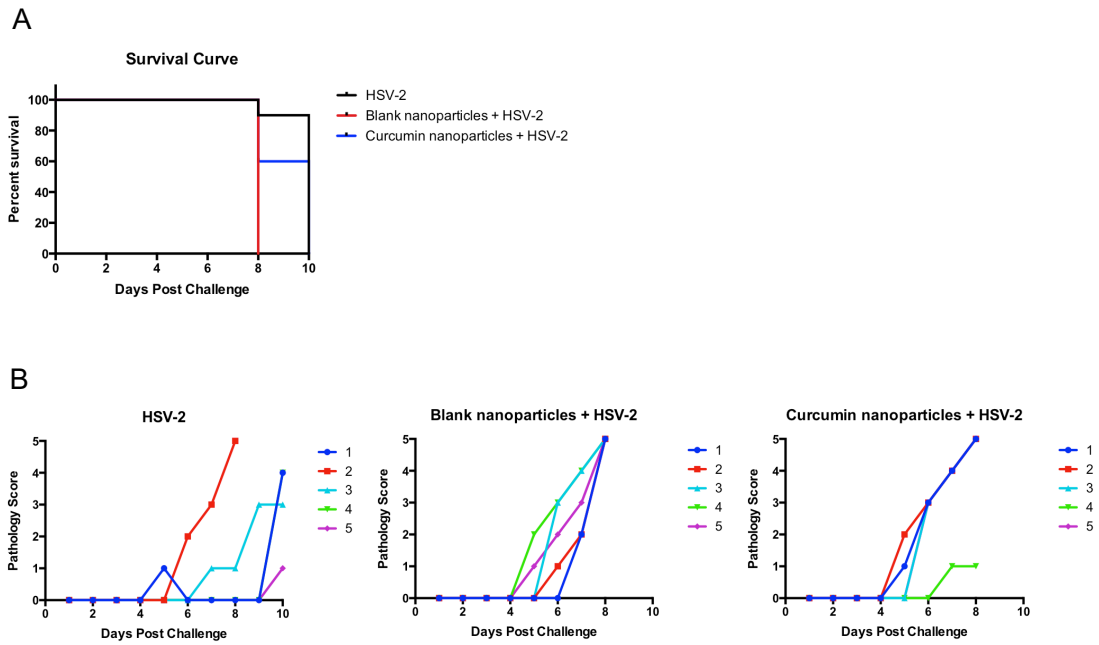

A
Survival Curve
Percent survival
Days Post Challenge
HSV-2
Blank nanoparticles + HSV-2
Curcumin nanoparticles + HSV-2
B
HSV-2
Blank nanoparticles + HSV-2
Curcumin nanoparticles + HSV-2
Pathology Score
Days Post Challenge
1
2
3
4
5

Figure 6. Anti-viral efficacy of curcumin-encapsulated nanoparticles following sub-lethal primary intravaginal HSV-2 challenge. Depo-treated mice received either H_2O, 0.6 mg vehicle-only nanoparticles or 0.6 mg curcumin-loaded nanoparticles 2 hours prior to inoculation with WT HSV-2 (10^3 pfu/mouse, n=5 per group). **(A)** Survival was monitored and **(B)** pathology scores were recorded for 7 days following challenge and plotted.

Table 1. Cumulative scores of tissue inflammation following oral and IP delivery of curcumin-loaded nanoparticles prior to inoculation with CpG.

Curcumin Preparation	Curcumin Dose	Route of Administration	Mouse	Grade	Score	Group Average
CpG only positive control (representative group)			1	++	2	1.67
			2	+	1	
			3	++	2	
H_2O negative control (representative group)			1	-	0	0.33
			2	-	0	
			3	+	1	
Curcumin-loaded nanoparticles	1.2 mg	Oral	1	+	1	1.67
			2	+	1	
			3	++	2	
			4	++	2	
			5	++	2	
			6	++	2	
		Intraperitoneal	1	++	2	0.8
			2	-	0	
			3	-	0	
			4	++	2	
			5	-	0	
	2.4 mg	Intraperitoneal	1	++	2	1.16
			2	+	1	
			3	++	2	
			4	+	1	
			5	+	1	
			6	-	0	
	4.8 mg	Intraperitoneal	1	-/+	0.5	1.5
			2	++	2	
			3	++	2	
Vehicle-only nanoparticles	1.2 mg	Oral	1	+	1	1.16
			2	++	2	
			3	++	2	
			4	+	1	
			5	+	1	
			6	-	0	
		Intraperitoneal	1	+	1	1.2
			2	++	2	
			3	+	1	
			4	++	2	
			5	-	0	

Table 2. Cumulative scores of tissue inflammation following intravaginal delivery of curcumin-loaded nanoparticles prior to inoculation with CpG.

Treatment	Mouse	Grade	Score	Group Average
CpG only positive control (representative group)	1	+++	3	3
Crude Curcumin	1	+++	3	3
Vehicle-only nanoparticles + CpG	1	+	1	1.67
	2	++	2	
	3	++	2	
Curcumin-loaded nanoparticles + CpG	1	-	0	0.5
	2	+	1	
	3	-	0	
	4	+	1	
H_2O negative control (representative group)	1	-	0	0

CHAPTER 3

APPENDIX

This appendix summarizes preliminary experiments that investigated the anti-viral effects of crude curcumin and curcumin polymer formulations when administered as a pre-treatment or in direct combination with HSV-2. This data was excluded from the manuscript because the curcumin preparations used were very crude, only partially solubilized and the efficacy observed was non-specific, likely due to physical barrier effects of the formulations.

Results

We initially conducted three preliminary experiments examining the anti-viral activity of crude curcumin administered in direct combination with HSV-2. In the first experiment, all of curcumin-treated mice survived the challenge dose, while only 40 percent of untreated controls survived (Appendix 1; Figure 1A, $p<0.05$). Strikingly, none of the curcumin-treated mice showed HSV-2 associated disease pathology (Appendix 1; Figure 1B) or viral shedding in vaginal washes (Appendix 1; Figure 1C), whereas 60 percent of untreated mice showed disease pathology and 80 percent of controls showed viral shedding, respectively. This experiment was repeated three times and similar results were observed, in that treatment with curcumin resulted in better outcomes in the context of lethal HSV-2 challenge compared to untreated controls.

Although these experiments offered promising results, the curcumin preparations used were very crude (more than 80% curcumin; Sigma Aldrich) and only partially solubilized. We subsequently explored the efficacy of various formulations of curcumin for intravaginal delivery. Dispersion of curcumin in an amorphous polymer matrix/carrier at solid state, which is one of the most widely and successfully applied methods to improve the solubility, dissolution rates and bioavailability of poorly soluble drugs, enhanced the solubility of curcumin by over 1000-fold in two different formulations. The first formulation, curcumin Soluplus®, uses the carrier polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer, a matrix polymer that possesses both hydrophilic and lipophilic properties and has a high viscosity when solubilized (Mogal,

2012). The second formulation, curcumin PVP K 30, uses the carrier polyvinylpyroline K 30, a hydrophilic matrix polymer that has a low viscosity when solubilized (Kaewnopparat, 2009). We tested the ability of lower doses of these formulations to offer similar or better protection as the crude curcumin in our model. We also tested vehicle-only preparations, Blank Soluplus and Blank PVP K 30, to determine the specificity of curcumin in our model. Curcumin and vehicle-only preparations were kindly provided by Dr. Garg at the School of Pharmacy and Medical Sciences at the University of South Australia. Given their increased solubility, we estimated that 100 μg/mouse of either formulation would exceed or match the biologically availability quantity administered in the previous experiments (1 mg). Mice were challenged with 5 x 10^4 pfu/mouse of WT HSV-2 alone or in direct combination with crude curcumin, curcumin formulations with enhanced solubility or their respective vehicle-only controls. All mice that received crude curcumin, curcumin Soluplus or curcumin PVP K 30 survived the viral challenge (Appendix 1; Figure 2A) with complete absence of disease pathology (Appendix 1; Figure 2B) and shedding (Appendix 1; Figure 2C). In fact, combining survival and shedding data from all experiments indicated significantly greater percent survival (Appendix 1; Figure 3A, $p<0.05$) and significantly lower percent mice shedding virus (Appendix 1; Figure 3B, $p<0.01$) in curcumin-treated groups compared to untreated controls.

Despite being tested at a much lower dose, the curcumin formulations offered the same level of efficacy as the crude curcumin, suggesting they do in fact have improved anti-viral activity. However, the vehicle-only preparations (Blank Soluplus and Blank PVP

K 30) also offered complete protection against viral challenge, indicating that the effects of both curcumin formulations with enhanced solubility (Soluplus and PVP K 30) were likely non-specific rather than targeted and anti-viral. It is unclear if the protection offered by the crude curcumin is also non-specific, but one way in which both the crude and formulations may offer non-specific protection is by physically obstructing viral access to the genital epithelium with this method of intervention. While it is still possible that curcumin is a direct-acting anti-viral, this method of intervention does not allow us to differentiate between physical barrier effects and anti-viral effects in our model.

Thus far, the efficacy of curcumin had only been investigated when delivered *in direct* combination with HSV-2; however, pre-treatment with curcumin is a far more clinically relevant method of intervention. It can also test the anti-viral effect of the formulations beyond the direct physical barrier effect. Mice received crude curcumin at 4, 6 and 10 hours prior to inoculation with 5 x 10^4 pfu/mouse of WT HSV-2. Unlike with the direct combination method of intervention, pre-treatment with curcumin did not offer any protection against viral challenge. None of the mice that received crude curcumin at 4, 6 and 10 hours prior to viral challenge survived (Appendix 1; Figure 4A), and all showing extensive disease pathology (Appendix 1; Figure 4B) and viral shedding (Appendix 1; Figure 4A) (pathology and shedding data for 4 hour time point not shown). Similarly, none of the mice that received curcumin formulations (Soluplus and PVP K 30) at 6 hours prior to viral challenge survived (Appendix 1; Figure 4). We concluded that pre-treatment with crude curcumin or the curcumin formulations is not efficacious in our model, and the

efficacy observed with the first method of intervention is likely a result of either physical barrier or direct-acting anti-viral effects. Interestingly, pre-treatment groups did show delayed progression to HSV-2 associated endpoint compared to untreated controls, whereby pre-treatment mice did not reach endpoint until days 10-20 while all untreated controls reached endpoint by day 7 (Appendix 1; Table 1).

Subsequent experiments, tested pre-treatment efficacy with an improved curcumin-encapsulated nanoparticle formulation with higher solubility than previously tested formulations, and we did not observe a similar delay in pathology progression (Chapter 3; Figure 5 and 6). These results offered a likely explanation for the delay in pathology observed in previous experiments. Crude curcumin, curcumin Soluplus and curcumin PVP K 30 were only partially solubilized compared to the nanocurcumin formulation. Furthermore, because of low solubility, the epithelial absorption was likely lower, the amount of unabsorbed curcumin retained within the vaginal lumen was likely higher, and physical barrier effects were likely greater compared to the nanocurcumin formulation. This may explain why we observed better outcomes following pre-treatment with crude curcumin, curcumin Soluplus, and curcumin PVP K 30. Nevertheless, we conclude that curcumin does not have anti-herpetic activity *in vivo* under the conditions tested.

Figure 1

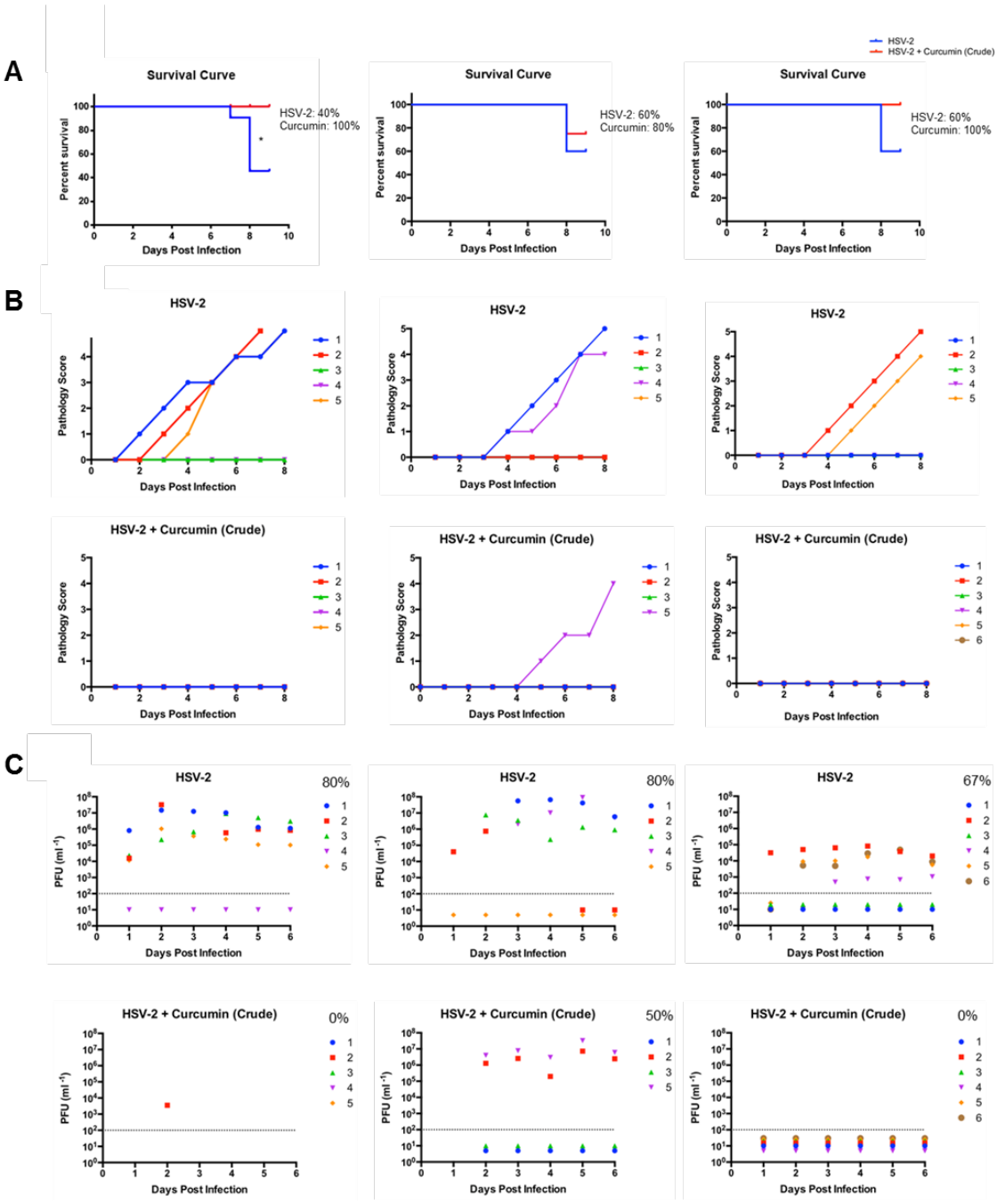

A
B
C
Survival Curve
Percent survival
Days Post Infection
HSV-2: 40%
Curcumin: 100%
HSV-2: 60%
Curcumin: 80%
HSV-2: 60%
Curcumin: 100%
HSV-2
HSV-2 + Curcumin (Crude)
Pathology Score
PFU (ml⁻¹)
80%
80%
67%
0%
50%
0%

Figure 1. Crude curcumin confers protection against primary intravaginal HSV-2 challenge. Depo-treated wildtype mice were exposed to the lethal dose of WT HSV-2 (5 x 10^4 pfu/mouse) alone or in the direct combination with 1 mg of crude curcumin (n=4-6/group), in three replicate experiments. **(A)** Survival was monitored and **(B)** pathology scores were recorded for 10 days following challenge and plotted. **(C)** Vaginal washes were collected for 6 days post-exposure from both groups and viral plaque assays were conducted, as described in Material and Methods. Plaques were counted and viral titers were expressed as pfu/mL. Each symbol represents a single animal. The dashed line in **(C)** shows the lower detection limit of the assay, and data points below this line indicate undetectable viral shedding. The % indicates survival in A and the maximum number of mice that shed virus on any given day **(C)**. Significance was analyzed using the Log-rank (Mantel-Cox) test (*p<*0.05)*.

Figure 2

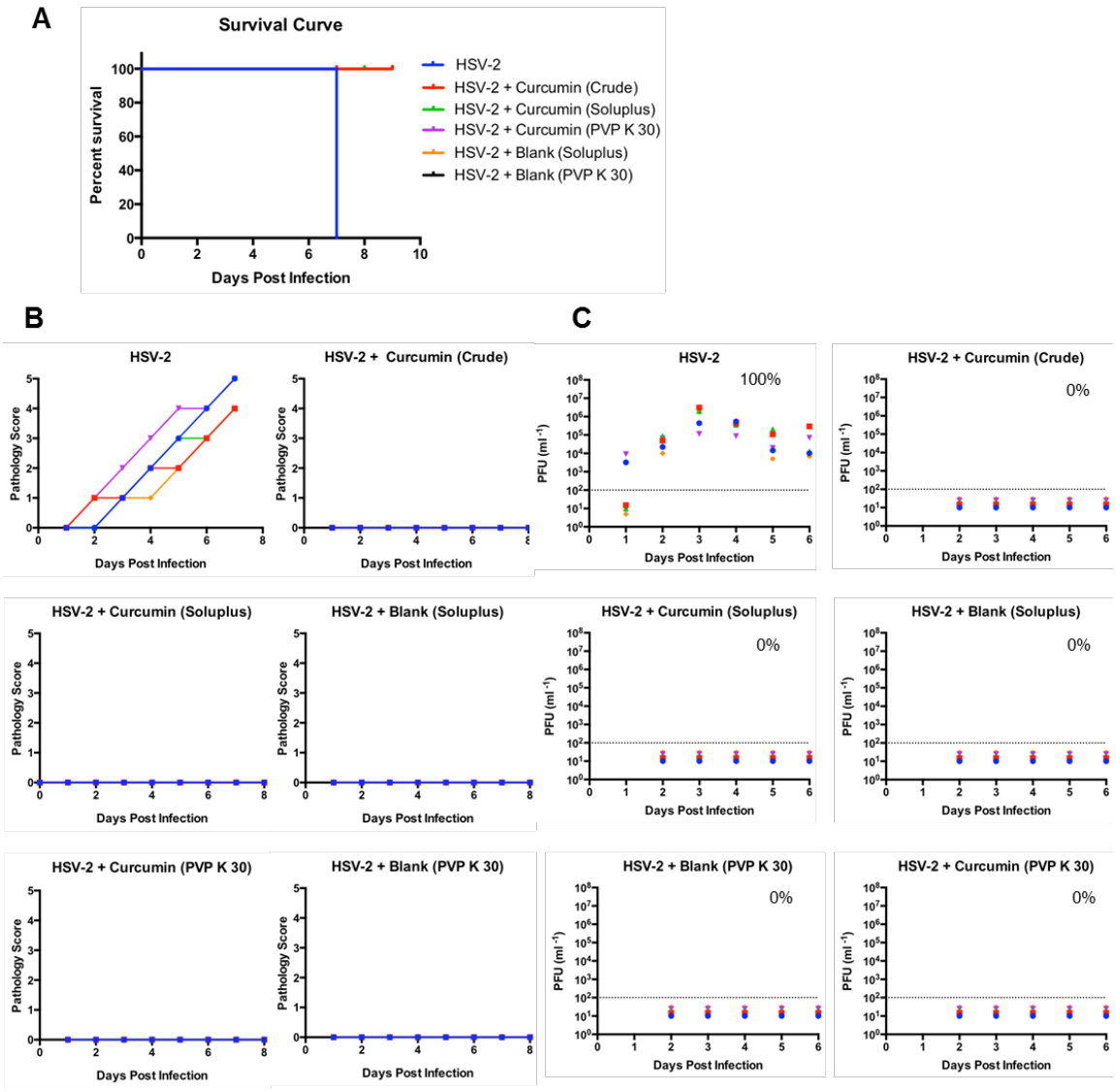

A
Survival Curve
Percent survival
Days Post Infection
HSV-2
HSV-2 + Curcumin (Crude)
HSV-2 + Curcumin (Soluplus)
HSV-2 + Curcumin (PVP K 30)
HSV-2 + Blank (Soluplus)
HSV-2 + Blank (PVP K 30)
B
C
HSV-2
HSV-2 + Curcumin (Crude)
HSV-2 + Curcumin (Soluplus)
HSV-2 + Blank (Soluplus)
HSV-2 + Curcumin (PVP K 30)
HSV-2 + Blank (PVP K 30)
Pathology Score
PFU (ml⁻¹)
Days Post Infection
100%
0%

Figure 2. Curcumin formulations confer non-specific protection against primary intravaginal HSV-2 challenge. Depo-treated wildtype mice were exposed to the lethal dose of WT HSV-2 (5 x 10^4 pfu/mouse) alone or in the direct combination with 1 mg of crude curcumin or 100 µg of formulations (n=5/group). Survival was monitored (A) and pathology scores were recorded (B) for 8 days post-exposure and plotted. (C) Vaginal washes were collected for 6 days post-exposure from both groups and viral plaque assays were conducted, as described in Material and Methods. Plaques were counted and viral titers were expressed as pfu/mL. Each symbol represents a single animal. The dashed line in (C) shows the lower detection limit of the assay, and data points below this line indicate undetectable viral shedding. The % indicates survival in (A) and the maximum number of mice that shed virus on any given day (C).

Figure 3

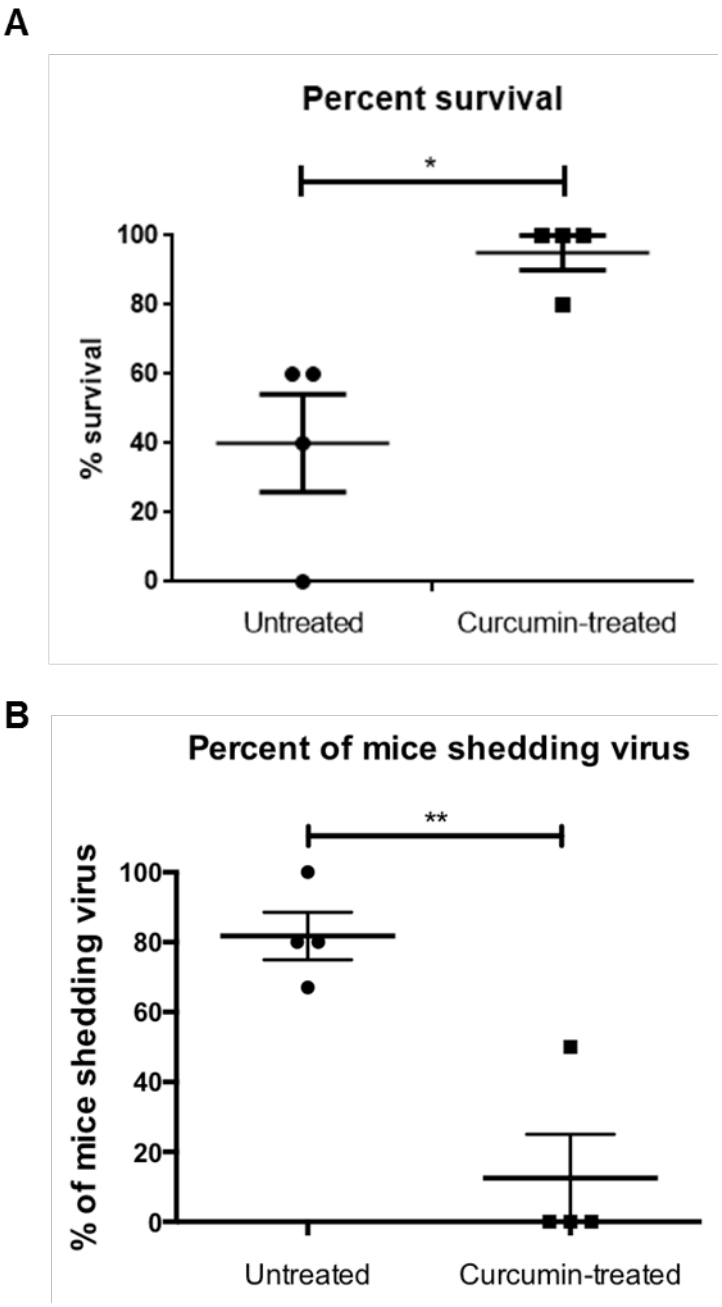

A
Percent survival
*
100
80
60
40
20
0
% survival
Untreated
Curcumin-treated
B
Percent of mice shedding virus
**
100
80
60
40
20
0
% of mice shedding virus
Untreated
Curcumin-treated

Figure 3. Curcumin-treated mice are less susceptible to primary intravaginal HSV-2 challenge as it relates to greater survival and lower percent of mice shedding virus. Depo-treated wildtype mice were exposed to the lethal dose of WT HSV-2 (5 x 10^4 pfu/mouse) alone or in the direct combination with 1 mg of crude curcumin (n=4-6/group). Survival was monitored **(A)** and vaginal washes were collected for 6 days post challenge from both groups and viral plaque assays were done **(B)**, as described in Material and Methods. Data was pooled from 4 separate experiments. Each symbol represents one experiment, and each experiment had n=4-6 mice. Significance was calculated by comparing mean ± SEM of percentage of survival **(A)** and mice shedding virus **(B)** between curcumin treated and untreated mice, and significance was analyzed using an unpaired T test $*\ p<0.05,\ **\ p<0.01$

Figure 4

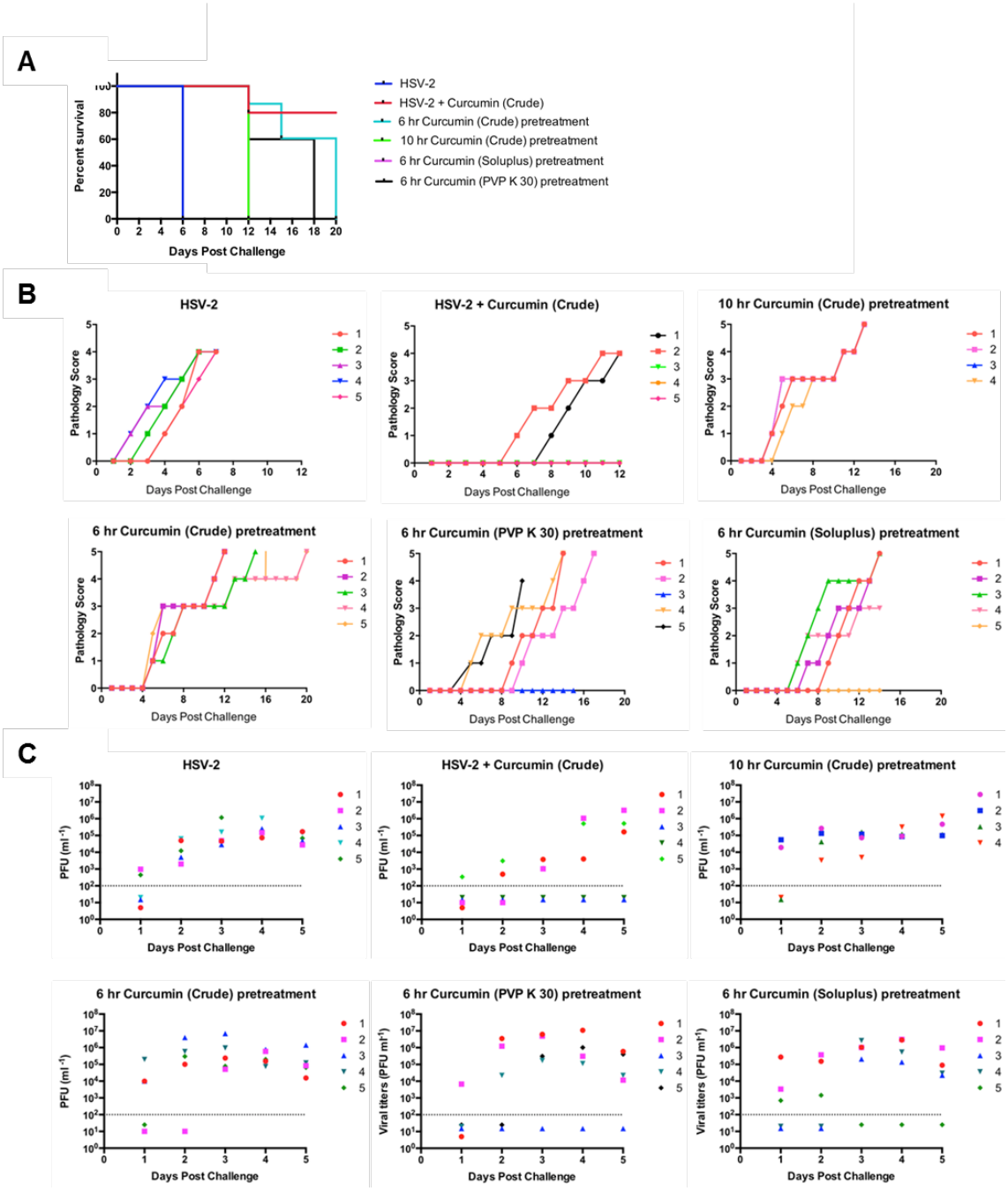

A
Percent survival
Days Post Challenge
HSV-2
HSV-2 + Curcumin (Crude)
6 hr Curcumin (Crude) pretreatment
10 hr Curcumin (Crude) pretreatment
6 hr Curcumin (Soluplus) pretreatment
6 hr Curcumin (PVP K 30) pretreatment
B
HSV-2
HSV-2 + Curcumin (Crude)
10 hr Curcumin (Crude) pretreatment
6 hr Curcumin (Crude) pretreatment
6 hr Curcumin (PVP K 30) pretreatment
6 hr Curcumin (Soluplus) pretreatment
Pathology Score
Days Post Challenge
C
HSV-2
HSV-2 + Curcumin (Crude)
10 hr Curcumin (Crude) pretreatment
6 hr Curcumin (Crude) pretreatment
6 hr Curcumin (PVP K 30) pretreatment
6 hr Curcumin (Soluplus) pretreatment
PFU (ml-1)
Viral titers (PFU ml-1)
Days Post Challenge

Figure 4. Crude curcumin and formulations Pre-treatment does not confer protection but delay clinical progression to HSV-2 associated endpoint following lethal primary intravaginal HSV-2 challenge. Depo-treated mice received either PBS, 1 mg of crude curcumin or 100 μg of curcumin formulations at 10 or 6 hours prior to exposure to WT HSV-2 ($5x10^4$ pfu/mouse, n=5 per group). (A) Survival was monitored and (B) pathology scores were recorded for 20 days following challenge and plotted. (C) Vaginal washes were collected for 5 days following exposure from all groups and viral plaque assays were conducted, as described in Materials and Methods. Plaques were counted and viral titers were expressed as pfu/mL. Each symbol represents a single animal. The dashed line in (C) shows the lower detection limit of the assay, and data points below this line indicate undetectable viral shedding.

Table 1. Pre-treatment with crude and solubilized curcumin decreases cumulative pathology following primary intravaginal HSV-2 challenge.

Treatment group	Pathology score	Number of mice	Number of days	Cumulative Pathology	Average pathology per mouse
HSV-2	5 5	2 3	14 13	140 195	95
10 hr Curcumin (Crude) pre-treatment + HSV-2	5	4	7	140	35
6 hr Curcumin (Crude) pre-treatment + HSV-2	5 5 5	1 2 2	5 8 1	25 80 10	23
6 hr Curcumin (Soluplus) pre-treatment + HSV-2	0 5 5 5	1 1 1 2	20 10 4 7	0 50 20 70	28
6 hr Curcumin (PVP K 30) pre-treatment + HSV-2	0 5 5 5	1 1 1 2	20 7 6 7	0 35 30 70	27

CHAPTER 4

DEPO-PROVERA LEADS TO SIGNIFICANT DECREASE IN *IN VIVO* MUCOSAL BARRIER FUNCTIONS

Danielle Vitali, Jocelyn M. Wessels and Charu Kaushic

There is a growing body of evidence implicating the role of the genital mucosal barrier in determining HIV susceptibility in women, yet the field still lacks a functional way of measuring genital mucosal barrier function. Thus, we decided to develop a comprehensive assay system that would allow experimental determination of *in vivo* genital barrier function in a preclinical murine model. The assay relies on the paracellular flow of fluorophore-conjugated macromolecules from the vaginal lumen into circulation. Following intravaginal delivery of ITC, fluorescence in the blood was quantified at various times post-delivery as a measure of genital barrier function. Differences in barrier function were compared throughout the murine estrous cycle and following intravaginal treatment with various immune stimulants. Our results indicate that the injectable hormonal contraceptive depot-medroxyprogesterone acetate (DMPA), a significant risk factor for HIV transmission, diminishes barrier function in the female genital tract beyond that seen during the naturally progesterone-high phase (diestrus) of the reproductive cycle. DMPA-treated mice showed significantly higher levels of FITC-dextran leakage, more severe epithelial thinning and markedly reduced expression of cell-to-cell adhesion proteins within the genital epithelium. These data offer additional evidence to support the current data that indicates that DMPA has detrimental effects in the genital tract that can enhance HIV-1 infection and argues for the development of new and safer contraceptive strategies that do not affect mucosal barrier function in the FGT.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments. Dr. Jocelyn M. Wessels provided technical assistance and contributed to the

generation of the data. I was responsible for data analysis, and Dr. Charu Kaushic and I wrote and edited the manuscript.

Depo-Provera leads to significant decrease in *in vivo* mucosal barrier functions

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Abstract

Objective: The female genital tract (FGT) mucosa is a major portal for entry of HIV into the body, responsible for initiating 40% of global infections. We have previously shown that exposure to HIV results in impairment in mucosal barrier function *in vitro.* Other studies have demonstrated that *pre-existing* disruption of the mucosal barrier in the FGT can also increase the risk of HIV infection, as seen with nonoxynol-9 (N-9). Despite the growing body of evidence implicating mucosal barrier function as a determinant of HIV risk, a functional assay to measure barrier function in the genital tract *in vivo* is still lacking. Thus, the purpose of this study was to develop a comprehensive assay system that would allow for the experimental determination of *in vivo* genital barrier function in a preclinical murine model.

Design and Methods: During various stages of the estrous cycle or following treatment with different immune stimuli (CpG, Poly I:C, N-9), female six to eight-week-old C57BL/6 mice were intravaginally administered FITC-dextran dye. Four hours following dye delivery, serum was collected and fluorescent spectrophotometry was performed to determine the percent FITC-dextran leakage from the vagina lumen into circulation. H&E and immunofluorescence staining of vaginal tissue sections was performed to assess epithelial thickness and expression of adhesion proteins. Cytokine analysis (IL-6, TNF-α and MCP-1) on vaginal tissue homogenates was also conducted.

Results: We successfully developed an *in vivo* assay system to detect changes in barrier function that occur throughout the estrous cycle, in response to intravaginal treatment with CpG, Poly I:C and N-9 and following subcutaneous injection with DMPA. Most strikingly, mice treated with DMPA showed significantly higher levels of FITC-dextran leakage, more severe epithelial thinning and markedly reduced expression of DSG1α compared to mice in diestrus, the naturally progesterone-high phase of the cycle.

Conclusions: Given the relevance of mucosal barrier function in HIV infection, the safety of topical microbicide formulations on the mucosal surface should be assessed using this assay before advancing into clinical trials. Furthermore, our results offer additional evidence to support the current data that indicates that DMPA has detrimental effects in the genital tract that can enhance HIV-1 infection and argues for the development of new and safer contraceptive strategies that do not affect mucosal barrier function in the FGT.

Introduction

Human Immunodeficiency Virus (HIV) infection remains one of the most serious health challenges in the world, and the fastest growing phase of this pandemic is currently by heterosexual transmission in women (UNAIDS, 2014)(UNAIDS, 2016). Adolescent girls and young women are at particularly high risk of HIV infection, accounting for 20% of new HIV infections globally (UNAIDS, 2016). In fact, the female genital tract (FGT) mucosa is a major portal for entry of HIV into the body, responsible for initiating 40% of global infections (Hladik and McElrath, 2008). Genital epithelial cells that line the FGT are the first cells to encounter the virus during sexual transmission. We have previously demonstrated that HIV directly impairs the genital mucosal barrier, leading to viral translocation that could initiate infection of underlying CD4+ HIV target cells in the FGT (Nazli et al., 2010). Here, the virus undergoes a short phase of local amplification for the successful establishment of HIV infection (Haase et al., 2015; Li et al., 2009). Thus, protecting the mucosal barrier could play a critical role in preventing HIV infection in women.

The upper FGT is lined by a simple columnar epithelium, a monolayer which relies on the presence of tight junction proteins, such as occludin and claudin-1, to seal the intercellular space (Blaskewicz et al., 2011) between adjacent cells and prevent pathogens from breaching the internal milieu (Blaskewicz et al., 2011). The lower FGT is lined by a stratified squamous epithelium, which relies on the presence of multiple epithelial layers to

provide a protective barrier (Blaskewicz et al., 2011). The most apical layers of the lower genital tract are terminally differentiated and devoid of cell-cell adhesion junctions, whereas the basal layers of the lower genital tract are metabolically active and contain several types of adhesion junctions, including adherens junctions, desmosomes and tight junctions (Blaskewicz et al., 2011) (Carias et al., 2013).

We have previously shown *in vitro* that HIV envelope protein gp120 interacts with Toll-like receptor (TLR)-2 and TLR-4 on GECs, resulting in the downstream induction of pro-inflammatory cytokines, such as notably tumor necrosis factor-α (TNF-α), and the rapid destruction of tight junction protein localization at the apical surface (Nazli et al., 2010; Nazli et al., 2013). The resulting impairment in mucosal barrier function allowed for significant viral translocation through the epithelium. Thus, the development of barrier protective agents should be investigated as a prophylactic strategy for the early window of susceptibility in the prevention of HIV transmission in women. *Pre-existing* disruption of the mucosal barrier in the FGT can also increase the risk of HIV acquisition, as seen with nonoxynol-9 (N-9), and recent studies have contributed to our understanding of how factors in the local microenvironment can influence this (Hoffman et al., 2004; Lozenski et al., 2012; Van Damme et al., 2002). For example, mouse studies have revealed that the injectable hormonal contraceptive depot-medroxyprogesterone acetate (DMPA), a significant risk factor for HIV transmission, downregulates vaginal cell-to-cell adhesion molecules, such as cadherins desmoglein-1α (DSG1α) (Quispe Calla et al., 2016).

Currently in the literature, there are well-established models to assess epithelial barrier function in both the lung and intestinal mucosa (Chen et al., 2014; Joly Condette et al., 2014; Wang et al., 2015). The basis of this method relies on the paracellular flow of fluorophore-conjugated macromolecules from the either the intestinal or pulmonary lumen into circulation and fluorescence within the blood is measured as a correlate of barrier integrity. However, an optimized model to assess genital barrier functions *in vivo* is still absent. The development of such a model would help with (a) testing barrier protective agents as HIV preventatives and (b) assessing the safety of topical microbicide formulations on the mucosal surface preclinically before advancing into clinical trials. The objective of this investigation was to develop a comprehensive assay system that would allow experimental determination of *in vivo* genital barrier function in a preclinical murine model.

Methods

Mice. Inbred, sexually matured, 6-8 week old female C57BL/6 mice were obtained from Charles River laboratories (Constant, Quebec, Canada). All mice were housed and maintained under standard temperature controlled conditions in the Central Animal Facility at McMaster University that followed a 12-hour light/dark cycle. Mice were given low-fat mouse chow and water ad libitum. All animal studies performed were approved by and were in compliance with the Animal Research Ethics Board (AREB) at McMaster University.

Depo-Provera injection and staging. Mice were subcutaneously injected with 2 mg of Depo-provera. Vaginal washes were collected daily for 4 consecutive days following Depo-provera injection by pipetting 30 μL of PBS into and out of the vagina 5-6 times. Sample fluid was smeared on glass slides and was examined by light microscopy to determine the stage of the estrous cycle as described previously. The following classification was used for identifying the stage of the cycle: estrus, >90% cornified epithelial cells; diestrus, >75% polymorphonuclear cells; -meta-estrus, 50% epithelial cells, 50% polymorphonuclear cells.

Functional assessment of in vivo genital barrier permeability. Genital permeability was evaluated in terms of the amount of fluorescein isothiocyanate dextran (FITC-dextran) that had crossed the genital epithelial barrier into the blood, adapted from protocols frequently used in intestinal permeability studies. Mice were anesthetized and treated with a single IVAG dose of either CpG ODN (30 μg), Poly I:C (100 μg), nonoxynol-9 (10% in saline; LKT Laboratories), mouse TNF-α (500 ng in 0.1% bovine serum albumin; Sigma Aldrich), peptidoglycan (0.5 mg in water; Sigma Aldrich) or flagellin (0.5 mg in water; Sigma Aldrich). After the given time period, mice were re-anesthetized and received FITC-dextran (60 – 625 μg; Sigma Aldrich) IVAG. After four hours, serum was collected. Fluorescence intensity was measured (excitation, 492 nm; emission, 525 nm) and serum levels of FITC were determined using a plotted standard curve. Percent FITC-dextran leakage was calculated as the ratio of FITC-dextran in the serum divided by the FITC-dextran delivered IVAG, as a measure of barrier permeability.

Multiplex Cytokine Assay. Mouse vaginal tissue was collected from mice and mechanically homogenized in PBS, using metal beads and the Gold BulletBlender system (Next Advance, Averill Park, New York, USA). Homogenates were centrifuged at 8000 RPM for 5 minutes, and the supernatants were collected and stored at -80° until required. Cytokines and chemokines were quantified in duplicate in the supernatants using the Magpix technology system and the Mouse Cytokine Magnetic Kit 96-Well Plate Assay (Millipore, Billerica, MA, USA), as per the manufacturer's instructions. The following cytokines and chemokines were measured: TNF-α, IL-6 and MCP-1, and the range of detection of this assay was between analyte concentrations 3.2 and 10,000 pg/mL

Histomorphology of the vaginal tract. Excised vaginal tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 7 μm for hematoxylin-and-eosin staining.

Immunofluoresence staining. Excised vaginal tissues were embedded and flash frozen in OCT, and 5 μm tissue sections were mounted on glass slides and fixed in methanol. After 3 PBS washes, sections were incubated 1 h with 10% normal donkey serum (Abcam, Cambridge MA) at 4°C, washed, incubated overnight at ambient temperature with rabbit anti-desmoglein-1 (clone EPR6766(B)) or monoclonal rabbit IgG isotype control (clone EPR25A), washed and incubated with 1 h with AlexFluor® 488-labeled donkey anti-rabbit

IgG labeled (all antibodies Abcam). All antibodies were diluted in PBS with 1% BSA and 0.05% Tween 20. Sections were stained with DAPI and evaluated by fluorescence microscopy.

Statistical analysis. Statistical analysis and graphical representation was performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test was used to calculate significant differences in FITC-dextran leakage and cytokine levels.

Results

Functional assessment of genital barrier function *in vivo*. We were able to test genital barrier function by administering 4 kDa fluorescein isothiocyanate-dextran (FITC-dextran) directly into the vaginal lumen and measuring the fluorescence within the blood at various time points after administration. We performed standardization experiments to determine the optimal dye quantity and time point to allow optimize the sensitivity to reflect barrier function alteration in the FGT (625 μg FITC-dextran and 4 hours following dye delivery, respectively). Using this functional assay, barrier function was compared throughout the various stages of epithelial thickness in the murine estrous cycle. During the estradiol-high phase of the estrous cycle (estrus-stage), the epithelium is thickest, and during the progesterone-high phase of the estrous cycle (diestrus-stage), the epithelium is at its thinnest state (Kress et al., 2004). Furthermore, Depo-medroxyprogesterone (DMPA), a progestin-only contraceptive, thins the epithelium and induces a prolonged state of diestrus when administered to rodents (Gillgrass et al., 2003).

WT C57BL/6 estrus, diestrus and DMPA-treated mice were administered FITC-dextran intravaginally and four hours following dye delivery, serum was collected for fluorescent spectrophotometry to determine the percent FITC-dextran leakage from the vaginal lumen into circulation. DMPA-treated mice showed significantly higher levels of FITC-dextran leakage compared to estrus and diestrus-stage mice, indicating that DMPA reduces barrier function and increases permeability in the FGT (Figure 1). We also assessed changes in barrier function following intravaginal treatment with Toll-like receptor (TLR) ligands CpG oligodeoxynucleotides (CpG ODN) and Poly I:C (polysinosinic:polycytidylic acid). Both CpG ODN and Poly I:C potently active the innate immune system and anti-viral inflammatory cascades within epithelial cells. DMPA-treated mice received a single intravaginal inoculation of Poly I:C or CpG 24 hours prior to dye delivery and showed significantly lower levels of FITC-dextran leakage compared to untreated DMPA-only controls. In fact, Poly I:C and CpG rescued barrier function to levels comparable to diestrus-stage mice. Lastly, we assessed barrier function following intravaginal treatment with HIV microbicide nonoxynol-9 (N-9), which was initially developed because of promising anti-viral activity *in vitro* but was unsuccessful in clinical trials because of considerable damage to the cervicovaginal epithelium. Intravaginal inoculation with N-9 (10%) resulted in approximately 1.5-fold greater dye leakage than DMPA-only controls and strikingly higher leakages levels compared to all other groups.

Correlation of vaginal barrier function with vaginal inflammatory cytokine response. Vaginal tissues were homogenized and levels of pro-inflammatory cytokines IL-6 and TNF-α as well as chemokine MCP-1 were measured. In general, TLR CpG and Poly I:C induced higher levels of IL-6, TNF- α and MCP-1 in the vaginal tissue compared to naïve groups. This emphasizes that innate immune activation does not necessarily correlate with loss in barrier function, since CpG and Poly I:C were able to overcome the effect of DMPA on barrier disruption. Thus inflammation can be independent of barrier permeability. It also suggests that inflammation-induced barrier disruption does not occur as a result of pro-inflammatory cytokines stimulation alone, contrary to previous *in vitro* studies (Nazli et al., 2010). Among hormonal groups, cytokine levels were relatively similar with the exception of the significant increase in TNF-α in diestrus mice.

Correlation of vaginal barrier function with vaginal histomorphological changes. Histomorphological studies of vaginal tissue showed varying epithelial thickness across experimental groups. Vaginal epithelium was stratified, squamous and keratinized in estrus-stage mice, characterized by approximately 30 to 40 cell layers of epithelial cells (Figure 3A), whereas in diestrus-stage mice, the epithelium was thin and characterized by 5 to 10 cell layers (Figure 3B). DMPA induced more severe epithelial thinning compared to diestrus-stage mice (Figure 3C), while single application of N-9 resulted in the most severe epithelial thinning, including sloughing of the superficial layers of epithelium and large regions of complete epithelial exfoliation (Figure 3D). Both Poly I:C (Figure 3E) and CpG (Figure 3F) induced rapid thickening of the vaginal epithelium, despite being under

the influence of DMPA. Given that higher levels of FITC-dextran leakage were observed following epithelial thinning and lower levels were observed following epithelial thickening, we conclude that epithelial thickness correlates with barrier function.

Correlation of vaginal barrier function with expression of desmosomal cadherins. To further explore alterations in genital barrier function, we harvested vaginal tissue from mice across experimental groups and assessed the expression of the desmosomal cadherin desmoglein-1α (DSG1α). DSG1α is a cell-cell adhesion molecule present in the FGT epithelium and has been shown to be important for maintaining the integrity and barrier function of cutaneous as well as intestinal epithelium. Compared to vaginal tissue from estrus (Figure 4A) and diestrus-stage (Figure 4B) mice, detection of DSG1α was almost entirely absent following treatment with DMPA (Figure 4C). On the other hand, CpG ODN (Figure 4D) and Poly I:C (Figure 4E) rescued tissue expression of DSG1α in DMPA-treated mice, while N-9 resulted in complete absence of DSG1α in areas of epithelial desquamation (Figure 4F). Taken together with the previous experiments, we can conclude that DMPA severely reduces genital barrier function, specifically diminishing genital tissue expression of DSG1α while TLR ligands can overcome this effect possibly by activating innate immune response.

Discussion

In this study, we established a simple, reliable and reproducible method to functionally assess alterations in genital permeability by instilling FITC-dextran intravaginally and measuring its concentration in the serum. This result from this assay were verified using other functional and morphological assessments that are traditionally used to measure barrier function in the genital tract (Catalone et al., 2004), and the results showed that changes in the FITC-dextran concentration in the serum were consistent with the changes observed in other assays. We found that the concentration of FITC-dextran in the serum decreased following treatment with CpG ODN and Poly I:C, which induce thickening of the genital epithelium according to histological and immunofluorescent parameters, and increased following treatment with N-9, which induces exfoliation of the genital epithelium according to histological and immunofluorescent parameters. Furthermore, unlike histological and immunofluorescent parameters, levels of IL-6, TNF-α and MCP-1 did not correlate with barrier dysfunction. Interestingly, the concentration of serum FITC-dextran was significantly increased and the expression of adhesion protein DSG1α was markedly reduced in mice treated with DMPA compared to those in diestrus, the naturally progesterone-high phase of the estrous cycle. Our results contribute to the body of evidence identifying DMPA as a risk factor for HIV acquisition in women and implicating reduced genital barrier function as a potential underlying mechanism.

The overall goal of this study was to develop a comprehensive assay system that would allow for the experimental determination of *in vivo* genital barrier function in mice. This has implications for the preclinical safety assessment of vaginal microbicides, the importance of which was identified following retrospective analyses of the testing of N-9 as an HIV microbicide (Van Damme et al., 2002). Early *in vitro* studies yielded promising results of its broad-spectrum activity against several sexually transmitted infections, including Herpes Simplex Virus Type-2 (HSV-2) and HIV, and quickly advanced N-9 into human clinical trials (Benes and McCormack, 1985; Bourinbaiar and Fruhstorfer, 1996; Hicks et al., 1985). However, the final phase of clinical trials demonstrated that high frequency use of this product was associated with an almost 2-fold greater risk of HIV acquisition (Van Damme et al., 2000; Van Damme et al., 2002). While these findings halted the implementation of N-9 into the market as a microbicide, they also emphasized that the safety of microbicide formulations on the mucosal surface must be assessed in preclinical animal models before advancing into clinical trials. Traditional methods to assess epithelial toxicity require that the animal is sacrificed and the cervicovaginal tract is excised following treatment for histological examination (Catalone et al., 2004). However, the measurement of FITC-dextran in serum after intravaginal instillation, as described in this study, can be used to functionally and quantitatively detect differences in genital barrier function without the animals having to be sacrificed. It can measure functional alterations in barrier related to both epithelial thickness and adhesion protein expression, whereas only epithelial thickness can be captured with histological examination. Overall, the method

described in this study is simple, reliable and reproducible and will prove highly valuable for evaluating the safety profile of future microbicides under preclinical investigation.

Genital barrier disruption has also been hypothesized to increase the risk of HIV transmission. Evidence of the significance of barrier function in HIV infection initially stemmed from studies of non-human primates. Recent work in rhesus macaque vaginal transmission models showed that depth of virus penetration into the squamous epithelium during the initial events of HIV entry is directly proportional to epithelial permeability (Carias et al., 2013). For viral transmission to occur, infectious virions from the donor must cross the epithelial barrier lining the female genital tract to find a susceptible cell in the host. Carias *et al* found that HIV can penetrate as much as 10 μm into the squamous epithelium, which is significantly increased upon tight junction disruption (Carias et al., 2013). We have previously shown that exposure to HIV directly impairs the genital epithelial barrier *in vitro*, allowing for significant viral translocation across the epithelial monolayers (Nazli et al., 2010). Furthermore, in female pigtail macaques, marked thinning of the epithelium during the progesterone-high phase of their menstrual cycle is associated with increase in susceptibility to SIV (Kersh et al., 2014).

Injectable hormonal contraceptives, DMPA and norethisterone enanthate (NET-EN), are high dose progesterone-only contraceptives used by approximately 8 million women in sub-Saharan Africa (Ross and Agwanda, 2012). With continued use, circulating E2 concentrations in DMPA users commonly fall into the postmenopausal range, ovulation

is suppressed and pregnancy is prevented (Bahamondes et al., 2014). Although it remains controversial in the literature, numerous observational studies have identified DMPA as a significant risk factor for the acquisition of HIV. In a recent prospective cohort study, the HIV incidence in South African women using injectable progestin-only contraceptives, including DMPA and NET-EN, was 2.93 times higher than the incidence in those not using long-term contraception (Byrne et al., 2016b). While the biological mechanism remains unclear, this study as well as others have implicated disruption of the epithelial barrier as one proposed mechanism through which DMPA may increase the risk of HIV transmission. Although there is evidence to suggest that higher levels of endogenous progesterone decrease barrier function (Kersh et al., 2014), such as during luteal phase of the cycle, we show loss in barrier function is even more extensive in mice treated with DMPA. Although the concentration of FITC-dextran in the serum was significantly higher in DMPA-treated mice, vaginal epithelial thickness was comparable among mice in diestrus and after DMPA treatment. This mirrors clinical findings that showed that mucosal thicknesses were equivalent among women in their luteal phase and women using DMPA (Bahamondes et al., 2014; Mauck et al., 1999; Mitchell et al., 2014b). However, we observed a striking absence of the expression of cell to cell adhesion molecules within the vaginal epithelium of DMPA-treated mice. These experimental findings imply that the effects of DMPA on the genital epithelium are subtler than what can be explained using traditional histological methods. They also highlight the extensive loss in barrier function associated with DMPA treatment and that corresponding increases in genital permeability to infectious virus particles could render a woman more susceptible to HIV infection.

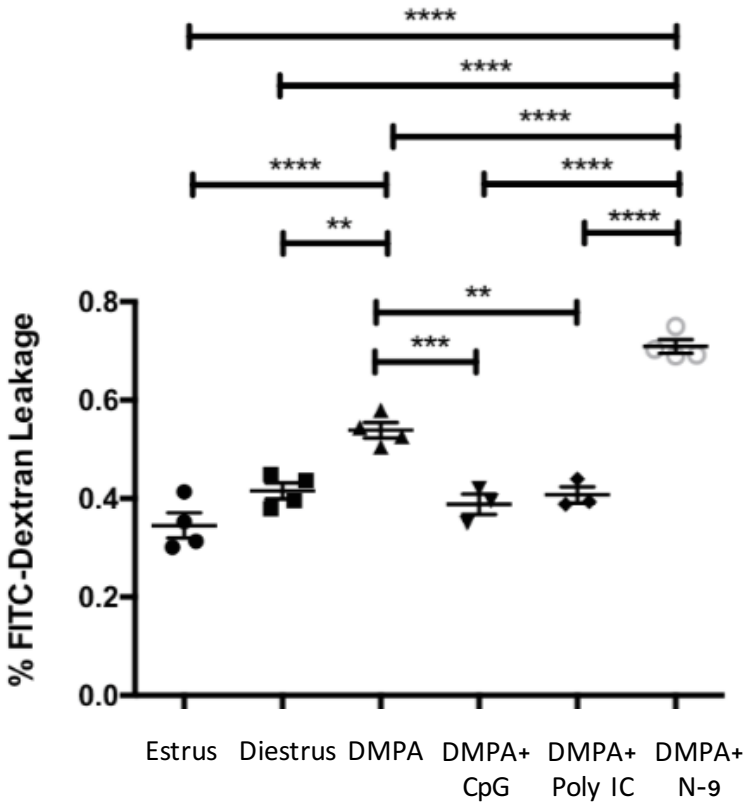
Although loss in barrier function observed in our investigation was produced with physiologically relevant doses of DMPA, as measured by serum levels of MPA in these mice, we do not know if the effect of DMPA seen in mice is also produced in women. Furthermore, our findings do not exclude the possibility that DMPA alters other mechanisms of the innate host defense that could also contribute to HIV risk, such as by decreasing antimicrobial peptide production or natural killer cell function or by increasing CD4+ target cell recruitment and activation in the genital tract. While the implication of our findings may be substantial, additional basic, translational and clinical studies are needed to fully understand the effects of progestin-based hormonal contraceptives on HIV susceptibility.

Conclusion

Taken together, we have successfully developed a comprehensive assay system that enables the experimental determination of *in vivo* genital barrier functions. Given the relevance of mucosal barrier function in HIV infection, the safety of topical microbicide formulations on the mucosal surface should be assessed using this system before advancing into clinical trials. Furthermore, our findings that Depo-Provera reduces genital barrier function may describe one of the underlying mechanisms through which it contributes to HIV risk in women. While the implications for our findings may therefore be substantial,

clearly additional basic, translational and clinical studies are needed to fully define the effects of Depo-Provera on susceptibility to HIV in the female genital tract.

Figure 1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% FITC-Dextran Leakage
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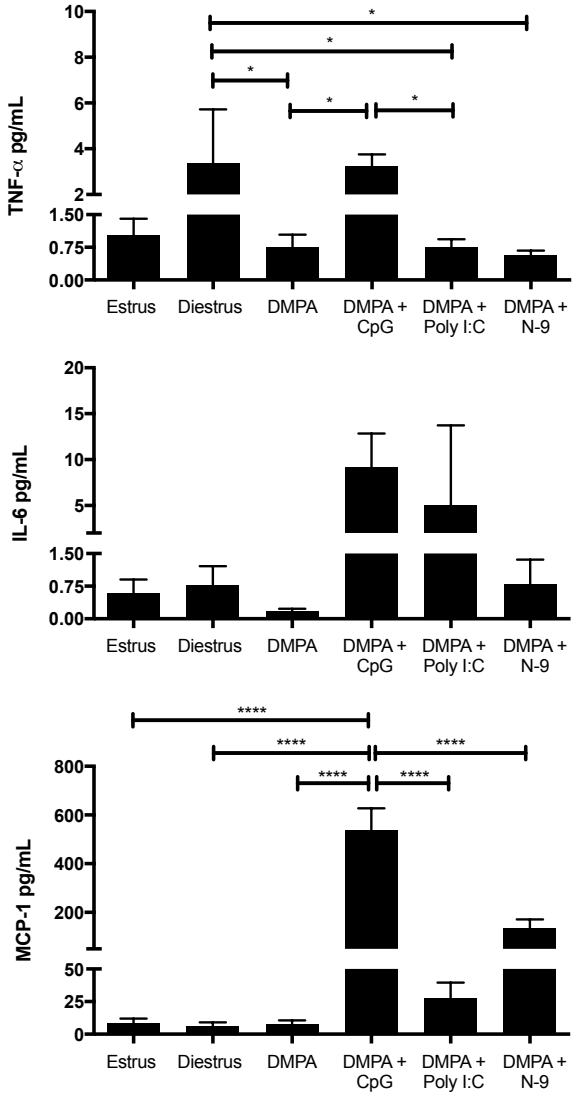
0.6
0.4
0.2
0.0
Estrus
Diestrus
DMPA
DMPA+ CpG
DMPA+ Poly IC
DMPA+ N-9

Figure 1. Functional assessment of mucosal permeability in the genital tract *in vivo*. *In vivo* genital barrier permeability was measured using the FITC-dextran assay, as described in Materials and Methods. Mice in the estrus and diestrus stages of the murine estrous cycle were identified following cytological analysis of vaginal smears, and DMPA-treated mice received a 2 mg subcutaneous injection of Depo-Provera. Treatment groups received either CpG (25 μg) or Poly I:C (100 μg) 24 hours prior to delivery of FITC-dextran dye or N-9 (10%) 4 hours prior to intravaginal delivery of FITC-dextran dye. Serum was collected 4 hours following dye delivery and fluorescence of FITC-dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices, Sunnyvale, CA) at an excitation of 490 nm and emission of 520. FITC-dextran leakage into the blood is expressed as a percentage of FTIC-dextran delivered. Data was analyzed by one-way ANOVA ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Each data point represents a single animal.

Figure 2


TNF-α pg/mL
10
8
6
4
2
1.50
0.75
0.00
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Estrus
Diestrus
DMPA
DMPA + CpG
DMPA + Poly I:C
DMPA + N-9
IL-6 pg/mL
20
15
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Estrus
Diestrus
DMPA
DMPA + CpG
DMPA + Poly I:C
DMPA + N-9
MCP-1 pg/mL
800
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Estrus
Diestrus
DMPA
DMPA + CpG
DMPA + Poly I:C
DMPA + N-9

Figure 2. Inflammatory cytokine expression in the vaginal tissue during various states of genital barrier function. Vaginal tissues from untreated mice in estrus or diestrus, mice treated with DMPA and mice that received intravaginal delivery of CpG, Poly I:C or N-9 were excised and homogenized using the Bullet Blender (Next Advance, New York). Magpix technology system was used to quantify the following cytokine and chemokines in duplicate in the homogenate supernatants: TNF-α, IL-6, MCP-1, and are expressed as pg/mL of cytokine/chemokine production. Data was analyzed by one-way ANOVA * *p<0.05,* **** *p<0.0001*. Data includes mean pg/mL of three to four animals per experimental condition.

Figure 3

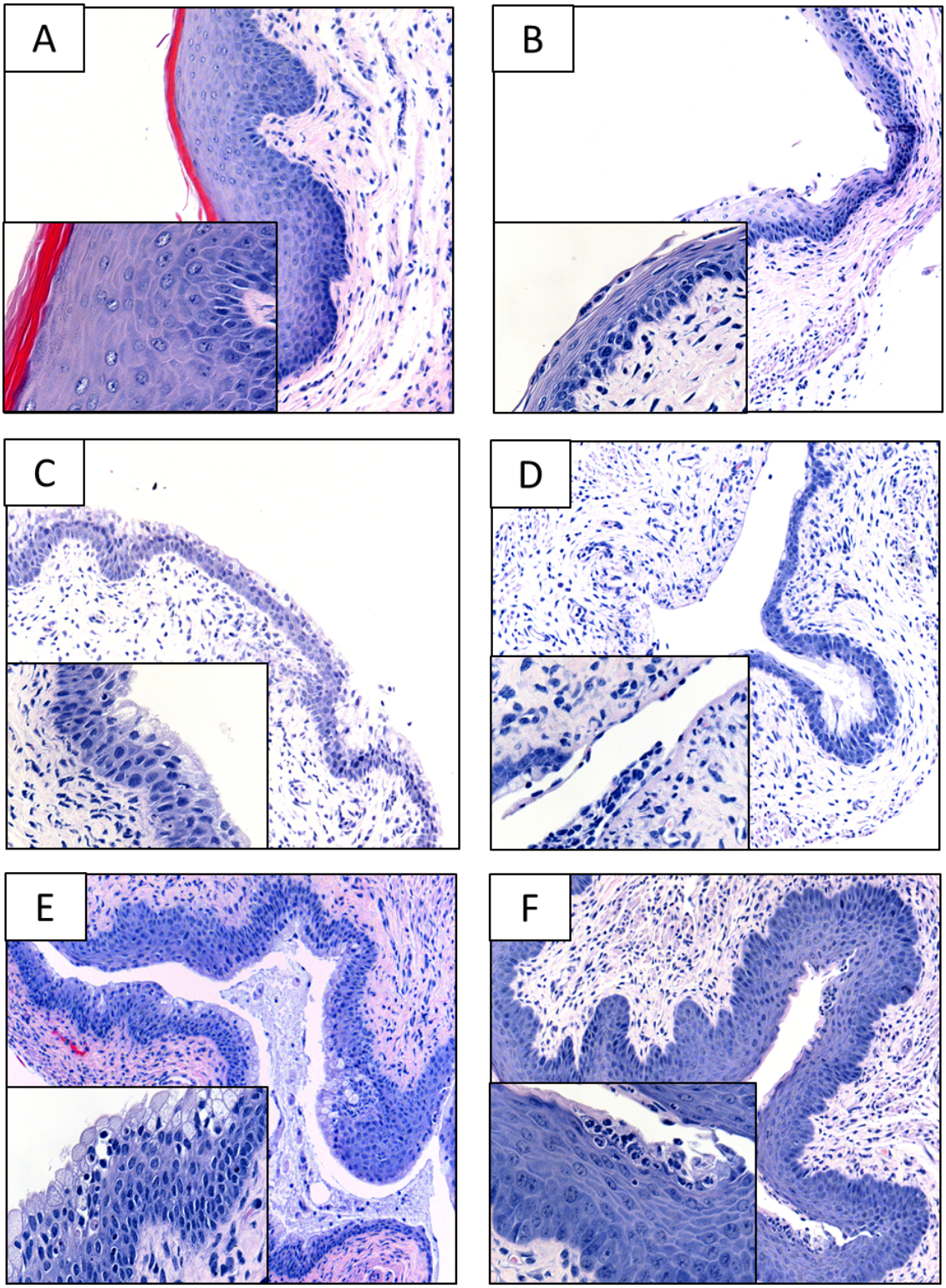


Figure 3. Histological assessment of genital barrier function. Vaginal tissues from untreated mice in (A) estrus or (B) diestrus, (C) mice treated with DMPA and mice that received intravaginal delivery of (D) N-9, (E) Poly I:C or (F) CpG were excised. Tissues were sectioned and stained with H&E, and representative images from multiple experiments with three to four animals per condition are shown. Image magnification 10x and 40x.

Figure 4

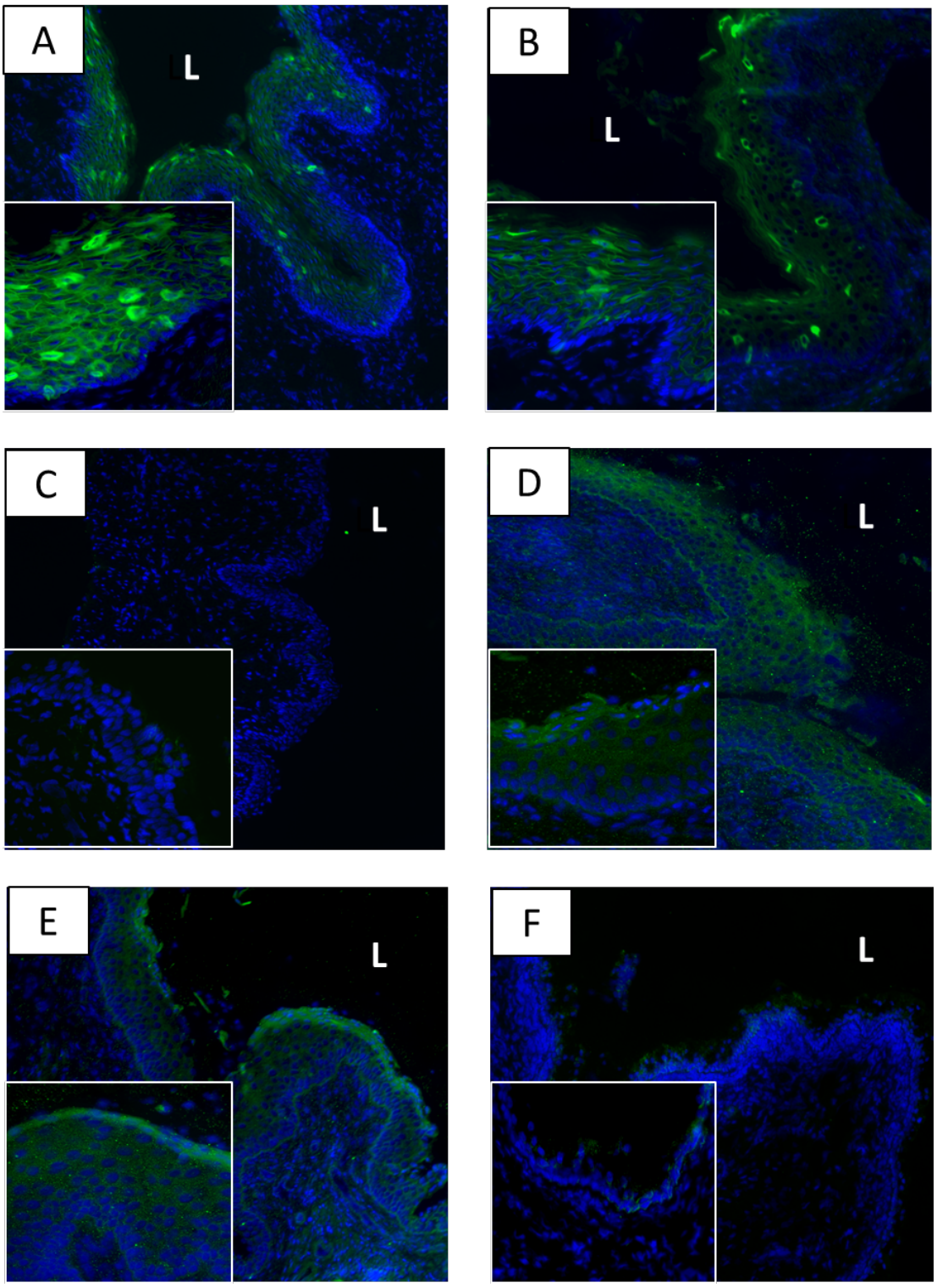


Figure 4. Immunofluorescence staining of cell-cell adhesion molecules to assess genital barrier function. Vaginal tissues from (A) untreated mice in estrus or (B) diestrus, (C) mice treated with DMPA and mice that received intravaginal delivery of (D) CpG, (E) Poly I:C or (F) N-9 were excised. Tissues were embedded and flash frozen prior to immunofluoresence staining to assess levels of desmoglein-1α (DSG1α) protein expression; DSG1α (green); DAPI (blue); L denotes vaginal lumen. Representative images from two independent experiments with three to four animals per condition are shown (image magnification 10x and 40x).

CHAPTER 4

APPENDIX

This appendix summarizes the initial optimization stages that explored the dye quantity and time points that allowed for sufficient assay sensitivity to detect changes in barrier function. Experiments conducted in attempt to develop a mouse model of inflammation-induced barrier disruption in the genital tract, using various inflammatory stimuli like TNF-α and flagellin, are also summarized here. These data were collected from early experiments during optimization, that did not include all experimental groups or controls and therefore were excluded from the manuscript.

Results

To quantify genital barrier disruption, we derived the FITC-dextran leakage assay from studies of intestinal permeability. Typically, FITC-dextran is administered to mice by oral gavage following the induction of intestinal barrier damage. Four hours following gavage, fluorescent activity in the blood is measured as the quantity of dye that leaked through the intestinal epithelium, which can be extrapolated as a measure of intestinal barrier function. In our study, initial experiments optimized this protocol to assess barrier function in the genital tract. Various dye quantities (Appendix 2; Figure 1A) and time points following dye delivery (Appendix 2; Figure 1B) were tested, and 625 μg of FITC-dextran and a 4 hour post-dye delivery time point were selected as the best parameters to detect changes in barrier function.

In vitro data from our lab indicated that curcumin's anti-inflammatory activity protects the genital epithelium from barrier disruption because of inflammation, which can be induced upon exposure to HIV (Ferreira et al., 2015b). To determine if curcumin could protect against and/or control barrier disruption *in vivo,* we first needed to develop a mouse model of inflammation-induced barrier disruption, in which we are able to detect increases in FITC-dextran leakage using our assay. We have shown that pro-inflammatory cytokine TNF-α increases the permeability of GECs *in vitro*, and thus, we initially assessed if this could be recapitulated *in vivo* (Nazli et al., 2010). Mice received a single intravaginal inoculation of TNF-α 24 hours prior to dye delivery, however, we were unable to detect

differences in leakage in the TNF-treated mice compared to saline-treated controls (Appendix 2; Figure 2A). It remains unclear whether TNF-α does not induce genital barrier disruption *in vivo* or if TNF-mediated disruption is below the limit of detection of this assay.

Later experiments tested various TLR ligands. Flagellin is the principle component of bacterial flagellum, which is used by many bacteria for propulsion, and is known to activate TLR5 on the surface of epithelial and immune cells. Although the effects of flagellin on the genital tissue *in vivo* are unclear, clinical data from patients undergoing hysterectomies suggests that TLR5 is present in the basal secretions of vaginal tissue and on the ectocervical epithelium. Thus, we examined if the inflammatory cascade induced in response to TLR5 stimulation by flagellin would promote genital barrier disruption. Mice received a single intravaginal inoculation of flagellin 24 hours prior to dye delivery, however, we were unable to detect differences in leakage in mice that received flagellin compared to controls that received saline (Appendix 2; Figure 2B). TLR ligands CpG and Poly I:C were also tested, but they increased rather than decreased genital barrier function because of rapid proliferation and thickening of the vaginal epithelium, which we observed during histomorphological assessment of the genital mucosa.

Overall, we were unable to develop a model of inflammation-induced genital barrier disruption in which we could test the ability of curcumin to maintain barrier function. However, we did successfully develop a comprehensive assay system in the process that

could be used to detect immunomodulatory and hormonal changes in genital barrier function (data summarized in Chapter 4). As expected, differences in genital barrier function were observed across DMPA, estrus and diestrus hormonal conditions, but there was no difference in genital barrier function between DMPA and ovariectomized mice (Appendix 2; Figure 3), likely because both induce similar epithelial thinning.

Lastly, we used our assay system to also assess barrier function following exposure to HSV-2. Genital herpes may facilitate HIV acquisition by disrupting the epithelial barrier, thereby increasing exposure of the virus to target cells in the underlying submucosa. Even in the absence of viral shedding and genital ulcerations, HSV-2 may also modify the mucosal environment by simulating the production of pro-inflammatory cytokines and chemokines, such as IL-6, IL-8 and TNF-α, by genital epithelial cells. We have eloquently shown *in vitro* that such responses are sufficient to induce barrier disruption and facilitate HIV translocation through the epithelium. To explore the *in vivo* relevance of this phenomenon, we measured genital barrier function in mice 18 hours following intravaginal HSV-2 challenge. We selected this as the optimal time to assess the effects of pro-inflammatory cytokines induced upon exposure, before viral shedding and epithelial ulceration begin. DMPA-treated mice were challenged with the lethal dose of HSV-2 and received FITC-dextran 18 hours later. There was no difference in dye leakage or adhesion protein expression between HSV-2 exposed and control mice (Appendix 2; Figure 4A and Figure 4B), and we conclude that either (a) the inflammation induced upon exposure to HSV-2 does not induce barrier disruption or (b) the disruption is subtle and below the limit

of detection of our assay. Although it is out of the scope of this study, future studies can use our assay to further explore HSV-2 mediated barrier disruption and its relationship to HIV acquisition *in vivo*.

Figure 1

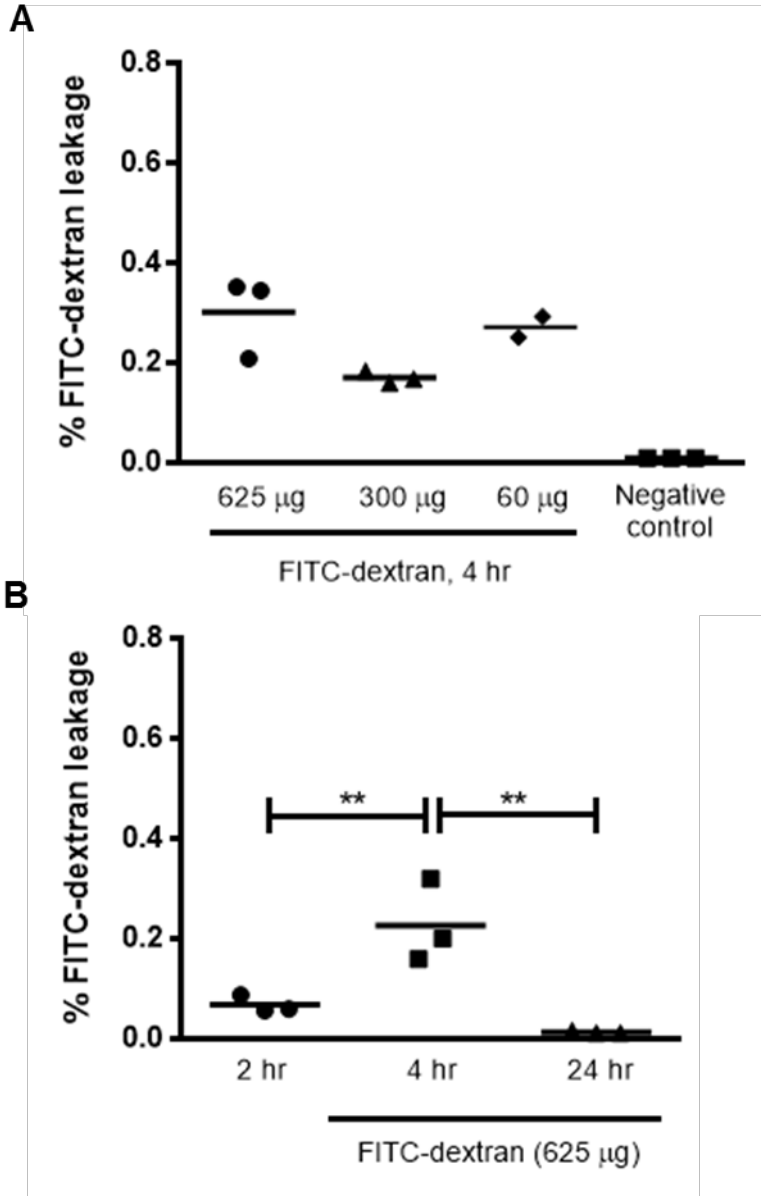

A
% FITC-dextran leakage
0.8
0.6
0.4
0.2
0.0
625 μg
300 μg
60 μg
Negative control
FITC-dextran, 4 hr
B
% FITC-dextran leakage
0.8
0.6
0.4
0.2
0.0
**
**
2 hr
4 hr
24 hr
FITC-dextran (625 μg)

Figure 1. Optimization of functional assay to assess mucosal barrier permeability in the genital tract *in vivo.* *In vivo* genital barrier permeability was measured using 4 kDa FITC-dextran dye, as described in Materials and Methods. **(A)** Depo-treated mice received either 625, 300, or 60 μg of FITC-dextran dye intravaginally and serum was collected 4 hours following dye delivery. **(B)** DMPA-treated mice received 625 μg of FITC-dextran dye intravaginally and serum was collected at 2, 4 or 24 hours following dye delivery. Fluorescence of FITC-dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices, Sunnyvale, CA) at an excitation of 490 nm and emission of 520 nm. FITC-dextran leakage into the blood is expressed as a percentage of FITC-dextran delivered. Data was analyzed by one-way ANOVA ** $p<0.01$. Each data point represents a single animal.

Figure 2

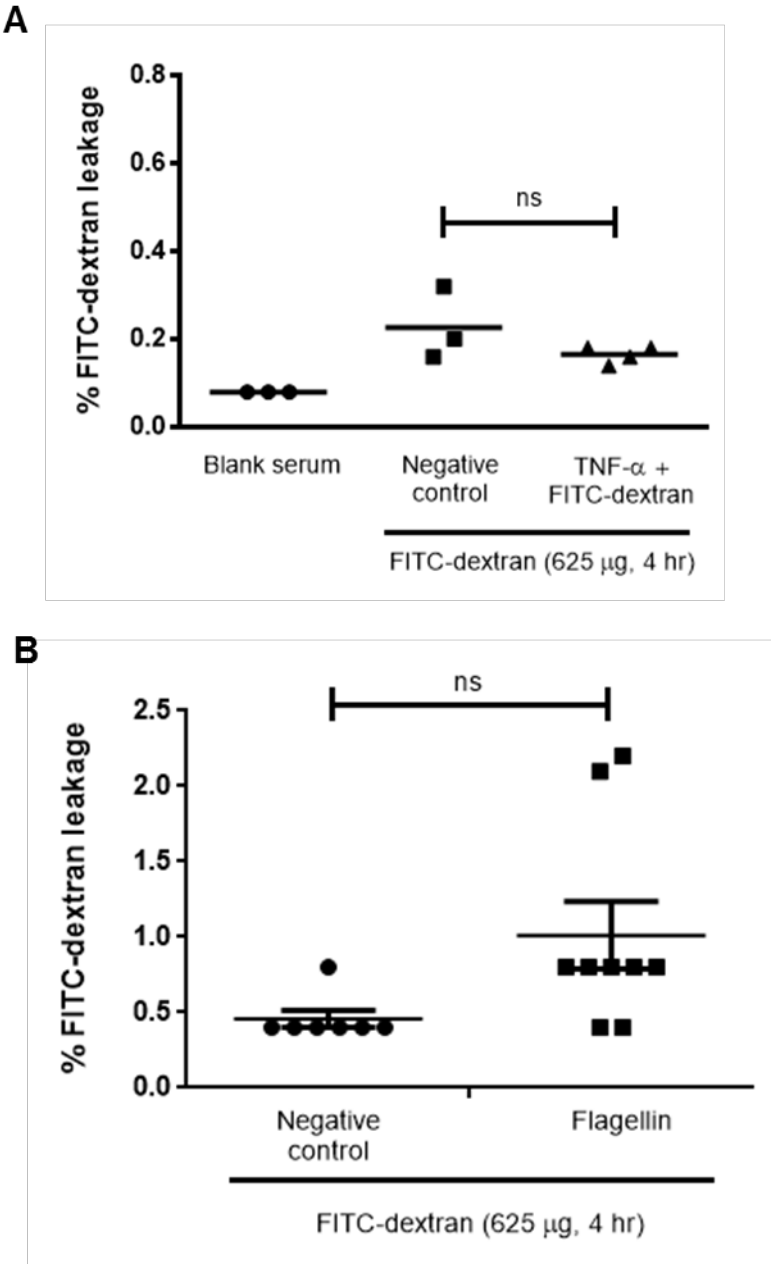

A
% FITC-dextran leakage
0.8
0.6
0.4
0.2
0.0
ns
Blank serum
Negative control
TNF-α + FITC-dextran
FITC-dextran (625 μg, 4 hr)
B
% FITC-dextran leakage
2.5
2.0
1.5
1.0
0.5
0.0
ns
Negative control
Flagellin
FITC-dextran (625 μg, 4 hr)

Figure 2. Immunostimulants TNF-α and flagellin do not alter genital barrier permeability. *In vivo* genital barrier permeability was measured following intravaginal treatment with TNF-α and flagellin using the FITC-dextran assay, as described in Materials and Methods. Depo-Provera treated mice received either **(A)** 500 ng of TNF-α or **(B)** 1.5 μg of flagellin 24 hours prior to delivery of FITC-dextran dye. % FITC-dextran was calculated as the concentration of FITC-dextran in the serum divided by the concentration of FITC-dextran delivered. Serum was collected 4 hours later and fluorescence of FITC-dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices, Sunnyvale, CA) at an excitation of 490 nm and emission of 520 nm. FITC-dextran leakage into the blood is expressed as a percentage of FITC-dextran delivered. Each data point represents a single animal.

Figure 3

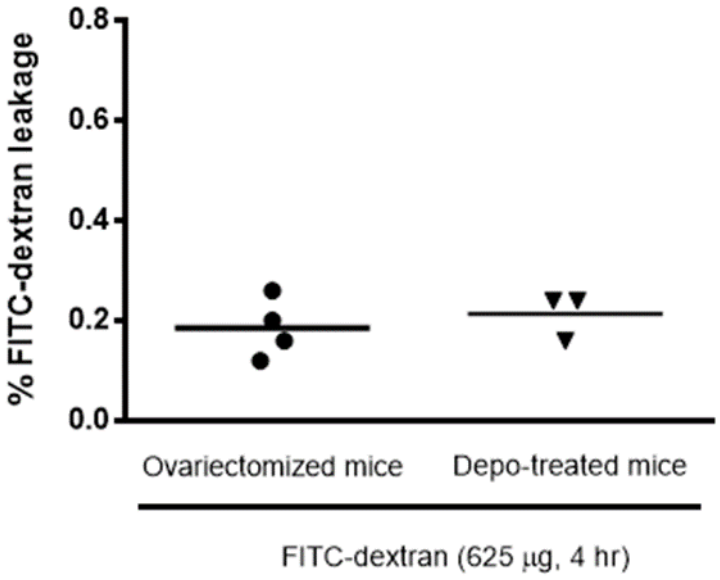
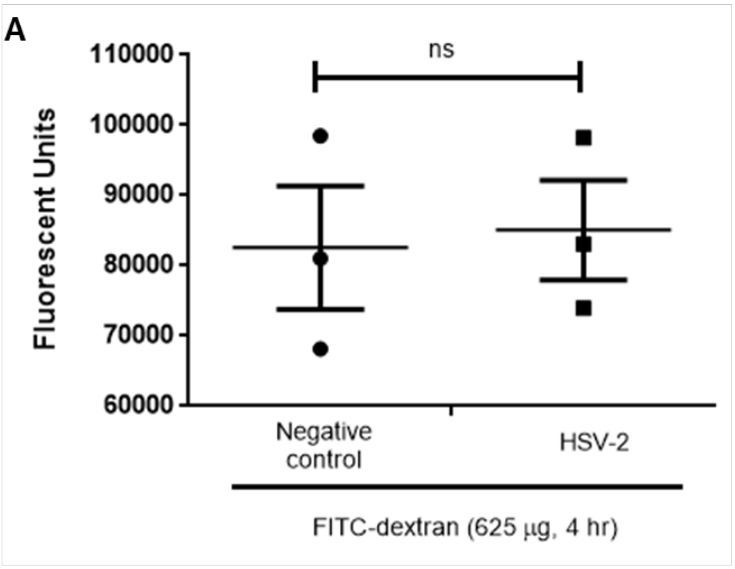

% FITC-dextran leakage
0.8
0.6
0.4
0.2
0.0
Ovariectomized mice
Depo-treated mice
FITC-dextran (625 μg, 4 hr)

Figure 3. DMPA-treated and OVX mice show similar genital barrier permeability. *In vivo* genital barrier permeability was measured in DMPA-treated and ovariectomized (OVX) mice using the FITC-dextran assay, as described in Materials and Methods. Mice received 625 μg of FITC-dextran dye intravaginally and serum was collected 4 hours following dye delivery. % FITC-dextran was calculated as the concentration of FITC-dextran in the serum divided by the concentration of FITC-dextran delivered. Serum was collected 4 hours later and fluorescence of FITC-dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices, Sunnyvale, CA) at an excitation of 490 nm and emission of 520 nm. FITC-dextran leakage into the blood is expressed as a percentage of FITC-dextran delivered. Each data point represents a single animal.

Figure 4


A
ns
110000
100000
90000
80000
70000
60000
Fluorescent Units
Negative control
HSV-2
FITC-dextran (625 μg, 4 hr)

B

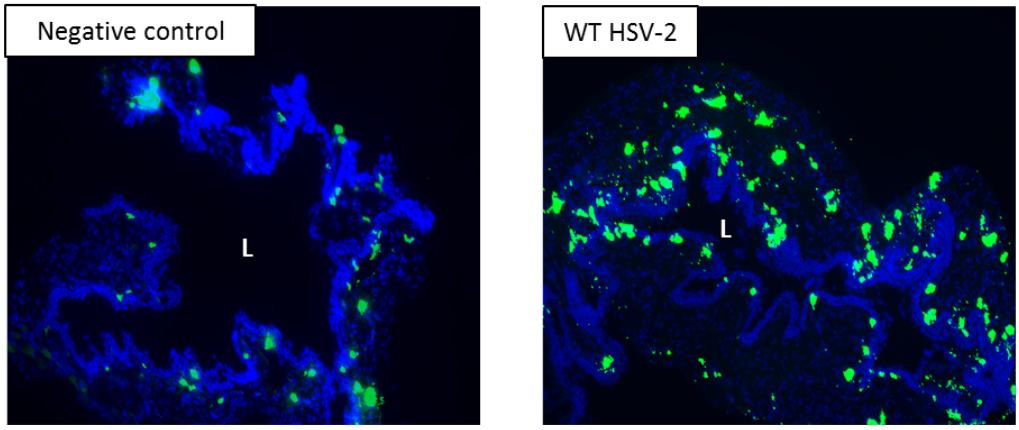

Negative control
L
WT HSV-2
L

Figure 4. HSV-2 does not alter genital barrier permeability within 24 hours of exposure. *In vivo* genital barrier permeability was measured in mice 18 hours following exposure to HSV-2 (333) using the FITC-dextran assay, as described in Materials and Methods. DMPA-treated mice were challenged with HSV-2 (10^4 pfu) and 18 hours following exposure, mice received 625 μg of FITC-dextran dye intravaginally. **(A)** Serum was collected 4 hours later and fluorescence of FITC-dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices, Sunnyvale, CA) at an excitation of 490 nm and emission of 520 nm. Each data point represents a single animal. **(B)** Vaginal tissues were embedded and flash frozen prior to immunofluoresence staining to assess levels of desmoglein-1α (DSG1α) protein expression; DSG1α (green); DAPI (blue); L denotes vaginal lumen. Representative images from a single experiment with three mice were experimental condition are shown (image magnification 10x).

CHAPTER 5: DISCUSSION

5.1 Overview of Women and HIV

Studies have consistently found that women are at much higher risk of contracting sexually transmitted infections compared to men (Wira and Fahey, 2008) (UNAIDS, 2014). For example, the prevalence of genital herpes infection is 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 (Looker et al., 2008). Similarly, women are also more likely to become infected with HIV, which remains one of the most serious health challenges in the world currently. Since the 1980s, HIV has shifted from a disease transmitted mainly by male to male sexual contact to a disease contracted mainly by female to male and male to female sexual contact. Currently, women make up more than half of the 35 million people worldwide living with HIV and the fastest growing phase of this pandemic is by heterosexual transmission in women (UNAIDS, 2014). Adolescent girls and young women are at particularly high risk of HIV infection, accounting for 20% of new HIV infections globally (UNAIDS, 2016). The FGT is a major portal for entry of HIV into the body, responsible for initiating 40% of global infections (Hladik and McElrath, 2008). In additional to the social, economic and behavioral factors that contribute to the increased prevalence of HIV in women, there are biological mechanisms underpinning this increased incidence. Furthermore, the acute events that follow HIV exposure in the FGT mucosa remain unclear. A better understanding of the microenvironment in the FGT is critical for developing strategies for the prevention of HIV transmission in women (Nguyen et al., 2014).

Despite the fact that the majority of HIV infections in females occur as a result of heterosexual intercourse with an infected male partner (UNAIDS, 2014), the precise mechanisms of sexual transmission in the female genital mucosa remain elusive. Heterosexual transmission models suggest that HIV in the male ejaculate must first overcome numerous innate and adaptive immune factors in the vaginal lumen (Carias et al., 2013). If successful, the virus will transverse the genital epithelium through tears in the squamous epithelium or transcytosis of the single cell layer of the endocervix, and ultimately infect underlying CD4+ target cells in the FGT (Carias et al., 2013). Here, the virus establishes a small founder population of productive infection that then expands systemically, likely *via* an influx of newly recruited target cells caused by an upregulation of chemokines (Haase, 2011).

Although there remains to be a clear consensus regarding the exact events during HIV transmission, the outcome following a viral exposure event is critically dependent on (a) mucosal inflammation and (b) the intactness of the epithelial barrier in the FGT. It is exceedingly clear that increased mucosal inflammation enhances the rate of sexual transmission of HIV in the FGT. Recently, Masson *et al* observed a three-fold increased risk of HIV infection in South African women with genital inflammation, defined by elevated levels of mucosal pro-inflammatory cytokines such as IL-8, IL-1β, IL-1α and TNF- α (Masson et al., 2015). This is relevant as an inflammatory cervicovaginal profile is correlated with the recruitment of HIV target cells to the portal of entry and the establishment of productive, systemic infection (Haase et al., 2015; Li et al., 2009).

Although far fewer clinical studies have examined the relevance of the genital epithelial barrier in HIV risk, infectious virions or infected cells from the donor must breach the epithelial barrier to find susceptible target cells in the host. Our lab has demonstrated that viral interaction with the genital epithelium results in increased impairment of barrier function resulting in enhanced viral translocation (Nazli et al., 2010). We have also shown that the effects of inflammation on HIV transmission extend beyond the recruitment of target cells. Mechanistic studies revealed that pro-inflammatory cytokines, produced by epithelial cells themselves in response to viral exposure, induce rapid destruction of tight junction proteins localized at the apical surface (Nazli et al., 2013). These results suggest that genital inflammation directly impairs barrier function and that the subsequent paracellular leaking of HIV across the genital epithelium could be a major source of HIV infection in the FGT (Nazli et al., 2013). Further studies indicated that other sources of local inflammation in the genital tract, such as presence of bacterial and viral co-infections and interaction with HIV infected semen, further enhance inflammation in the FGT, facilitating HIV infection and replication (Ferreira et al., 2011; Kafka et al., 2012). Clinical studies have since supported these findings. The proteomic profile of women with elevated cytokine expression in the genital tract contained a signature of proteases and actin cytoskeletal pathways, whereas protease inhibitor and other barrier function proteins, including epidermal cell differentiation and cornified envelope pathways, were decreased (Arnold et al., 2016).

Despite the implications to our understanding of HIV transmission and prevention, few studies to date have furthered the investigation into the relevance of genital epithelial barrier function to HIV infection in women. We have previously investigated the use of interventions that impede tissue inflammation and maintain barrier as prophylactic strategies for the prevention of HIV transmission *in vitro*, including IL-22 (Kim et al., 2012). Subsequently, we tested and demonstrated the promising anti-viral, anti-inflammatory and barrier promoting activity of curcumin, which provided a strong rationale for testing curcumin *in vivo* (Ferreira et al., 2015b). Thus, the work undertaken in this thesis characterizes (a) the anti-viral and anti-inflammatory activity of curcumin in the female genital tract *in vivo* and (b) an assay system to experimentally determine genital barrier function *in vivo*. Such an assay system, which can experimentally test barrier function *in* vivo will be very useful for us and other researchers, and will allow for the future testing of barrier promoting agents like curcumin and more detailed investigation into how loss of barrier function is implicated in HIV infection.

5.2 Summary of Results

HSV-2 is a common HIV co-infecting agent and curcumin exhibited significant anti-herpetic activity in our *in vitro* genital epithelial cell culture model (Ferreira et al., 2015b). As such, we were interested in assessing the anti-herpetic activity of curcumin *in vivo* using a well-established mouse model of intravaginal HSV-2 infection. We conducted three preliminary experiments examining the anti-herpetic activity of crude curcumin when

administered in direct combination with HSV-2. Overall, treatment with the crude curcumin resulted in better outcomes in terms of survival, pathology and shedding compared to controls. However, subsequent experiments which tested various polymer formulations of curcumin revealed that this was a result of non-specific physical barrier effects. Protection was lost when we tested the formulations in a pre-treatment regime, although we did observe delayed progression to HSV-2 associated endpoint. When we tested the efficacy of an improved curcumin nanoparticle formulation in the context of both sub-lethal and lethal doses of virus, we still did not observe protection. Overall, our studies indicate that regardless of formulation, curcumin is not an effective anti-herpetic therapeutic in our mouse model of primary intravaginal HSV-2 infection.

Although we did not observe anti-viral activity, based on our observations and the well described *in vitro* anti-inflammatory activity of curcumin, we next characterized the anti-inflammatory activity of curcumin *in vivo*, given the relevance of genital inflammation in HIV infection. The anti-inflammatory efficacy was assessed in the context of immunostimulatory TLR9 agonist CpG, which has been well-documented to induce inflammatory pathology within the genital tissue (Ashkar et al., 2004; Sajic et al., 2003), and qualitative as well as quantitative readouts of inflammation were used. When the curcumin-encapsulated nanoparticles were delivered *via* oral or IP routes, we did not observe any anti-inflammatory effects, likely because of insufficient tissue penetration in the FGT. Conversely, local delivery of the curcumin-encapsulated nanoparticles resulted in retention within the genital epithelium, and we observed significant anti-inflammatory

activity, which was absent when crude formulations of curcumin were compared. Our findings revealed a potent and promising anti-inflammatory efficacy of curcumin in the genital tissue that is dependent on both tissue penetration by the formulation and local route of delivery. When topically applied to the genital tract as a nanoformulation, curcumin can significantly inhibit inflammatory processes within the vaginal tissue *in vivo.* This can be beneficial given that FGT inflammation has been implicated in the recruitment of HIV target cells, impairment of barrier function and increased HIV risk in women.

The second portion of this thesis focused on the development of a system that could be used to assess genital barrier function in an *in vivo* mouse model. Currently, thre are no models that can assess barrier impairment and increased permeability *in vivo* in the FGT. Development of such a model will allow future studies to address the effect of HIV on barrier disruption in the genital tract *in vivo*, including the etiology of mucosal barrier disruption following HIV exposure and the effect of protecting mucosal barrier function prior to HIV exposure using compounds such as curcumin, as prophylactic treatment for HIV prevention. A series of experiments optimized a novel *in vivo* assay to functionally detect changes in genital barrier function. Briefly, FITC-dextran dye was delivered directly into the genital tract and four hours later, serum was collected for fluorescent spectrophotometry. Dye leakage into the bloodstream was used as a measure of genital barrier function. This assay was combined with three additional parameters, including cytokine analysis, histology and immunofluorescence, to comprehensively assess genital barrier function under a variety of hormonal and immunomodulatory conditions in the

genital tract. Using this system, we have demonstrated increased barrier function and expression of adhesions proteins within the genital epithelium of estrus-stage, Poly I:C and CpG treated mice, and conversely, reduced barrier function and expression of adhesions proteins in Depo and N-9 treated mice.

These data also contribute to the growing body of evidence implicating the use of injectable contraceptive Depo-Provera as a risk factor for HIV infection in women (Melhado-Kimura et al., 2017; Morrison et al., 2015). The relevance of studying epithelial dynamics within the murine genital tract has been called into question, primarily because epithelial thickness fluctuates dramatically throughout the murine estrous cycle while it remains relatively constant in the human menstrual cycle (Bahamondes et al., 2014; Kress et al., 2004; Mauck et al., 1999; Mitchell et al., 2014a). Furthermore, treatment with Depo induces extensive thinning of the genital epithelium in mice, yet there is no convincing evidence to suggest that this happens in women. However, in our study, we observed almost complete absence of cell to cell adhesion proteins in the remaining layers of genital epithelium in Depo-treated mice regardless of the thickness of the vaginal epithelium. This suggests that there are more subtle effects of Depo on genital barrier function in mice, in additional to reducing epithelial thickness, and could explain why women on Depo are significantly more susceptible to HIV infection than those on other forms of hormonal contraception.

5.3 Implications of this study

Based on our results, we posit that given the anti-inflammatory activity of curcumin *in* vivo, it could play a significant role in preventing mucosal transmission of HIV. Early mucosal inflammation is necessary for viral transmission in the FGT (Li et al., 2009). We have demonstrated that an inflammatory cytokine production induced upon exposure to HIV compromises the epithelial barrier (Nazli et al., 2010). Others have demonstrated that an inflammatory profile, characterized by the presence of IL-8, IL-1β, IL-1α and TNF-α is necessary for the recruitment of target cells to the portal of entry (Li et al., 2009; Masson et al., 2015), indicating that inflammation can facilitate viral penetration of the genital epithelium and the establishment of productive HIV infection in the underlying submucosa. *In vitro* studies have also demonstrated that inflammatory pathways can directly drive HIV replication (Ferreira et al., 2011). Thus, anti-inflammatory prophylaxis may be beneficial in decreasing HIV infection or replication.

In addition to the anti-inflammatory effects of curcumin, previous studies provide evidence that curcumin may block HIV by directly interfering with HIV replication cycle. Curcumin-loaded apotransferrin nanoparticles have been successfully used to block the synthesis of HIV viral cDNA (Barthelemy et al., 1998). Other *in vitro* studies have demonstrated that curcumin can inhibit the enzymatic activity of recombinant, purified HIV protease (Vajragupta et al., 2005)and integrase (Mazumder et al., 1995) and can directly block the HIV-LTR promoter in T cells (Ferreira et al., 2015b). Combining curcumin with

existing antiretrovirals, such as the protease inhibitor indinavir (IDV), reduced viral infectivity relative to IDV alone (Riva et al., 2008). Collectively, these results suggest that curcumin may interact and block HIV replication *via* multiple and perhaps redundant pathways and that there may be utility in supplementing current antiviral therapies with curcumin to reduce HIV infection and replication.

Curcumin may also play a significant role in preventing or reducing chronic immune activation. Microbial translocation due to mucosal barrier impairment is not only associated with HIV acquisition but is strongly believed to be one of the main contributors to chronic immune activation, characterized by increased inflammatory markers and immune cell activation that persists even in HAART-treated individuals (Brenchley et al., 2006; Burgener et al., 2015; Klatt et al., 2013b; Tincati et al., 2016). Immune activation is believed to be one of the main driving forces of CD4+ T cell depletion and promoter of HIV replication. Repeated exposure to HIV in mucosal tissues from shed virus may continuously provide a source for generating inflammation in mucosal tissues that facilitates barrier breakdown and microbial translocation. Because immune activation is typically observed during the chronic stages of infection, the window of opportunity to intervene and limit these processes likely occurs during the initial stages of HIV infection. Blocking HIV-mediated barrier disruption by curcumin may prevent microbial translocation and the initiation of immune activation, suggesting that curcumin treatment, may also contribute to decreasing the chronic inflammatory state that contributes to HIV pathology. We have also previously shown that genital barrier disruption following HIV

exposure can result in microbial translocation at the genital mucosa and increased levels of immune activation during acute infection (Nazli et al., 2010). This immune activation can increase chemokine production and the influx of HIV target cells during the early transmission stages of HIV infection, which may speed up the rate of viral dissemination and shorten the window of opportunity to slow viral spread. Curcumin could also be used to curb this acute immune activation and slow viral spread from the genital mucosa to the rest of the body.

To answer the critical need for effective female-controlled methods of protection against STIs, continued efforts are being directed towards the development of potential microbicides, such as curcumin. Unfortunately, clinical trials involving the microbicides COL-1492 (N-9), Savvy (C31G), Ushercell (cellulose sulfate), Carraguard (carrageenan) and PRO 2000 failed to demonstrate any efficacy despite promising effects in pre-clinical studies (Cutler and Justman, 2008; Van Damme et al., 2000; Van Damme et al., 2008). The consequences of these trials emphasized the urgent need for more stringent pre-clinical protocols. This was particularly apparent following retrospective analyses of the development of N-9 (Van Damme et al., 2002). Pre-clinical assessments of N-9 failed to predict its adverse effects on the cervicovaginal mucosa that would ultimately lead to increased HIV risk in high frequency users in clinical trials (Van Damme et al., 2002). These findings implicated the role of genital barrier function in determining HIV susceptibility, and raised new questions about the mechanisms by which potential topical microbicides can fail. In our study, we found that dye leakage was nearly 2-fold greater in

the mice that received a single application of N-9 compared to controls, and histology and immunofluorescence images show severe epithelial exfoliation even 4 hours post-application. These are observations that have been qualitatively been reported in previously published studies (Catalone et al., 2004; Lozenski et al., 2012).

Microbicide candidate molecules must be rigorously evaluated in pre-clinical models of safety and efficacy to ensure that only candidates with favorable risk benefit ratios are advanced into human clinical trials. The pre-clinical pipeline starts with *in vitro* assays that measure cytotoxicity in cell or tissue cultures or cervicovaginal and colorectal tissue explants. The use of differentiated monolayers to measure transepithelial electrical resistance (TEER), as a measure of monolayer integrity, has been frequently used in the microbicide field (Begay et al., 2011; Dezzutti et al., 2012; Kenney et al., 2013; Kizima et al., 2014; Rohan et al., 2010). The assay can be used to assess integrity of differentiated vaginal, ectocervical, endocervical or rectal epithelial monolayers(Fernandez-Romero et al., 2015). However, these *in vitro* systems do not allow for assessment of prolonged or repeated exposure to a microbicide candidate, which appeared to be the source of risk with N-9 use (Fernandez-Romero et al., 2015). They also cannot comprehensively assess microbicidal impact on the inflammatory responses and immune microenvironment that can only be seen in intact mucosal tissues. The local delivery of vaginal microbicides demands a closer look at the integrity of the cervicovaginal mucosa and the possible development of inflammatory cascades after microbicide application. Thus far, histological analysis and cytokine analysis of vaginal washes have been performed to observe the

morphology and inflammatory state of the cervicovaginal tissue, respectively. However, neither of these methods can be used to functionally assess the effects of the candidate microbicide on permeability.

The assay optimized and described in this thesis provides a new system to comprehensively evaluate the safety of future microbicide candidate molecules based on cervicovaginal tissue integrity. This assay specifically addresses the need for an *in vivo* model system that can be used to provide pre-clinical results predictive of clinical trial outcomes. As a comprehensive approach, we have combined four parameters that assess genital barrier function based on epithelial thickness as well as adhesion protein disruption. Thus, we propose that candidate microbicides undergo rigorous testing using this comprehensive system during the pre-clinical pipeline of development. We showed significantly higher FITC-dextran leakage in mice that received a single topical application of N-9 compared to the placebo, and histological and immunofluorescence assays revealed extensive epithelial exfoliation. We also showed that our assay system can be used to detect the subtler differences in barrier function in mice that are treated with synthetic progestin Depo-Provera compared to mice in the naturally progesterone-high phase of the estrous cycle. The FITC-dextran assay revealed significantly greater leakage in Depo-treated mice and more in-depth analysis revealed lowered expression of desmoglein. This suggests that Depo-Provera can compromise the integrity of the cervicovaginal tissue by reducing the expression of adhesion proteins that bind individual epithelial cells, and could therefore facilitate the passage of HIV during sexual intercourse with an infected partner. This data

could inform clinical regulations regarding the use of progestin-based hormonal contraceptives in high-risk women. It could also inform the selection of novel microbicidal formulations with a promising safety profile and the best risk/benefit ratios for Phase 1 clinical testing, along with other *in vitro, ex vivo* and *in vivo* tests.

5.4 Limitations

Although we observed promising anti-viral properties mediated by curcumin *in vitro,* this did not translate *in vivo* under the conditions tested. The reason for the lack of efficacy against HSV-2 is unclear but it highlights the need for testing new interventions in more physiologically relevant but complex in vivo models than pure epithelial cells in culture. Furthermore, the anti-inflammatory effects of curcumin are more likely to be beneficial for the prevention HIV than HSV-2, as HIV risk is closely correlated with genital inflammation and unlike HSV-2, HIV does not directly infect epithelial cells.

Although we propose to use curcumin to suppress inflammatory pathways within the genital tract that contribute to barrier disruption in HIV infection, we were unable to test this *in vivo.* This was not because the system developed in this study was not useful or curcumin did not show protective activity. Rather, the repertoire of immunomodulatory factors that were tested all induced mucosal inflammation, but none resulted in compromised genital barrier function. Rather, increased barrier function occurred because of the protective mechanism of epithelial thickening. Therefore, we were unable to test the

ability of curcumin to maintain barrier function in the presence of barrier-compromising inflammation. This was an interesting observation, since it distinguishes between innate inflammation that could be beneficial and protective compared to inflammation that impairs the barrier. While we hypothesize that the anti-inflammatory effects of curcumin will be barrier protective, this remains unclear because of the limitations of our current system to induce inflammation in the genital tract *in vivo*. A mouse model of inflammation-induced barrier disruption will need to be developed in order to assess this, similar to the dextran sulfate sodium (DSS)-induced colitis model used for intestinal barrier studies (Chassaing et al., 2014).

It should also be noted that use of an anti-inflammatory agent like curcumin at a mucosal site considered high-risk for pathogen exposure may be accompanied by undesirable side effects. In addition to inhibiting inflammation that impairs the barrier, the broad-spectrum anti-inflammatory effects of curcumin also impair innate inflammation that could be beneficial and protective. Indeed, inflammation is a necessary part of the immune cascade that deals with incoming threats, and complete suppression of this immune arm could theoretically increase susceptibility to other sexually transmitted infections with non-inflammatory etiologies. Thus, there may be hesitation in promoting the use of curcumin in women who are at considerable risk of contracting STIs such as HSV-2, chlamydia or gonorrhea. Ideally, the anti-inflammatory formulation would be strong enough to block the inflammatory pathways that cause barrier disruption and CD4+ T cell recruitment but

would still enable basal levels of inflammation to control viral or bacterial pathogens. While curcumin reduced the induction of pro-inflammatory cytokines and chemokines, it did not completely abrogate their production, suggesting that it may be an attractive candidate that can balance these two necessities. Nevertheless, future studies are needed to evaluate the safety profile of topically applied curcumin in genital tract of women. This includes its effects on other factors within the genital microenvironment, including the cervicovaginal microbiome and virome, antigen presentation, T cell activation, antimicrobial peptide production, in addition to its effects on susceptibility to other types of infections.

There are also practical limitations to the use of curcumin as a microbicide that should not be ignored, particularly because of the social and cultural implications surrounding intercourse. Following topical application, the formulation will reside within the vaginal lumen as a bright, yellow, semi-viscous substance. Once absorption occurs, the genital epithelial cells remain stained brightly yellow for 24 to 48 hours post-treatment. This begs the question, will women be inclined to use a microbicide that leaves behind undesirable, albeit harmless, side effects? Although this may seem like a trivial issue, there are well-established issues surrounding adherence and compliance in microbicide clinical trials that are rooted in aesthetics, convenience and culture. For example, despite being highly effective, the female condom is rarely used by women because of issues related to lack of discreetness and reduced pleasure. Should curcumin advance into clinical trials, the formulation will likely need to undergo changes that are able to quench its color without

reducing its efficacy. It may also be worthwhile to consider adding curcumin to personal lubricants or vaginal rings, products that will already be regularly used by our target groups.

5.5 Future Directions

Our results provide many promising directions for future research related to the prevention of HIV in women. Previous work in our lab has demonstrated that HIV envelope protein gp120 interacts with TLR2 and TLR4 on the surface of genital epithelial cells *in vitro,* resulting in the downstream induction of pro-inflammatory cytokines, such as TNF-α and IL-6, and the rapid destruction of tight junction protein localization at the apical surface (Nazli et al., 2010; Nazli et al., 2013). This resulted in impairment of barrier function, which was measured *via* transepithelial resistance (TER) and FITC-dextran leakage, and significant viral translocation across the epithelium. These results suggest that HIV can induce barrier disruption that facilitates viral transmission into the submucosa where it encounters susceptible CD4+ target cells. Yet, the *in vivo* relevance of this phenomenon remains unclear. Humanized mouse models have significantly facilitated HIV research by modeling HIV infection in a small animal model. This model has since been an invaluable tool for pre-clinical evaluation of HIV prevention approaches and is expected to accelerate their implementation into clinical trials. Two main models, humanized BLT mice and RAG2-/- γc-/- mice are the most widely used models for HIV studies. We have recently optimized a mouse model of intravaginal HIV infection, using humanized BALB/c RAG2-/- γc-/- mice reconstituted with human CD34+ enriched cord blood. These mice

show excellent immune reconstitution in the bone marrow, spleen, thymus, lymph nodes and genital tract, and have demonstrated functional innate and adaptive immune responses against HSV-2 and Salmonella, as well as NK cell responses for malignant cell detection (Firoz Mian et al., 2011; Mian et al., 2011; Pek et al., 2011). Our lab has also used this model to follow viral dissemination and CD4+ cell depletion following intravaginal inoculation with HIV, as well as assess target cell frequency HIV viral inoculation dose as determinants of HIV infection (Nguyen et al, in revision, 2017). Thus, we propose that our system to evaluate barrier function could be used in this humanized mouse model to directly assess effect of barrier protection or impairment on HIV infection. To examine the ability of HIV to mediate barrier disruption in the genital tract *in vivo,* FITC-dextran dye should be administered at various time points following intravaginal inoculation with HIV (ADA, 10^5 -10^7 infectious units/mL of NL4.3 Bal-Env virus). The correlation between HIV-mediated barrier disruption and levels of endogenously produced TNF-α should also be assessed by measuring TNF-α in vaginal washes collected post-challenge. This experiment could then be repeated with intravaginal treatments of TNF-α neutralizing antibody prior to HIV challenge to determine if blocking endogenously produced TNF-α prevents HIV-mediated barrier disruption and/or HIV infection. This will reveal whether barrier disruption induced in the genital tract upon viral exposure is necessary for the establishment of HIV infection.

Data from our previous *in vitro* studies indicated that pre-treatment of primary genital epithelial cells with curcumin prevent HIV-mediated disruption of the mucosal

barrier by inhibiting the production of pro-inflammatory cytokines and maintaining tight junction expression across the genital epithelium (Ferreira et al., 2015b). Thus, we expect that curcumin will be an effective prophylactic strategy for the prevention of HIV infection and/or the control of HIV replication. Although we demonstrated its potent anti-inflammatory activity in the genital tract *in* vivo, we were unable to assess the ability of curcumin to prevent inflammation-mediated barrier disruption because of limitations within the model. However, future experiments should assess the ability of curcumin-encapsulated nanoparticles to prevent infection in our humanized mouse model of intravaginal HIV challenge. Vaginal washes and plasma should be collected at various time points, such as 1, 3 and 5 weeks post-inoculation, to measure HIV viral load by RT-PCR using the Abbott HIV-1 Viral Load Assay, a clinical assay which measures viral RNA copies/mL.

If curcumin does not prevent HIV infection *in vivo*, the ability of curcumin to limit immune activation could also be investigated. As described previously, this is an additional consequence of HIV infection that strongly correlates with disease progression and is likely explanation for the increased levels of immune activation observed during the acute phase of HIV infection. Immune activation in the presence of curcumin treatment could be measured by examining levels of prototypical markers, such as sCD14 and LPS, in the serum by ELISA. At the termination of experiments, spleen and lymph node cells could be used to measure the CD4 to CD8 ratio by flow cytometry, which is usually reversed during chronic HIV infection. CD8+ T cells from the spleen, lymph nodes and mucosa

could also be analyzed for expression of CD38 and HLA-DR, both of which are characteristic of activated CD8+ T cells during HIV infection. Alternatively, future experiments could test if the combination of curcumin with other anti-viral treatment (Pre-exposure prophylaxis, PrEP) is more effective than either treatment alone in preventing HIV infection. Studies of Tenofovir vaginal gel in the VOICE trial have cast doubt on the efficacy of the vaginal PrEP alone approach (Celum and Baeten, 2012; Karim and Karim, 2011; Rossi, 2011). The underlying lack of efficacy in this study compared to the previous CAPRISA trial, that showed 39% less infections in women applying 1% Tenofovir gel, are unclear and could be due to pre-existing inflammation. Therefore, combination of PrEP with an anti-inflammatory treatment, such as curcumin, could potentially increase the efficacy of the PrEP anti-virals in women.

These studies will provide further understanding of the role of the mucosal barrier in HIV transmission and pathogenesis. This approach to HIV prophylaxis is completely novel and could open a new area for prevention strategies. The results of testing in the validated pre-clinical humanized mouse model could accelerate the implementation of curcumin into clinical trials.

CHAPTER 6: CONCLUSION

Approximately half of all individuals living with HIV/AIDS are currently women, and it is estimated that the female genital tract is the main site of HIV acquisition globally, accounting for approximately 40% of all new HIV infections. Despite this very clear gender imbalance, very little is known about the early events of HIV transmission and pathogenesis in the female genital tract. Epithelial barrier function, mucosal inflammation and HSV-2 infection are all factors that have both been implicated in HIV risk and early disease progression. Given its potent anti-inflammatory activity, we investigated the ability of curcumin to prevent mucosal inflammation and HSV-2 infection in a mouse model. We also sought to further our understanding of the genital epithelial barrier and developed an assay system that allowed us to test the effect of various immune and hormonal factors on genital barrier function *in vivo.* The results of our study have implications for the prevention of HIV transmission and early disease progression. Curcumin is able to curb mucosal inflammation in the genital tract, which could decrease target cell populations and prevent the likelihood of successful transmission or dampen acute immune activation and slow viral spread. We also added to the growing body of evidence implicating Depo-Provera, which severely reduces genital barrier function *in vivo*, as a risk factor for HIV acquisition in women. This work lays the foundation to examine the underlying mechanisms through which HIV can directly impair the epithelial barrier and the endogenous factors, such as hormones, the microbiota and inflammation, that affect genital barrier function *in vivo.* It also acts as rationale to shuttle anti-inflammatory and barrier protective agents into the prophylactic development pipeline as innovative ways to protect women from acquiring HIV infection.

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