INVESTIGATING THE ROLE OF LAMP3 IN HIV-1 UPTAKE AND TRANSCYTOSIS ACROSS VAGINAL EPITHELIAL CELLS

INVESITGATING THE ROLE OF LAMP3 IN HIV-1 UPTAKE AND TRANSCYTOSIS ACROSS VAGINAL EPITHELIAL CELLS

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree Master of Science

McMaster University

Hamilton, Ontario, Canada

DESCRIPTIVE NOTE

MASTER OF SCIENCE (2020) McMaster University
(Medical Sciences, Infection and Immunity) Hamilton, Ontario

TITLE: Investigating the Role of LAMP3 in HIV-1 Uptake and Transcytoses Across Vaginal Epithelial Cells

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NUMBER OF PAGES: xi, 98

Abstract

Background: It is estimated that 38 million people currently live with HIV-1. Of the 38 million currently infected, 19.2 million women are infected compared to 17 million men, indicating a clear disproportion. As such, understanding mechanisms that result in increased susceptibly in women is critical to develop more efficacious prevention strategies. The epithelial cells that line the lower female reproductive tract are the first line of defense against invading pathogens. The current consensus in the field is that HIV-1 can cross the mucosal epithelium in two ways: paracellular passage and transcytosis. There are several endocytic pathway associated proteins within vaginal epithelial cells that may have a role in viral transcytosis, however, little is known about their role. One of these is Lysosomal Associated Membrane Protein 3 (LAMP3), which has been shown to be upregulated following viral exposure and involved in viral trafficking. The mechanisms regarding the early events of transmission of HIV-1 across vaginal epithelial cells remains unclear and warrant further investigation. This study was designed to examine the mechanism of how HIV-1 crosses vaginal epithelial cells and potential interactions of LAMP3 and HIV-1.

Method of Study: We utilized an *in vitro* cell culture system to grow vaginal epithelial cells in in both an air-liquid interface (ALI) and liquid-liquid interface (LLI). We investigated barrier function in vaginal epithelial cells after 24 hours of exposure to HIV-1 using FITC leakage assays, measurement of transepithelial resistance, and confocal imaging of barrier proteins. We also assessed the potential role of LAMP3 in HIV-1 uptake and transcytosis by using LAMP3 knockdown (KD) and LAMP3 overexpression (OE) cell lines. Barrier functions were assessed, as well as HIV-1 transport into the basolateral compartments was measured using a P24 ELISA and TZM-bl assay in KD and OE cells compared to WT-Vk2.

Results: We found that in HIV-1 treated ALI cultures barrier function was not impaired, however, in LLI cultures the barrier was disrupted. We also found that HIV-1 uses the endocytic pathway in Vk2 cells. Additionally, we found that alterations in LAMP3 expression do not alter barrier function in vaginal epithelial cells. Moreover, LAMP3 gene expression is induced upon exposure to HIV-1 and knockdown of LAMP3 significantly increased the amount of HIV-1 present in the basolateral supernatants compared to WT-Vk2 cells grown in LLI cultures.

Conclusions: This work contributes to a better understanding of how HIV-1 crosses the vaginal epithelium, and host-factors that may play a role in HIV-1 trafficking in vaginal epithelial cells. These findings can be built upon to develop more effective prevention measures for HIV-1 acquisition in women.

Acknowledgements

I am forever thankful to my supervisor Dr. Kaushic for providing me the opportunity to be part of her lab. Her outstanding mentorship and supervision throughout my time has been greatly appreciated and her commitment to the success of the students is admirable. I will implement the skills I have learned from you and apply them to future work I obtain throughout my life. Your demonstration of endless energy, assertiveness, knowledge, and critical thought have been inspirational.

Thank you to my committee members Dr. Carl Richards and Dr. Amy Gillgrass for their advice and guidance. Thank you to Dr. Richards for his modelling of endless curiosity, humor, and dedication to science, and to Dr. Gillgrass for her divergent thinking, approachability, and fostering the love of science.

A specific thank you to Dr. Aisha Nazli, the senior research scientist in the lab, for her constant inspiration, guidance, assistance with troubleshooting, teaching me everything I know and helping with all the data analysis. You are truly an amazing scientist! Also, thank you for the tasty snacks throughout my time in the lab!

To the Kaushic lab members who have been nothing but helpful and supportive of my time here. To Dr. Puja Bagri, Dr. Allison Felker, Haley Dupont and Ramtin Ghasemi who welcomed me into the lab with open arms. The memories we spent in and outside the lab will be cherished forever. Thank you to undergraduate Ryan Chow for teaching me new methods and helping with data analysis when I first arrived in the lab. To the more recent members Sidney Pa, Tushar Dhawan, Dr. Christina Hayes, and Ingrid Schwecht for providing constant laughs, support, as well as all the fun-filled snack and coffee runs.

Lastly, to my friends and family for the constant pep talks, inspiration, and encouragement. Although you had no idea what I was talking about majority of the time, the wine nights, and late-night snaps always made me feel better! Thank you.

DECLARATION OF ACADEMIC ACHIEVEMENT

All experiments were conceived and designed by Andrew Rempel, Dr. Charu Kaushic, and Dr. Aisha Nazli. Andrew Rempel wrote this thesis dissertation with contributions from Dr. Charu Kaushic.

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AIDS: Acquired Immunodeficiency Syndrome

ALI: Air-Liquid Interface

AMPs: Antimicrobial Peptides

DMPA: Medroxyprogesterone Acetate

FITC: Fluorescein Isothiocyanate

FRT: Female Reproductive Tract

GEC: Genital Epithelial Cell

HIV-1: Human Immunodeficieny Virus

IFNs: Interferons

IL: Interleukin

IU: Infectious Unit

JAM-1: Junctional Adhesion Molecular 1

kDa: Kilodalton

KSFM: Keratinocyte Serum Free Media

LAMP: Lysosomal Associated Membrane Protein

"LDH: Lactate Dehydrogenase "

LGT: Lower Genital Tract

LLI: Liquid-Liquid Interface

LPS: Lipopolysaccharides

LRT: Long Terminal Repeat

MIP-1α: Macrophage Inflammatory Protein 1 alpha

NHP: Non-human primate

OD: Optical Density

PAMPS: Pathogen-Associated Molecular Patterns

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PRRs: Pattern Recognition Receptors

RANTES: Regulated on activation, normal T cell expressed and secreted

SDF-1: Stromal cell derived factor

SEM: Standard Error of the Mean

SIV: Simian Immunodeficiency Virus

SLPI: Secretory Leukocyte Protease Inhibitor

STI: Sexually Transmitted Infection

TER: Transepithelial Resistance

TERs: Toll Like Receptors

TNF-α: Tumor Necrosis Factor alpha

TRIM5α Tripartite motif 5a

TZ: Transition Zone

UNAIDS: United Nations joint program on HIV/AIDS

ZO-1: Zonula Occluden

CHAPTER 1: INTRODUCTION

1.1 HIV-1 Infection in Women:

1.1.1 Epidemiology

Human immunodeficiency virus (HIV-1) continues to be a major health pandemic, despite global efforts focused on prevention measures and major advancements in treatment for individuals living with HIV-1. In 2019 it was estimated that 38 million people were living with HIV-1, 690,000 people died from acquired immunodeficiency syndrome (AIDS) related illnesses, and an estimated 1.8 million new HIV-1 infections occurred[1]. Globally, Africa remains the epicenter of the HIV-1 epidemic with roughly 23.8 million people living with HIV-1, accounting for nearly 75% of all global infections[1]. When analyzing demographics of affected individuals, it is clear that women are more susceptible to HIV-1 than men. UNAIDS reports that, 5500 young women aged 15-24 become infected with HIV-1 weekly and are twice as likely to acquire HIV-1 than men the same age range. In sub-Saharan Africa, five in six new infections are among young women ages 15-19 and in 2019 girls accounted for 59% of all new HIV-1 infections[1]. Therefore, focusing efforts on this key population that is disproportionately affected by HIV-1 is critical to control the HIV-1 epidemic. Unfortunately, many women living with HIV-1 or at risk of acquisition do not have adequate access to prevention, care, and treatment. This is due in part to negative attitudes towards sex outside of marriage, and the restricted social independence of women and young girls which can reduce their ability to access sexual health clinics and HIV-1 services[2]. Despite the various treatment options and preventative therapies available, new HIV-1 infections remain high in young women and thus immediate action is needed to minimize acquisition of HIV-1 in this target population.

1.1.2 Structure of HIV-1

Epidemiologic and phylogenetic analysis indicates that, HIV-1 was most likely introduced into the human population around 1920-1940 through non-human primate immunodeficiency viruses from Central African chimpanzees [3]. HIV-1 is a member of the lentivirus genus, which includes retroviruses that possess complex genomes and structural proteins[4]. HIV-1 can persistently infect humans without raising any significant alarm by suppressing the innate and adaptive immune systems [4]. The HIV-1 genome consists of two identical single stranded RNA molecules surrounded by a capsid protein[5]. The genome of HIV-1 is generated by reverse transcription of viral RNA into viral DNA and inserted into the human genome. The DNA genome is bound at both ends by long terminal repeat (LTR) sequences where the 5' end codes for the promotor of transcription of the viral genes. Reading from the 5' to 3' end, after the 5' LTR promoter, follows the gag gene, coding for the proteins of the outer core membrane (p17), the capsid protein (p24), the nucleocapsid (p7) and a stabilizing protein[5]. The pol reading frame follows which codes for the enzymes protease (p12), reverse transcriptase (p51), and RNase H (p15), and integrase (p32). Neighboring the pol gene, the env reading frame follows which contains two envelope glycoproteins (gp120 and gp41)[5]. Lastly, the 3' LTR promoter remains. Along with the structural proteins, the HIV-1 genome codes for several regulatory proteins: transactivator protein (TAT), RNA-splicing-regulator (Rev), negative regulating factor (Nef), Viral infectivity factor (Vif) virus protein r (Vpr) and virus protein unique (Vpu) all of which have an impact on viral replication, virus budding and pathogenesis[5].

1.1.2.1 HIV-1 Entry and Infection

HIV-1 can infect a variety of immune cells including CD4+ helper T-cells and macrophages, as well as dendritic cells that express the CD4 molecule on their surface along with

coreceptors CXCR4 or CCR5[6]. Strains of HIV-1 that utilize the chemokine receptor CCR5 for entry can infect macrophages and CD4+ T-cells expressing CCR5 coreceptor. These strains are termed R5, or CCR5-tropic HIV-1 strains. In contrast, HIV-1 isolates that replicate primarily in CD4+ T cells and macrophages but use the chemokine receptor CXCR4 for entry are termed X4, CXCR4-tropic or, T-tropic strains. Those that can enter cells using either co-receptor are termed R5X4 or dual tropic. HIV-1 entry into cells is mediated through chronological interactions between HIV-1 envelope protein gp120 and the primary CD4+ cell surface receptor along with co-receptors CCR5 or CXCR4. When HIV-1 approaches a target cell gp120 binds to CD4, after binding to CD4 gp120 undergoes a conformational change that allows the exposure of its V3 loop segment, which coordinates co-receptor recognition[7]. Interestingly, co-receptor preference has been associated with specific viral traits. CXCR4-tropic viruses have been associated with rapid disease progression, late infection phase emergence associated with an advanced state of immunodeficiency, as well as increased host cell pathogenicity[6],[8]. In comparison to CXCR4, CCR5 tropic HIV-1 is efficiently transmitted through blood, mother to child or sexually and generally dominates initial HIV-1 infection[8]. R5 is more likely transmitted sexually due to the CCR5 expressing CD4T cells and macrophages that reside in the mucus membranes of the genital tract. Moreover, Langerhans cells that extend dendrites that reach the surface of the epithelium can directly bind to HIV-1 and can assist with CD4 T Cell infection[9]. X4 tropic HIV-1 emerges approximately 5 years after initial HIV-1 infection with this tropism switch occurring in about 50-60% of individuals[10]. The selective transmission of R5 over X4 is still not well understood. Therefore, unravelling interactions of HIV-1 within the female reproductive tract and its asymmetric co-receptor usage would help understand HIV-1 pathogenesis and lead to better understanding of how to prevent viral entry.

1.2 Female Reproductive Tract

1.2.1 Structure of the Female Reproductive Tract

The leading route of HIV-1 transmission in women is through unprotected heterosexual vaginal intercourse[11]. The current consensus on infection in the female reproductive tract (FRT) is that genital epithelial cells, are not productively infected due to their lack of CD4 surface receptors, and act as a barrier to protect underlying target CD4+ T cells and macrophages[12]. Thus, the epithelium represents an important first line of defense against HIV-1 virions[13, 14]. Therefore, it is imperative that we understand the structure and morphology of the FRT and how it may influence susceptibility or confer protection against HIV-1 acquisition.

The FRT is a complex environment suited to meet specific functional demands by serving as the site for fetal allograft, while simultaneously protecting against invading pathogens, such as HIV-1. The FRT can be divided into two main parts, the upper and lower. The upper FRT consists of the endocervix, uterus, fallopian tubes and ovaries and offers a physically thin protective barrier comprised of a single layer of columnar epithelial linked together by tight junction proteins[15, 16]. Tight junctions join neighboring cells and are critical in allowing and preventing paracellular passage of molecules[17].

The lower FRT is comprised of the ectocervix and vagina and is lined with stratified squamous epithelial cells covered by mucus and colonized by bacteria[15]. It is the first location that HIV-1 encounters when transmitted sexually from an infected male partner[16]. The most basal cells are mitotically active and are held together by tight junctions. The most apical layers of

vaginal epithelial cells take on a superficial role and do not contain organelles, tight junctions, or a nucleus[18].

Between the upper and lower tracts lies the transformation zone (TZ) where the squamous epithelium of the lower vaginal tract transitions into simple columnar epithelium[16]. The TZ represents a very small portion of the FRT tract and is considered to be a minor area of exposure. Although a small area, the TZ zone has a physically thin barrier similar to that of the upper FRT and a large amount of underlying immune cells making it a susceptible target for HIV-1 transmission[16].

Although HIV-1 virions can traverse both the upper and lower FRT, there is no consensus on the most probable area of HIV-1 transmission in the FRT. However, the stratified squamous cells of the lower vaginal tract are the first cells to encounter HIV-1 during heterosexual intercourse, and comprise the largest surface area of the FRT exposed to HIV-1 in male ejaculate[16]. Additionally, during intercourse physical damage such as micro tears can compromise the robust vaginal barrier of the lower FRT facilitating HIV-1 transmission and a more efficient way to reach target cells in the underlying epithelium[19, 20].Vaginal epithelial cells have previously been shown to uptake and internalize HIV-1 in-vitro. A study done by Young et al., found that sperm from healthy donors incubated with dual tropic HIV-1 added to an immortalized vaginal epithelial cell line was found to be effectively internalized into these cells [21]. Moreover, a study done by Stieg et al., found that infecting rhesus macaques intravaginally with an R5 tropic stain of simian immunodeficiency virus (SIV) resulted in infection of underlying target cells throughout both the lower and upper FRT[22].

While the epithelial barrier is a robust defense against HIV-1, studies have shown that HIV-1 can traverse intact epithelial layers and gain access to target cells expressing the surface receptor

CD4 and co receptors CCR5 or CXCR4[23]. As the lower FRT represents the greatest surface area exposed to HIV-1 during intercourse and transmission and is the first point of contact with HIV-1, understanding how HIV-1 crosses vaginal epithelial cells warrants further investigation to better develop preventative strategies against HIV-1.

1.2.2 Genital Epithelial Cells of the Lower FRT

The stratified squamous epithelium of the vaginal tract has a large surface area of about 360cm^2 and is the first site of pathogen contact[24]. Understanding the vaginal epithelium structure, its interactions with HIV-1, and the influence of hormone on epithelial function is imperative to better understand HIV-1 transmission and develop more efficacious prevention strategies.

As aforementioned, the lower vaginal tract is comprised of a distinct stratum of epithelial cells including a mitotically active basal layer, the superbasal layer and a superficial top layer of dead cornified cells, that are constantly shed and replaced by underlying cells[18, 25, 26]. The complex layers of the lower FRT provide a robust barrier against pathogen entry[27]. However, as vaginal cells differentiate and undergo cornification, they lose many of their nuclei and organelles, along with the loss of tight junctions, which may facilitate viral entry between superficial layers [18, 28].

The structure of the vaginal epithelium constantly changes with age and is affected by hormonal and environmental stimuli. Major functions of epithelial cells include secretion of various proteins like antimicrobial peptides (AMPs), selective trafficking of molecules such as salt ions, and protection against pathogens. During adulthood, the vaginal epithelium becomes thicker and develops a distinct cornified layer and is influenced by hormones such as estrogen[18, 29, 30]. The normal female menstrual cycle is divided into 2 distinct phases: the follicular and luteal phase[31]. During the follicular phase, in which the maturing follicle produces estradiol, follicle

stimulating hormone and luteinizing hormones are released which allow the stimulation of egg development and promotes endometrial growth[32]. Following, ovulating during the luteal phase, the corpus luteum secretes progesterone to stabilize the endometrial lining and prepare for implantation of the egg into the lining of the endometrium[32]. Patton et al., examined vaginal tissue during the menstrual cycle and found that there was a significant reduction in the number of vaginal epithelial cell layers over the menstrual cycle, which could increase the chances of HIV-1 acquisition[30]. Additionally, hormonal contraceptives are often used by women to prevent pregnancy, and work to inhibit the natural cyclical hormones that would occur during the menstrual cycle. Hormonal contraceptives mostly differ by their progesterone components and have been linked to increased HIV-1 acquisition due to multiple underlying mechanisms including alterations in local mucosal immunity [31, 33].

Epithelial cells of the lower FRT are metabolically active cells that play a critical role in the regulation of homeostasis in the vaginal tract and protection from viral pathogens. Studies have demonstrated that HIV-1 can be taken up by the stratified squamous epithelium[34, 35]. Epithelial cells lack the classical CD4+ receptor needed for infection, therefore, alternative mechanisms by which HIV-1 can breach the vaginal epithelial layer which warrant further investigation.

1.2.3 HIV-1 Transmission in The Lower Female Reproductive Tract

Women account for more than half of all HIV-1 infections worldwide, with sexual transmission being the primary route of infection in women[36]. Biological, cultural, and societal factors all contribute to the risk of HIV-1 acquisition in women. Among biological factors, age, viral load, presence of co-infections such as bacterial and viral sexually transmitted infections, as well as anatomy, innate immune response, hormonal contraceptive use, and epithelial integrity of the vaginal tract have been linked to increased risk[36]. Although the understanding of HIV-1

transmission in the female reproductive tract (FRT) has improved, there is still insufficient information to fully understand the biological mechanisms that result in increased susceptibility in women. In order for HIV-1 exposure to result in productive infection, it must traverse the epithelial barrier and infect target cells. HIV-1 infection can be present in cell associated and cell-free virus forms and has the ability to cross the vaginal epithelium, however the mechanisms of viral entry and intracellular trafficking across the vaginal mucosa remains incompletely understood[36].

As epithelial cells lack the CD4 receptor necessary to result in infection, alternate mechanisms for entry have been proposed. Several host factors not involved with fusion have been implicated for HIV-1 transmission and infection. For example, the syndecan family of receptors has been shown to bind and adsorb HIV-1 through interactions between heparin sulfate chains, preserving the infectivity of HIV-1 and enhancing cell-cell transmission of virus produced from peripheral blood mononuclear cells (PMBCs) [37]. Using immortalized genital epithelial cell lines, Stoddard et al., demonstrated that gp340 expressed on genital epithelial cell binds HIV-1 and facilitates viral transmission[38]. Moreover, the mannose receptor on vaginal epithelial cells was shown to bind with HIV-1 gp120 with high affinity, inducing the production of proteinases, which in turn facilitated HIV-1 transmission across vaginal cells[35].

It was also found that the thickness of the mucosal layer may play a protective role from HIV-1 invasion, and that certain hormones such as medroxyprogesterone acetate (DMPA) correlate with increased HIV-1 transmission across the mucosa[39-41]. Although the mechanism is not entirely clear regarding increased susceptibility of women using DMPA, Marx et al., showed that exogenous progesterone administered to rhesus macaques thinned vaginal epithelial tissue and increased susceptibility to SIV[40]. They further demonstrated that treating rhesus macaques with exogenous estrogen increased vaginal epithelium thickness and protected against SIV[40]. Previous

work in our lab has investigated the effect of DMPA in, *in-vivo* mouse models and a vaginal epithelial cell line. In our mouse models we found that not only did DMPA thin the vaginal barrier but mice treated with DMPA also had a 100 fold increased susceptibility to HSV-2 infection compared to a saline suspension of progesterone[42, 43]. The mice treated with DMPA also demonstrated inhibitory effects on immune responses to HSV2. Specifically, lowering of HSV-2 immunoglobins IgG and IgA in vaginal washes, thus demonstrating DMPA treatment leads to increased HSV-2 susceptibility and alterations in local immune response[42, 43]. More recently, in our in-vitro vaginal epithelial cell, Vk2, model, we found that in the presence of DMPA barrier function is decreased, and the chemokine RANTES is produced in larger amounts (Lam, Master's Thesis). Thus, linking a role of hormonal contraceptives such as DMPA in increased HIV-1 transmission.

Moreover, studies have investigated how disruption of natural defenses such as microtears during intercourse in the female reproductive tract may influence HIV-transmission. Investigation by colposcopy and Lugol's staining 6 hours after consensual vaginal intercourse revealed that 61% of patients showed microtrauma in the vaginal mucosa, which could potentially increase the chances of HIV-1 transmission[44]. Additional studies investigating douching, breaks in the epithelial lining, and cytological changes from human papilloma viruses have also been implicated in HIV-1 transmission [45],[46]. Despite these studies, there are still large gaps in the knowledge surrounding increased susceptibility to HIV-1 infection in the female population that requires more research. A deeper understanding of the epithelial cells that line the FRT and mucosal immunity is key to the development of better preventative measures to combat HIV-1 transmission.

1.3 Innate Immune Response of GECs

The mucosal surfaces of the FRT contain numerous chemical, and physical defenses against pathogens and responds rapidly after encountering foreign antigens[47]. Epithelial cells of the FRT not only act as a physical barrier, but can also produce mucus, and release antimicrobial peptides as well as cytokines and chemokines to assist in pathogen clearance[48].

1.3.1 Mucus

Cervical and vaginal epithelial cells act as barriers to HIV-1 entry during heterosexual transmission and are coated with a layer of mucus that confers an added level of protections against pathogens[49, 50]. Mucus in the vaginal tract provides not only a physical barrier but contains chemical components such as mucins that help provide protection against HIV-1 infection[51]. Mucus is found on epithelial cell surfaces in all sites of the FRT and is secreted by epithelial cells of the cervix and uterus. Mucus in the FRT is comprised primarily of about 92-95% water, but also contains numerous proteins, lipids, nucleic acids, cell debris, ions, and mucins all which contribute to the gel forming properties[52]. Mucus acts as a physical barrier to invading pathogens by providing a layer of sustained lubrication and moistening of epithelial cell surfaces. Hope et al., digitally tracked HIV-1 virions and found that HIV-1 diffusion was significantly hindered in vaginal mucus and that virus transport was envelope dependent[51]. Moreover, Lai et al., tracked HIV-1 virions in cervicovaginal mucus from health donors and found that HIV-1 progressed at a rate 15 times slower in mucus compared to water[53]. Additionally, mucus also contains defense proteins such as immunoglobin A, lactoferrin and lysozymes that can help interfere with HIV-1. For example, lactoferrin, a multifunctional glycoprotein shown to have antibacterial and antiviral properties is present within cervical mucus[54]. Rolando et al., found that when lactoferrin from

0.31 to 50 uM was added in a dose-dependent manner to human or bovine PBMCs it inhibited HIV-1 p24 antigen for all HIV-1 isolates used[55].

1.3.2 Pattern Recognition Receptors (PRRs)

Epithelial cells of the vaginal tract function as sentinels that recognize and respond to antigens swiftly to eradicate foreign invaders[56]. If mucus is unable to prevent the trapping and neutralization of a viral or bacterial particle, binding of a pathogen to the epithelium can lead to innate cell signaling cascades that result in cell death, necrosis, apoptosis, or internalization of the foreign antigen.

Upon cell surface contact with a viral pathogen, epithelial cells are able to recognize and respond to viruses though PRRs, specifically toll like receptors (TLRs). TLRs alert the immune system to the presence of foreign antigens through pathogen-associated molecular patterns (PAMPs) on pathogens surfaces. TLRs then activate signal transduction pathways by the interaction with specific ligands, leading to the production of various chemokines and cytokines[57]. Recognition of PAMPs by TLRs ensures that upon entry into the vaginal tract, viral pathogens rarely escape surveillance by the innate immune system. TLRs are often located on the cell surface, however, can also be expressed in the membranes of endosomes and lysosomes[58]. Cell surface TLRs include TLR1-6, whereas intracellular TLRs include TLR3, TLR7-9. Columnar epithelial cells of the upper genital tract express TLRs1-9, whereas vaginal epithelial cells express TLR1-3, and TLR5-9 [56, 59]. TLRs 1,2, 4, and 6 recognize microbial products such as lipopolysaccharides (LPS) and glycoproteins present on bacteria and viruses, respectively, whereas TLRs 3, 7, 8 and 9 detect nucleic acids of pathogens[60, 61]. TLR1, 2, and 6 form heterodimers with one another and can distinguish between molecular structures of diacyl and triacyl lipopeptides[56]. They can also recognize various lipoproteins from gram-positive and gram-

negative bacteria. Interestingly, TLR2 is also implicated with viral glycoprotein recognition such as cytomegalovirus and herpes simplex virus 1 in the FRT[62]. Constitutive TLR1 and TLR6 expression has been detected in epithelial cells of both the ecto- and endo-cervix[56]. It was found that lipopeptide binding to TLR1 and 2, result in the production of MIP-13α and TNF- α by endometrial epithelial cells[56]. TLR3, is constitutively expressed in the fallopian tubes, endometrium, cervix, and vagina and has been shown to recognize dsRNA and mediates various antiviral responses[56]. After contact with a viral pathogen, TLR3 recognizes dsDNA and produces proinflammatory cytokines and chemokines as well as type I interferons (IFNs) to assist in pathogen clearance[63]. Conflicting results of TLR4 expression in the FRT have been reported, with some researchers stating its presence in both the ecto- and endo- cervix where others find it only present in the endocervix[64, 65]. Binding of endotoxins to TL4 has been found to lead to the rapid activation of NF- κB and cytokine expression in the fallopian tube and stromal fibroblasts[66]. TLR5 has been noted to recognize flagellin and serves as a sensor for pathogenic bacteria that is able to cross the epithelium. Its expression has been documented in the fallopian tubes, vagina, ectocervix and endocervix. Activation of TLR5 leads to proinflammatory cytokines and chemokines in epithelial cells of the cervix and vagina[56]. TLRs 7 and 8 are expressed in epithelial cells of the fallopian tubes, endometrium, cervix and vagina and have been implicated in the recognition of viral ssRNA from viruses such a HIV-1 and stomatitis[56]. Although there is little information on the interaction of TLR7 and 8 with HIV-1 in vaginal epithelial cells, upon TLR8 endosomal interaction with HIV-1,T cells induced cytokine secretion, and enhanced HIV-1 replication[67]. Lastly, TLR9 expression has been reported in epithelial cells of the fallopian tube, endometrium, cervix and vagina and recognizes CpG motifs of DNA viruses such as herpes simplex virus[56]. CpG oligonucleotides upregulated the production of chemokine interleukin 8

(IL-8) in epithelial cells of the fallopian tube and endocervix[56]. Nazli et al., demonstrated that HIV-1 glycoprotein 120 signals through PRRs, TLR2 and TLR4 in endometrial cells, resulting in the upregulation of NF- κB and type I IFN pathways. Following this, proinflammatory cytokines were produced and disruption of tight junctions was observed, indicating a potential mechanism by which HIV-1 could initiate innate immune activation in the FRT[68-70].

1.3.3 Antimicrobial Peptides

In addition to the many PRRs present on vaginal epithelial cells, genital epithelial cells also release antimicrobial peptides (AMPs)[71] . Expression of AMPs in epithelial cells occurs both constitutively and in response to external stimuli such as injury or infection. AMPs are a diverse class of proteins used as a first line of defense against bacteria, yeast, fungi, and viruses[72]. The amino acid composition of AMPs is variable, however, they can be classified into 5 main groups: α-helical, β-sheet, mixed α-helices/β-sheets, cyclic enriched with specific amino acids, and fragmented[73]. In mammals, AMPs have been noted to interact with both the innate and adaptive immune systems and are derived mainly from granules of neutrophils and in secretions from epithelial cells of mucosal surfaces [74, 75]. A key difference, however, is that epithelial cells secrete AMPs into the host-environment whereas phagocytic cells secrete AMPs mainly within intracellular compartments[74]. AMPs generally exert their neutralization effects by disrupting and preventing the growth of microorganism, for example, inhibiting protein synthesis, cell wall synthesis, nucleic acid synthesis, and metabolic pathways in bacteria[76]. However, AMPs can also impede viruses by binding to viral glycoproteins thus effectively blocking viral entry. AMPs can directly act on the viral particle or interfere with its replication cycle thus effectively neutralizing the virus[77].

Epithelial cells of the upper and lower FRT have been demonstrated to secrete AMPs[78, 79]. There have been several identified AMPs secreted by GECs that are known to help inhibit HIV-1 infection including secretory leukocyte protease inhibitor (SLPI), human β-defensins 1-4, and lysozymes[80]. SLPI is shown to inhibit HIV-1 infection in purified monocytes and T cells *in-vitro*. While the mechanism is unclear, is it hypothesized that SLPI assists in the disruption of the infection process rapidly after virus budding[80]. Moreover, SLPI is found to inhibit HIV-1 infection of lymphocyte and monocyte derived tumor cell lines by both primary and laboratory adapted HIV-1 isolates[81]. Similarly, lysozyme, another AMP secreted by genital epithelial cells is reported to possess anti-HIV-1 properties. It inhibits HIV-1 infection of target cells through HL9 and HL18 peptide regions, thus, effectively blocking entry[80].

1.3.4 Cytokines and Chemokines

In addition to the AMPs released by genital epithelial cells, cytokines and chemokines contribute widely to the local immune response and clearance of pathogens. Cytokines are small proteins released by cells to assist in the communication and interactions between other cells whereas chemokines attract immune cells such as leukocytes to areas of damage or inflammation, and are also involved in antimicrobial responses against bacteria, viruses and fungi[82]. Genital epithelial cells have been documented to release both cytokines and chemokines in response to viral and bacterial pathogens including type I interferons, TNF-α, interleukin-1α (IL), IL-6, IL-12, IFN-y, IL-10, macrophage colony-stimulating factor, transforming growth beta 1, leukoproteinase inhibitor, and macrophage inflammatory protein 1β [83, 84].

Cytokines and chemokines associated with the FRT have been shown to both upregulate and block HIV-1 infection. In the same study mentioned above, Nazli et al., showed that HIV-1 gp120 induces TLR2 and 4 innate immune activation resulting in the activation of NF-κB and

induction of proinflammatory cytokines resulting in endometrial epithelial cell barrier disruption[68]. In contrast, macrophage inflammatory proteins have been noted to be natural ligands of CCR5 and can interfere with HIV-1 replication in PBMCs[85, 86]. Walker et al., found that increased levels of macrophage inflammatory proteins resulted in resistance to R5-tropic HIV-1[85]. Previous students in the Kaushic lab have assessed cytokines and chemokines, including RANTES, IL-1, and MIP-1α, in response to hormonal changes and certain vaginal bacterial species. In primary endometrial GECs, it was found that DMPA treatment caused increased HIV-1 uptake and the induction of chemokines such as RANTES[12]. In a separate study it was found that the co-culture of dysbiosis-associated bacterial species and vaginal epithelial cells resulted in significantly more RANTES present in the apical cell culture media. Similarity, addition of the progestin-based hormonal contraceptive DMPA to vaginal cell cultures also resulted in increased RANTES production (Dupont, Lam, Master's Theses, Mac Sphere). RANTES is a chemoattractant primarily for T cells and is released in response to many viral infections[87]. Thus, increased production of RANTES may result in more CD4+ T cells infiltrating into the site of epithelial cell-pathogen interaction consequently increase the chance of HIV-1 acquisition in the FRT[87].

Overall, the epithelial cells of the FRT are equipped with various defensive mechanisms to invading pathogens. Unfortunately, despite the numerous innate immune mechanisms that GECs elicit to impede HIV-1 infection in the vaginal tract, HIV-1 is still capable of crossing epithelial cells barriers to infiltrate and infect target cells.

1.4 Research Models of HIV-1 Infection

A vast database of information has been collected on many aspects of HIV-1 including transmission, and virus-host cells interactions that contribute to HIV-1 pathogenesis in the FRT[88]. Without the use of the animal, *ex-vivo*, and *in-vitro* models this would not have been possible.

1.4.1 Animal Models of HIV-1

Animal models offer obvious advantages in HIV-1 study by allowing more comprehensive investigation of the disease, testing of drugs and vaccines, as well as understanding the immune response to HIV-1[89]. One of the major limitations in searching for treatments for HIV-1 has been the lack of models that fully recapitulate all the salient features represented in humans[89]. Small animal models were used to attempt the study of HIV-1, however, initial attempts to infect rodents with HIV-1 were unsuccessful due to the lack of a human CD4+ receptor. Their use as models were quickly deemed inadequate to study HIV-1[89]. As transgenic animal technologies have developed, lab animals, such as humanized mice, have been created to express vital proteins necessary for HIV-1 replication. Unfortunately, these models require extensive time to establish large colonies, can suffer from graft versus host disease after reconstitution and are expensive.[89, 90]. To better model long term pathogenesis many non-human primates (NHP) models have been used to study simian immunodeficiency virus (SIV), a genetically similar retrovirus to that of HIV-1[89, 91, 92]. For example, it was found that in hysterectomized macaques HIV-1 was able to cross an intact vaginal mucosa demonstrating that this site is vulnerable to viral transmission[93]. Miller et al., further complimented these findings using rhesus macaques as a model for heterosexual transmission of HIV-1. SIV was infused onto the vaginal mucosa of mature and immature female rhesus macaques using a soft plastic feeding tube. The researchers found that application of SIV to an intact genital mucosa resulted in successful SIV-1 transmission[94]. Unfortunately, there are differences between SIV infection in NHP and HIV-1 infection in humans. For NHP studies to be accurate they require many animals to achieve sufficient statistical power and obtaining a large number of NHP is both time consuming and costly. Furthermore, animal studies contain variables

that are challenging to control which may affect efficiency of the study. Thus, despite being very informative, *in-vivo* modeling studies have limitations.

1.4.2 *Ex-vivo* Culture Models of HIV-1

To answer specific research questions regarding epithelial cells that may be difficult to address in animal models, such as molecular mechanisms that result in infection, *in-vitro* and *ex-vivo* models are useful. Many labs have use trans well culture systems which better represents the FRT, compared to typical cell culture techniques. For instance, the "raft" culture system, uses blocks of cervico-vaginal human tissue cultured on collagen sponges in an air liquid interface[95]. Using the raft model has major benefits. The preservation of tissue architecture can last for up to 3 weeks, cell types are retained within the tissue without the need for exogenous stimulation, and key surface molecules needed for HIV-1 entry and infection are also maintained[95]. *Ex-vivo* explants can support HIV-1 replication without exogenous stimulation that is necessary for HIV-1 infection in isolated lymphocytes as well as assist in the study of early HIV-1 transmission events across the epithelium without the genetic manipulation that many immortalized cell lines contain[95]. Cervico-vaginal and other *ex-vivo* tissues can be used to evaluate potential therapeutic compounds in pre-clinical tests. For example, Fletcher et al., found that microbicides PRO 2000 and dextrin sulfate, inhibited infection by X4 and R5 HIV-1 isolates in human cervical explants[96]. Additionally, Collins et al., developed an organ culture model derived from squamous cervical tissue to study HIV-1 transmission across the mucosal barrier[97]. In the organ culture system, a circular piece of squamous cervical tissue is placed into a transwell chamber sealed with agarose. After the addition of either cell-free or cell associated virus, basolateral supernatants were collected from various culture days and HIV-1 transmission was assessed. They found that HIV-1 was able to cross the vaginal epithelium. As the model uses tissues rather than cell lines it more

accurately resembles the natural physiological architect of the lower FRT[97]. Furthermore, tissue explants can also be used to isolate cells of interest, such as epithelial cells, cultured and grown in transwells to study HIV-1 transmission[70, 98-100]. Nazli. et al., demonstrated that exposure of *ex-vivo* cultured primary endometrial epithelial cells to HIV-1 lead to breaching of the mucosal barrier and increased leakage of HIV-1 across the epithelium[70]. They also found that HIV-1 gp-120 was a driver in barrier impairment and that neutralization of gp120 significantly abrogated the effect of HIV-1[70]. Of course, each method has limitations and cannot recapitulate every aspect of the FRT. For example, *ex-vivo* culture viability and integrity can vary between samples, and generally cannot be maintained for long periods of time. Therefore, using immortalized cell lines, or primary epithelial cell cultures can help address some of the limitations posed by *ex-vivo* cultures.

1.4.3 *In vitro* Culture Models of HIV-1

The use of *in-vitro* models provides an unlimited supply of cells and bypasses limited supply and short term culture of primary human tissues[101]. The use of immortalized cell lines has contributed greatly to the knowledge of HIV-1 transmission and infection in the FRT. For instance, Dalgleish et al., used T cells to identify the CD4 antigen as an essential and specific component for the receptor of the causative agent of AIDS[102]. This then led to the discovery of HIV-1 chemokine receptors CXCR4 and CCR5 as critical cell-surface co-receptors for HIV-1 entry[103, 104]. Further building on this knowledge, immortalized cell lines were also essential to the discovery that epithelial cells were an unlikely target of HIV-1 infection as they lack the CD4 receptor needed to infect cells[13, 105]. In addition, the use of epithelial cell lines such as ectocervical (Ect-1), vaginal (Vk2) and endocervical (End-1) cells has contributed to the understanding of interactions between of cell free HIV-1 and the FRT epithelium. Benefits of the Vk2, Ect-1 and End-1 cell lines is that they exhibit the same phenotype and cytokeratin expression as human tissues in *in-vitro* culture

offering a good alternative to *ex-vivo* culture systems[106]. More specifically, the cell lines express genes such as cytokeratin's 8, 10, 13, and 19 as well as macrophage colony stimulating factor, TGF-β1, and IL8. In addition to expressing many of the same genes and cellular products as primary vaginal epithelial cells the End-1 possesses characteristics of the simple columnar epithelium in the upper FRT, while the Ect-1, and Vk2 cell lines express characteristics of stratified squamous epithelium found in the lower FRT. Micsenyi et al., examined the interactions between Ect1 and End1 immortalized epithelial cell lines and a fluorescent recombinant strain of HIV-1[106]. They found that both cell lines internalize cell-free HIV-1 and were not productively infected by HIV-1[12, 13, 107, 108].

1.4.4 Vk2/E6E7 Cell Line as a Model to Study Host-Pathogen Interactions

Many *in vitro* studies have utilized the Vk2 cell lines to study pathogen interactions, vaginal irritants, and hormonal influences on vaginal cells[109-111]. The Vk2 cell line was established in the late 1990's from a premenopausal woman undergoing vaginal repair surgery. Cells were then immortalized using the retroviral vector containing the human papillomavirus 16/E6E7 antigen[112]. The Vk2 cells line expresses phenotypes resembling normal vaginal epithelial cells and are physiologically relevant to study the lower female genital tract mucosa. Many studies have used the Vk2 cell line to study interactions of vaginal cells with external stimuli. However, many of these studies utilize liquid-liquid interface systems which results in a single monolayer of cells, whereas the vaginal epithelium naturally exists as stratified layers[113-115]. Our lab previously developed a multilayer culture method using Vk2 cells that more accurately resembles the FRT[116]. Vk2 cells were grown in transwells and apical media was removed to create an air-liquid interface culture system. Vk2 cells grown in ALI culture conditions resulted in multilayered cultures compared to monolayers in LLI cultures. This system has successfully been

used to examine the effects of female sex hormones on vaginal epithelial cell growth and susceptibility to the sexually transmitted infection, herpes simplex virus type 2 [116]. For the purpose of my thesis and to understand the trafficking of HIV-1, we utilize both LLI and ALI cultures.

1.5 Epithelial Cell Interaction with HIV-1

Mucosal surfaces such as those in the anorectal, and genital tract continue to be one of the primary routes of new HIV-1 infections. Since epithelial cells are the first cells to contact HIV-1 upon entrance into the FRT it is imperative we understand how HIV-1 interacts with GECs.

1.5.1 Epithelial Cell Barrier Function and Disruption by HIV-1

As previously mentioned, HIV-1 cannot productively infect epithelial cells in the FRT[117]. Therefore, it must cross the epithelial barrier in order to access underlying target cells and establish productive infection[117]. However, the mechanisms that allow HIV-1 to cross the epithelial barrier are not fully understood.

Three major types of cell-to-cell structural components that occur between epithelial cells including tight junction, adherens junctions and desmosomes. Tight junctions are critical for the proper functioning of genital epithelial cells in the FRT and play a pivotal role in maintaining morphological and physiological features. Adherens junctions form a continuous adhesion belt below tight junctions connected by bundles of actin filaments[28]. Lastly, desmosomes form specialized adhesion protein complexes that maintain physical integrity of tissues[28]. Tight junctions form the intercellular barrier between adjacent epithelial cells in the upper genital tract and the most basal layer of cells in the lower FRT, which assists in the regulation of molecules across the epithelium[118]. The three major transmembrane proteins comprising tight junctions are occludin, claudins and junction adhesion molecule proteins[118]. Tight junctions perform vital

functions including, adhesion between adjacent cells, which creates a protective and functional barrier for key processes such as osmotic balance[119]. Occludin is a tetra-span protein containing two extracellular loops, known to assist in the regulation of paracellular permeability between cells[119]. Moreover, the extracellular loops of occludin were shown to interact with claudin and junction adhesion molecule in a human epithelial intestinal cell line, and disruption of these interactions prevented formation of tight junctions[120]. Similarly, claudins are also a tetra-span transmembrane protein with two extracellular loops that assist in the recruitment of occludin to tight junctions. Claudins play a major role in the passage of molecules across cells, acting as gate keepers for ion passage[121]. Zonula occuldens (ZO) comprise the transmembrane proteins of occludin and claudins, which are associated with cytoplasmic proteins zonula occludens-1 (ZO-1), 2 and 3[122]. ZO proteins contain binding domains to tight junction proteins as well as the actin cytoskeleton, indicating a major involvement in barrier formation[119]. Indeed, mutations of ZO-1 in mouse epithelial cell lines resulted in significantly slower formation of junctions between cells indicating the importance of ZO-1 in proper assembly of junctional domains[123]. Junctional adhesion molecule 1 (JAM-1) is another crucial transmembrane component of tight junctions with a variety of functions including cell polarity, cell permeability and control of the passage of nutrients and solutes across epithelial surfaces[124].

Despite the efficacy of the epithelial barrier in protection against pathogens, HIV-1 is capable of disrupting tight junction proteins resulting in subsequent translocation of the virus[125, 126]. *In-vitro* studies investigating HIV-1 transmission in Vk2 cells found that gp120 bound to the human mannose receptor, a receptor found on the surface of some epithelial cells shown to interact with HIV-1, with high affinity[35]. As a result of this binding, metalloproteinases were produced leading to the degradation of tight junction proteins and the extracellular matrix causing weakening of the

epithelial barrier, therefore facilitating transport across vaginal epithelial cells[35]. Furthermore, Nazli et al., found that both X4 and R5 strains of HIV-1 were able to upregulate proinflammatory cytokine production by upper genital tract epithelial cells and reduce transepithelial resistance, a measure of monolayer integrity following exposure to HIV-1 for 24 hours. This coincided with visual disruption of tight junction proteins claudin 1,2,4, occudin, and ZO-1 as shown using immunofluorescent staining[70]. These results were further complimented by Bashir et al., who saw that gp120 resulted in significant reduction in TER values in ectocervical cell line ECT1 and endocervical cell line HEC1A[127]. Understanding how HIV-1 interaction affects the barrier proteins of Vk2 cells is imperative to better comprehend HIV-1 transmission.

1.5.2 The Role of the Endocytic Pathway in HIV-1 Transmission

In addition to HIV-1 affecting junctional proteins and weakening epithelial barriers, it has also been proposed that HIV-1 may use the endocytic machinery of epithelial cells to breach the epithelial barrier and gain entry through the genital mucosa to reach target CD4+ T cells[98, 128, 129]. Endocytosis is a cellular process by which substances are brought into the cell. Many pathogens use the endocytic pathway to protect them from detection by immune cells, however, some viruses, including HIV-1 may enter cells through different mechanisms such as membrane fusion [130]. During membrane fusion, viruses bind to receptors on the cell surface and directly fuse with the host membrane, resulting in the release of their inner content into the host cell. Some viruses that enter cells through membrane fusion include Ebola virus, and HIV-1 (target CD4+ T cells)[131, 132]. Entry via endocytosis can occur with both enveloped and non-enveloped viruses. The endocytic pathway may be used for transport, or as a method to bind directly to the cell to release their content, as in membrane fusion. Endocytic entry of viruses occurs in a stepwise manner involving attachment of the virus to cell receptors, clustering of receptors, interaction and

activation of signaling pathways, formation of endocytic vacuoles, trafficking of viral cargo to endosomal compartments, sorting and evasion to the cytosol and nucleus[130]. Endocytic vesicles help to traffic the incoming viral particles deep into the cytoplasm unhindered by obstacles such as the cytoskeleton and can be subdivided into four categories clathrin-mediated, caveolae, pinocytosis and phagocytosis[130, 133]. Clathrin mediated endocytosis is versatile and has a range of different functions and advantages including, regulating surface expression of proteins, sampling cell environment for growth and abnormalities, absorbing nutrients into the cell and trafficking of membrane components to the lysosome[134]. During clathrin mediated endocytosis, cargo ligands in the luminal compartment of the vesicle bind to receptors on the host-cell membrane[134]. The cargo ligand receptor will then recruit various adapter proteins to the membrane of the cell where budding forms[134]. A mature clathrin pit forms and will be cleaved from the plasma membrane, where it uncoats and fuses to a sorting endosome[134]. Once fused, the associated cargo is then trafficked to other areas of the cell. Another type of endocytosis is caveolae mediated endocytosis. Caveolae are highly specialized membrane domains with critical roles in lipid metabolism and cell signaling[135]. After a virus binds to a host cell, some viruses can cluster receptor molecules such as integrins within lipid rafts[135]. Accumulation of viral particles in caveolae causes a cascade of biochemical reactions, followed by rearrangement of the cortical actin cytoskeleton and internalization of the particle[135]. Pinocytosis is a type of endocytosis in which small particles outside the membrane of the cell are internalized through invagination of ruffled cell membranes. Viruses belonging to vaccinia, adeno, picorna and other virus families have been documented to take advantage of pinocytosis to gain access to the cytoplasm[136]. The virus particles trigger signaling pathways that cause actin-mediated membrane ruffling[136]. These vesicles are then trafficked to early endosomes for hydrolyzation[136]. Lastly, phagocytosis, is the process by which

a cell membrane surrounds a large particle resulting in a vacuole[137]. Although, under a branch of endocytosis, phagocytosis is mainly used to ingest solid matter, bacteria, and viruses which results in the formation of phagosomes. Phagosomes are formed by a macrophages, neutrophils, and dendritic cells as they engulf a foreign particle. Phagosomes can fuse with lysosomes, forming phagolysosomes which results in the digestion of the foreign material by hydrolytic enzymes and reactive oxygen species[138]. It is mostly used by immune cells such as macrophages and monocytes where they ingest foreign material and destroy it. Phagocytosis of particles is initiated by receptors including Fcγ, complement receptors and mannose receptors[137]. It is important to note that lysosomes are membrane bound organelles that play a part in membrane repair, and contain digestive enzymes, used to break down macromolecules, as well as viruses and bacteria, delivered from late endosomes[139]. Clement et. al., found that during entry into Chinese hamster ovarian cells, HSV-1 virions associated with the plasma membrane followed by phagocytic uptake and rearrangement of the actin cytoskeleton, indicating a potential method by which a virus can exploit phagocytosis to gain entry into the cell[137]. Which pathway a virus uses depends on several factors including the signals needed for activation and receptors present on the host cell and virus.

HIV-1 can enter and exit cells without releasing its inner contents which is known as transcytosis. Transcytosis is defined as the transport of a particle from one side of the cell to another and is used by multicellular organisms to move material through cells without modifying the cargo. HIV-1 has utilized this method to its advantage to cross the epithelial cell barrier and infect the underlying cells [140]. However, debate has ensued whether transcytosis of HIV-1 is a mere artifact of cell culture systems, or if it truly does occur in genital epithelial cells. While some studies have suggested that transcytosis in genital epithelial cells does not occur, others have clearly demonstrated the uptake and transcytosis of HIV-1 across genital epithelial cells[23, 141, 142].

Transcytosis of HIV-1 in epithelial cells is linked to the endocytic pathway and as genital epithelial cells do not express the HIV-1 CD4 receptor, they do not become productively infected, rather HIV-1 must traverse this barrier to infect target cells[143, 144]. As such alternate receptors have been implicated in the attachment and entry of HIV-1 to epithelial cells[35]. Stoddard et al., found that gp340 expressed on genital epithelial cells binds HIV-1 and facilitates viral transmission[38]. Several other studies have also supported the notion that HIV-1 has to ability to transcytose and utilizes the endocytic pathway. For example, it was shown in primate models that the application of HIV-1 to intact cervicovaginal epithelia leads to HIV-1 infection of susceptible immune cells[145, 146]. Furthermore, Gupta et al., investigated whether pH, and envelope specific monoclonal and polyclonal antibodies resulted in changes of uptake and transcytosis across epithelial cells of endometrial cells using the HEC-1 cell line[147]. They found that a more acidic environment with the combination of envelope specific antibody increased transcytosis 30 times compared with neutral pH[147]. Additionally, Yasen et al investigated the role of initial HIV-1 internalization into oral and genital epithelial cells and found that HIV-1 was sequestered into the late endosomes[98]. Electron microscopy work in our lab demonstrated that HIV-1 entry into primary genital epithelial cells takes place via endocytosis [12]. To further support that HIV-1 uses endocytosis to cross epithelial cells, treatment of primary genital epithelial cells with dynasore, an inhibitor of clathrin mediated endocytosis resulted in significant abrogation of HIV-1 uptake further confirming that HIV-1 uses endocytosis to epithelial cells[12]. Kinlock et al investigated the intracellular pathway that HIV-1 exploits to transcytose Vk2 cells, however, they did not visualize all endocytic compartments, only used X4 strain of HIV-1, and investigated whether native or heat inactivated virus was trafficked differently when studies have supported a selective transmission of R5 over

X4 variants of HIV-1[143, 148]. Therefore, a better understanding of how HIV-1 crosses Vk2 cells is needed in order to develop better preventative strategies.

1.6 Lysosomal Associated Membrane Proteins and LAMP3

Lysosomes are organelles of eukaryotic cells crucial to the degradation of extracellular material as well as intracellular components that are delivered through endocytosis[149]. A lysosome has several major functions including the breakdown of macromolecules, cell membrane repairs, and response against foreign antigens. To conduct the variety of tasks performed by lysosomes, they possess several highly specialized lysosome associated membrane proteins (LAMPs)[149].

LAMPs are integral membrane proteins constitutively expressed in many cell types such as epithelial cells, and dendritic cells, however, their complete biological function remains unknown. However, LAMP proteins are thought to play a role in maintaining pH, autophagic pathways, lysosomal integrity, lysosomal motility, lysosomal exocytosis and catabolism[149]. Structurally, LAMP proteins consist of an internally homologous lysosome-luminal domain, separated by a proline hinge region[150]. The C terminal extremity contains a transmembrane region, followed by a short cytoplasmic tail[150]. In each of the two domains there is a conserved disulfide bond. In mammals there are three main LAMP proteins: LAMP1, LAMP2 and LAMP3. Although LAMP proteins are mainly located within the lysosomes, they may be expressed in different parts of the cell including the cell surface, membrane compartments like the endoplasmic reticulum, golgi or transport vesicles[149]. For example, LAMP proteins interact with TAPL, a polypeptide translocation machinery responsible for transport of cytosolic peptides for lysosomal degradation[149]. Moreover, the short cytosolic tail of LAMP proteins in required for lysosomal targeting and regulation of lysosomal motility[149]. A deficiency of LAMP proteins shifts the

cellular localization of lysosomes to the periphery, reducing the capacity to fuse with forming auto- or phagosomes[149].

Among the most abundant LAMP proteins are LAMP1 and LAMP2, which represent 50% of all membrane proteins on the lysosomal membrane and are typical transmembrane proteins[149]. Mice deficient in either LAMP1 or LAMP2 are viable and fertile, however, mice deficient in both contain an embryonic lethal phenotype, demonstrating that LAMP proteins may overlap in their functions[151]. Interestingly, LAMP2 may contain more specific functions as a deficiency in LAMP2 results in lysosomal glycogen storage disease[151]. LAMP3, also known as CD63 is a member of the tetraspanin superfamily and is a virus inducible gene[149]. An important distinguishing factor of LAMP3, is that is belongs to the tetraspanin superfamily which may indicate unique functions of LAMP3 compared to its counter parts LAMP1 and LAMP2. Tetraspanins are a family of proteins with four transmembrane domains that play distinct roles in cell physiology including cell-cell adhesion, cell signaling activation and tumor invasion[152]. Structurally, LAMP3 consists of around 416 amino acid residues with over 90% of the protein located within the lumen of lysosomes. A key function of LAMP3, due to its tyrosine-based internalization motif, may assist the facilitation of proteins and their targeting to the late endocytic organelles[149].

A more recent emerging role of LAMP proteins is their role in viral infections[149, 153-156]. For example, LAMP3 has been found to be significantly induced in human lung epithelial cells upon exposure to influenza A virus. Knockdown of LAMP3 in lung epithelial cells (A549) by silencing RNA resulted in significantly less influenza A virus production[157]. Moreover, LAMP1 was found to increase the efficiency of Lassa virus infection in 293T cells. LAMP1 knockout cell lines were developed and subjected to Lassa virus, the researchers found that knockout of LAMP1

reduced entry into 293T cells and that LAMP1 promotes Lassa virus entry in less acidic environments[154]. Additionally, LAMP3 has been implicated in early HIV-1 infection in macrophages, as treatment of macrophages with anti LAMP3 treatment inhibited HIV-1 infection in R5 isolates [158, 159]. Moreover, LAMP3 was found to regulate Epstein-Barr virus LAMP1 exosomal packaging in Rat1 cells and has been found to mediate both early and post integration steps of HIV-1 in human macrophages[160]. LAMP3 has also been associated with regulating expression of CXCR4 on the cell surface of T-lymphocytes and B cells by stimulating its degradation through the lysosomal pathway[161]. Further delineation of the role of LAMP3 and its interactions with HIV-1 in other cell types may lead to the development of novel therapeutic agents.

Chapter 2: Rationale and Hypothesis

Women represent over half of individuals living with HIV-1, with approximately 40% of transmission events in women occurring in the FRT[1]. The vaginal epithelial cells that line the lower FRT comprise the largest surface area exposed to HIV-1 containing ejaculate, and the first contact point of HIV-1 during heterosexual transmission. Despite the relatively low transmission rate of HIV-1 per coital act and the innate defense mechanisms in the FRT to prevent HIV-1 acquisition, HIV-1 can penetrate an intact epithelial barrier and gain access to target cells to establish productive infection[13, 23, 142]. The early events underlying transmission of HIV-1 in the FRT remain unclear and warrant further investigation.

Although HIV-1 has been studied extensively and our understanding of transmission in the vaginal tract has improved, extensive gaps in our knowledge exist regarding the trafficking of HIV-1 in the lower FRT. It has been established that HIV-1 can enter vaginal epithelial cells by endocytosis, however, host-factors that may influence the trafficking processes during HIV-1 endocytosis in vaginal epithelial cells remain unclear[143]. LAMP3, a virus inducible gene and trafficking protein present within vaginal epithelial cells has recently been implicated in the enhancement and inhibition of several viruses[157, 160, 162]. To our knowledge, the role of LAMP3 and its involvement in trafficking of HIV-1 in vaginal epithelial cells has yet to be investigated.

With this context, the goal of this thesis was to investigate potential interactions of LAMP3 and HIV-1 in immortalized vaginal epithelial cells (Vk2) using both air-liquid interface (ALI) and liquid-liquid interface (LLI) culture systems. Based on the limited amount of literature available, we hypothesized that **LAMP3 will assist in HIV-1 uptake and transcytosis in vaginal epithelial cells**. We addressed our hypothesis in two aims:

Aim 1: Determine how HIV-1 crosses vaginal epithelial cells using an immortalized cell line (Vk2)

Aim 1.1 Assess the effect of HIV-1 on barrier function in WT-Vk2 cells

Aim 1.2 Study endocytic uptake and transport of HIV-1 in Vk2 cells

Aim 2: Determine the role of LAMP3 in uptake and transcytosis of HIV-1 across vaginal epithelial cells using WT-Vk2, LAMP3 Knockdown (KD) Vk2 and LAMP3 Overexpression (OE) Vk2 cell lines

Chapter 3: Materials and Methods

3.1 Cell Media Preparation:

Keratinocyte serum-free growth medium (KSFM) (Thermofisher, Cat.17005042) was supplemented by the addition of epidermal growth factor (EGF, McMaster University Media Centre) to a final concertation of 0.1ng/mL, bovine pituitary extract (BPE, McMaster University Media Centre) to a final concentration of 0.05mg/mL. 200uL of 1M CaCl2 was added for every 500mL of KSFM prepared. 5 mL of pen/strep was supplemented in all media. All prepared media was stored at 4°C.

3.2 Preparation of Hormone Containing Media and Hormone Incubation of Cells:

DMPA (Sigma-Aldrich) was prepared to 10^{-9} M standard concentration, in KSFM cell media. Prepared hormone supplemented media was kept at 4°C and wrapped in foil due to light sensitivity. Vk2 cells were grown either in the presence or absence of DMPA by adding 300 µL of prepared hormone media to the apical compartment and 700 µL to the basolateral compartment of the transwell culture system. The media was changed every 48-hours until the cells became confluent. Hormone media was added for at least 7 days prior to use for experiments

3.3 Vk2/E6E7 Air-Liquid Interface Cell Culture

Vk2 cells beginning at passage 3 (ATCC CRL-2616™) were cultured in keratinocyte serum free media (KSFM) (Thermofisher, Cat.17005042) until 80% confluency. The T-75 flask containing the Vk2 cells was washed with phosphate buffered saline (PBS), and trypsinized using 1X trypsin-EDTA (McMaster Media Centre) for 10 minutes. Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) (Thermofisher, Cat. 12634- 010) containing 10% fetal bovine serum (FBS) was added to the trypsinized cells to neutralize the trypsin. The cell containing solution was then

centrifuged at 1,500 rpm for 10 minutes. The supernatant was decanted, and the cell pellet was resuspended in supplemented KSFM. Cells were then enumerated via trypan blue exclusion assay using a hemocytometer. 60,000 cells were then seeded in the apical side of 0.4um transwells (VWR, Cat. 82050-022) in 24 well plates. The apical side of the cell culture was topped up to 300uL with KSFM. 700uL of KSFM was added to the basolateral side. The 24 well plates were shaken briefly by hand to ensure that the cells were distributed evenly in each of the transwells. Cells were then incubated at 37 degrees Celsius, 5% carbon dioxide. One day after seeding, the apical media was aspirated to induce air-liquid interface conditions. Basolateral media was replaced with fresh KSFM media or KSFM media containing DMPA (10^{-9} M) as specified. Basolateral media was changed every 2 days.

3.4 Vk2/E6E7 Liquid-Liquid Interface Cell Culture

Vk2 cells (ATCC CRL-2616™) were cultured in keratinocyte serum free media (KSFM) (Thermofisher, Cat.17005042) until 80% confluency. The flask containing the Vk2 cells was washed with phosphate buffered saline (PBS), and trypsinized using 1X trypsin-EDTA. Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) (Thermofisher, Cat. 12634- 010) containing 10% fetal bovine serum (FBS) was added to the trypsinized cells to neutralize the trypsin. The cell containing solution was then centrifuged at 1,500 rpm for 10 minutes. The supernatant was decanted, and the cell pellet was resuspended in KSFM. Cells were then enumerated via trypan blue exclusion assay using a hemocytometer. 60,000 cells were then seeded in the apical side of 0.4um transwells (VWR, Cat. 82050-022) in 24 well plates. The apical side of the cell culture was topped up to 300uL with KSFM. 700uL of KSFM was added to the basolateral side. The 24 well plates were shaken briefly to ensure that the cells were distributed evenly in each of the transwells. Cells were then incubated at 37 degrees Celsius, 5% carbon dioxide. Basolateral

and apical media was changed every 2 days. Basolateral media was replaced with fresh KSFM media or KSFM media containing DMPA (10^{-9} M) as specified.

3.5 Vk2/E6E7 Overexpression and Knockdown Cell Lines

The LAMP3 over expression vector was constructed by cloning the coding sequence of LAMP3 into the pHR-CMV-ΔN-IκBα-IRES-Puromycin vector. The open reading frame LAMP-3 gene (accession number NM_014398) was PCR amplified using primers 5'- CGG CAC AGG TAG GTT TCT CT – 3', and 5' –ATT TCC CAA CAT CCA TCC TG – 3' containing BamHI and EcoRI restriction enzyme sites at each end and cloned into pHR-CMV-ΔN-IκBα-IRES-Puromycin essentially by replacing the ΔN-IκBα region with LAMP3. The resulting plasmid was designated as pHR-CMV-LAMP3-IRES-Puromycin. For CRISPR/Cas9-mediated knockdown of LAMP3 gene, single-guide RNAs targeting LAMP3 were designed using CRISPR Design Tool (http://crispr.mit.edu/). Targeting sequence lies in the first exon of LAMP3 gene (forward, 5′- CAC CGC ATC GTG CAA AAT TAC GGC C 3'; reverse, 5′- AAA CGG CCG TAA TTT TGC ACG ATG C 3'. Oligo DNAs for the single-guide RNAs were annealed and inserted into lentiCRISPR v2 vector digested with BsmBI restriction enzyme as described . All clones were confirmed by DNA sequencing using a primer 5′- GGACTATCATATGCTTACCG-3′ from the sequence of U6 promoter that drives expression of sgRNAs. Cassettes of single-guide RNAs-Cas9 or pHR-CMV-LAMP3-IRES-Puromycin were introduced to VK2 cells by using lentivirus-mediated gene transfer.

3.6 Single cell clone isolation

Limiting dilution technique was performed and used to isolate single cell clones of both LAMP3 overexpression and LAMP3 knockdown cell lines. Briefly, LAMP3 KD and LAMP3 OE cell lines were isolated by splitting them as described in 3.2. After removal of cells from the centrifuge, individual cells were isolated by dilution and placed into a 96 well plate with 100 uL of media. Cells were checked under the microscope to ensure a single cell was isolated and placed into an incubator at 37^{o}C and left undisturbed for 7-14 days. After 7 days cells were checked for growth, if no colonies were observed they were placed back into the incubator for another 7 days. Once the cells were expanded and before they became over-confluent, cells were trypsinized and expanded to 6 well plates and the process above was repeated until a T75 flask was reached. .

3.7 Lactate Dehydrogenase Assay for Vk2 Cell Viability with the addition of Dynasore:

1 hour after the addition of dynasore, an endocytic inhibitor, the apical media from Vk2 cells was collected. The apical media collected was utilized to run a lactate dehydrogenase (LDH) assay (Thermofisher, Cat. #88953) according to the manufacturer's instructions. To begin, the lysis buffer and stop solution were acclimated to room temperature. 50uL of apical media was taken from Vk2 cells added to a 96 well plate in duplicate. Next, 50uL of reaction mixture was added to each well. The plate was incubated at room temperature for 30 minutes and protected from light. 50 uL of stop solution was added to each well and absorbance was read. A maximum lysis control was obtained by adding in 50 μL of lysis buffer provided in the LDH kit to ALI cultured Vk2 cells, diluted with KSFM to a final volume of 100 μL before being assayed for LDH. The absorbance was measured at 490 nm and 680 nm. To determine LDH activity, the value obtained from 680 nm (background) was subtracted from 490 nm (absorbance value).

3.8 Transepithelial Resistance Measurements:

Vk2 cells were grown in ALI or LLI cultures as described above. 100uL of KSFM was added onto the apical side of ALI grown Vk2 cells, and incubated for 1 hour at 37°C, 5% CO2 before transepithelial resistance (TER) measurements were taken using a volt-ohm meter (World Precision Instruments). After TER measurements were taken, the KSFM on the apical side was aspirated. To evaluate the effect of HIV-1 on TERs of Vk2 cells, day 7 (prior to the addition of virus) was used as a reference point for an intact barrier. TER was shown as Percent Pre-treatment of TER The calculations are as follows: TER at Day 8 (addition of HIV-1) / TER at day 7 (prior to addition of any virus) x 100% = % pretreatment TER.

3.9 FITC-Dextran Leakage Assay:

On day 8 of culture, 24 hours after the addition of KSFM to the apical side of each cell culture (with or without virus), the apical media was removed in both ALI and LLI cultures. 300 μL of KSFM containing 10 kDa FITC-Dextran (2.4mg/mL) (Sigma Aldrich, Cat. FD10S250MG) was added onto the apical side of each transwell. After 24 hours, the basolateral media (50 μL) FITC-Dextran concentration was measured using a spectrophotometer at an excitation wavelength of 490nm, and emission wavelength of 520nm. A FITC-Dextran standard curve was created and plotted in order to correlate fluorescence and FITC-Dextran concentration. The dextran leakage in the basolateral compartment is expressed as a percentage of dextran added to the apical compartment. Calculation: (dye in basolateral well/dye in apical well)*100 = % of dextran leakage

3.10 RNA Extraction and LAMP3 Quantification by PCR:

Total RNA was extracted from Vk2 cells cultured in air-liquid-interface trans-wells, using the RNeasy mini kit lysing buffer according to manufactures guidelines. Vk2 cells were lysed and

processed for RNA isolation using RNeasy kit (Qiagen Cat #74104). Briefly, cells were lysed directly in the transwell using the disruption buffer provided. Next, 500 uL of 70% ethanol was added to the lysate and mixed by pipetting, and up to 700 ul of the sample was placed into an RNeasy spin column provided in the kit. The sample was spun using a tabletop centrifuge for 15 seconds at 10,000 rpm. The flow through was discarded. Next, 700 ul of buffer RW1 was added to the RNAeasy spin column and centrifuged with the same parameters as above. The flow through was discarded. Next 500 ul of buffer RPE was added to the RNeasy spin column and centrifuged with the same parameters above. The previous step was repeated. Next, the RNeasy spin column was placed into a 2 mL collection tube and centrifuged at full speed for 1 minute to dry the tube. Lastly, the RNeasy spin column was placed in a 1.5 mL collection tube and 30 uL RNAse-free water was added to the spin column and centrifuged at 10,000 rpm to elute the RNA. Purified RNA quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE). LAMP3 Gene accession number NM_014398 was PCR amplified using oligonucleotides with the sequences 5'-CGAGGATCC+ CGG CAC AGG TAG GTT TCT CT – 3', and 5' – CGCAATTC+ ATT TCC CAA CAT CCA TCC TG – 3'. The qPCR settings were as followed: Hot start 95ºC 3 minutes, followed by 40 cycles at: 95ºC 3 seconds, 60ºC 30 seconds, 1 cycle at 72ºC for 2 minutes. Hold at 4ºC.

3.11 Extraction of Protein for Western Blotting by Dr. Atif Zahoor and Ryan Chow:

Whole-cell extracts were prepared by trypsinizing Vk2 cells and centrifuging at 13000 g for 15 minutes. Cell pellets were resuspended in RIPA lysis buffer. Whole-cell lysates were clarified by centrifugation at 13000g for 10 minutes at 4°C to remove cell debris. Protein quantification was performed by BCA method as per manufacturer's recommendations (Thermo Scientific, Rockford, IL, USA). Briefly, working reagent was made for microplate samples. 25 uL of each standard and

unknown sample were pipetted into a microplate well. Next, 200 uL of working reagent was added to each well and mixed thoroughly using a plate shaker for 30 seconds. The plate was covered and incubated for 30 min. Lastly, the plate was cooled to room temperature and absorbance was measured at 562 nm of a plate reader.

3.12 Immunofluorescent Staining of Epithelial Cell Cultures:

LLI cultured Vk2 cells will were fixed with 4% paraformaldehyde for five minutes (Electron Microscopy Sciences, Hatfield, PA). The cells were permeabilized in transwells and treated with a blocking solution of 5% BSA (Sigma Aldrich), and 5% goat serum (Life Technologies) in 0.1% Triton X-100 (Sigma Aldrich) with primary antibody together for one hour at room temperature as indicated in the table below (Table 1). The primary antibody was then removed, and the cells were washed 3 times with PBS. The appropriate secondary antibody was diluted in blocking buffer as indicated in the table below (Table 1) and 100 µL was added to each well and incubated at room temperature for one hour. Transwell insert membranes containing the LLI cultures were cut out using a scalpel and mounted on slides using VectaShield with DAPI (already added upon purchasing)mounting fluid (Vector Laboratories Inc.) and left to set overnight. Multiple images (at least two Z-stacks) were taken at 60x in triplicate samples, using the Nikon confocal microscope.

Table 1: Reagents used for Immunofluorescence

Primary Antibody	Primary Antibody Dilution	Primary Antibody Company and Catalogue Number	Secondary Antibody/Reagent	Secondary Antibody Dilution	Secondary Antibody Company	Fluorochrome
Mouse anti-human LAMP3	1/100	Sigma-Aldrich --	Goat anti-Mouse IgG	1/1000	ThermoFisher Cat: A32742	Alexa Fluor 594

polyclonal antibody		Cat: SAB1401635				
Goat anti-human HIV-1 polyclonal antibody	1/100	Abcam – Cat: ab20460	Donkey Anti-Goat IgG	1/1000	Abcam Cat: ab6566	CY5
Rabbit anti-human EEA1 polyclonal antibody (early endosome)	1/100	Abcam Cat: ab2900	Goat anti-Rabbit IgG	1/1000	Thermofisher Cat: A-11008	Alexa Fluor 488
Rabbit anti-human Rab7 polyclonal antibody(late endosome)	1/100	Sigma-Aldrich Cat: R4779	Goat anti-Rabbit IgG	1/1000	Thermofisher Cat: A-11008	Alexa Fluor 488
Mouse anti-ZO-1-1A12 monoclonal antibody	1/200	ThermoFisher Scientific cat: MA3-39100-A488	N/A	N/A	N/A	FITC

3.13 In vitro exposure of Vk2 cells to HIV-1:

HIV-1 strains ADA (R5) or IIB (X4) were prepared by infection of adherent monocytes from human peripheral blood mononuclear cells and from the chronically infected H9 cell line, respectively, as described previously[70]. Briefly, cell lines were grown as cell suspension in RPMI medium containing 10%FBS in a T75 flask. Media was changed every 2 days and cells were split once a week. During supernatant collection HIV-1 stocks were purified and concentrated by the Amicon Ultra-15 ultracentrifugation filtration system (Millipore, Billerica, Massachusetts). R5 and X4 were added to the apical side of confluent Vk2 ALI or LLI cultures at a concentration of $6x10^5$ IU/mL in 100 µl and cultured at 37°C for 4 or 24 hours. The inoculum was removed, and cells were washed thoroughly with PBS and incubated with 0.05% trypsin for 3 min at room temperature to remove any non-internalized virus. Following this, 100 ul of fresh KSFM was added to the apical side of Vk2 cells. To inhibit endocytosis, Vk2s were pretreated with 100 ul of

dynasore at a concentration of 100uM before the addition of HIV-1 (Sigma Aldrich) for one hour at 37ºC[143].

3.14 p24 ELISA

Cells were grown in ALI culture conditions as described in materials and methods section 3.3 for 7 days. On day 7 HIV-1 was added to Vk2 cells for 4 hours of HIV-1 exposure. After 4 hours the inoculum was removed, and cells were washed thoroughly with PBS and incubated with 0.05% trypsin for 3 min at room temperature to ensure removal of all non-internalized virus. Following this, 100ul of fresh KSFM was added to Vk2 cells. 24 hours later 200 ul of media was removed from basolateral compartments and subjected to p24 ELISA following manufacturers protocols (HIV Type 1 p24 Antigen ELISA (96 Determinations), Zeptometrix Cat. #0801111, Buffalo New York). Briefly, 450 ul of basolateral supernatant was treated with 50 ul of lysing buffer suppled in the kit in a 96 well plate. Using the 96 well plate provided in the kit, the number of wells used was washed using 1X plate wash buffer provided in the kit. The plate was thoroughly blotted until no droplets in the well remained. 200 ul of each specimen was added into duplicate wells using the 96 well plate provided. The plate was covered with a plate sealer and incubated overnight at 37ºC. The following day wells were aspirated and washed. 100 ul of streptavidin-peroxidase working solution was added into each well, sealed, and incubated for 30 minutes at 37ºC. The plate was aspirated and washed. Next, 100 ul of substrate working solution provided in the kit was added into each well and incubated uncovered for 30 minutes at room temperature. Next, 100 ul of stop solution provided in the kit was added to each well. Within 15 minutes, the optical density of each well was read at 450 nm using a microplate reader.

3.15 TZM-bl Cell Line Culture

TZM-bl (NIH, Cat. #8129) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum with 5 mL of pen/step until 80% confluency. The flask containing the TZM-bl cells was washed with phosphate buffered saline (PBS), and trypsinized using 1X trypsin-EDTA for 10 minutes. DMEM containing 10% FBS was added back to the flask and the cell containing solution was then centrifuged at 1,500 rpm for 10 minutes. The supernatant was decanted, and the cell pellet was resuspended in 10% DMEM. Cells were enumerated using via trypan blue exclusion assay using a hemocytometer. 50,000 cells were then seeded in a 24 well plate. Plates were shaken briefly by hand to ensure even distribution of TZM-bl cells.

3.16 TZM-bl Assay

TZM-bl cells were grown in 24 well plates as described in materials and methods section 3.5 After 24 hours, 200 ul of HIV-1 containing media was removed from the basolateral side of Vk2 cells seeded in transwells and placed onto TZM-bl cells containing 20ul of 20ug/ml DEAE-dextran. 2 hours later cells were overlayed with 800 ul of 10% DMEM and after 48 hours cells were fixed in a 1% formaldehyde and 0.2% glutaraldehyde solution for 10 minutes. After being fixed, cells were stained with a potassium ferrocyanide/ferricyanide solution containing X-gal. The TZM-bl cell line is a HeLa cell line engineered to express CD4 and CCR5 and CXCR4 and contains integrated reporter genes for β-galactosidase under control of the HIV-1 long term repeat (LTR). Thus, HIV-1 infects TZM-bl cells through receptors, Tat is then produced which acts on HIV-1 LTR to induce β-galactosidase. Therefore, cells infected with HIV-1 result in blue cells. Cells positive for HIV-1 (stained dark blue) were enumerated.

3.17 Statistical Analysis

GraphPad Prism version 5 (GraphPad Software) was used to compare 3 or more experimental conditions unless otherwise stated, by one-way analysis of variance (ANOVA). When an overall statistically significant difference was measured ($p<0.05$), Tukey's test was performed to adjust the p-value for multiple comparisons.

Chapter 4: Results

AIM 1 -Determine How HIV-1 Crosses Vaginal Epithelial Cells

Rationale: To understand why women are at an increased susceptibility to HIV-1, it is fundamental to study the interactions between HIV-1 and the epithelial cells that line the FRT. It has been established that HIV-1 can enter epithelial cells through several mechanisms including paracellular leakage and or transcytosis. During intercourse microtears may result aiding in the translocation of HIV-1 to underlying immune cells thus increasing the chances of HIV-1 acquisition. Other studies have shown that HIV-1 has the ability to transcytose genital epithelial cells as another mechanism of access to underlying immune cells[141, 143]. However, many studies did not rule out paracellular leakage or conducted experiments in LLI cultures which does not accurately represent the multilayered structure of the FRT[143]. Endocytosis is suggested to be the predominant method of viral entry into different epithelial cell types and that endocytosis of HIV-1 may lead to productive infection of susceptible cells present in the lamina propria underlying the epithelium. Endocytosis of HIV-1 has reported to occur in primary genital columnar epithelial cells, polarized tonsil, and foreskin cells[12, 98]. However, studies have revealed the precise trafficking mechanism that HIV-1 uses to cross vaginal epithelial cells. As the stratified squamous epithelial cells of the lower FRT comprise the largest surface area exposed to HIV-1 carrying semen, we decided to further investigate the interactions of HIV-1 and vaginal epithelial cells using an immortalized vaginal epithelial cell line[19]. Therefore, we aimed to understand the precise mechanism of how HIV-1 crosses vaginal epithelial cells using a more biologically relevant model (ALI cultures), and confirmed these results using a LLI culture system.

Aim 1.1 Assess the effect of HIV-1 on barrier function in Vk2 cells

4.1.1 Establish Measurements of Baseline Barrier Function in Untreated Vk2 Cells

An intact and robust vaginal epithelial barrier is imperative to prevent pathogenic organisms from crossing the lamina propria. While it has been established that HIV-1 has the ability to impair the mucosal barrier of the upper genital tract to cross the epithelium by paracellular transport, whether or not similar mechanisms of crossing are seen in vaginal epithelial cells *in vitro* remains unclear. Therefore, we assessed the effect of HIV-1 on barrier function in Vk2 cells and established baseline measurements for growth and leakage in Vk2 cells. We utilized both transepithelial resistance (TER) measurements to assess growth and barrier function of Vk2 cells and FITC-Dextran leakage assays to determine paracellular leakage. In ALI cultures, Vk2 cells were grown in in KSFM for 12 days, during which barrier integrity was monitored by TER measurements every 24 hours. TER measurements reached a maximum value on day 9, with decreasing values occurring on days 10, 11 and 12 (Figure 1A). As such, all experiments were completed, prior to day 9. Depo-Medroxyprogesterone acetate (DMPA), a progestin-based hormonal contraceptive, is used in our lab as a positive control for leakage in Vk2 cells, as it is known to disrupt barrier function (Jeff Lam, Master's Thesis Mac Sphere). The addition of 20 ng/mL of DMPA treatment to Vk2 cells grown in ALI cultures showed significant leakage ($p<0.05$) compared to untreated Vk2 cells and 5 ng/mL of DMPA (Figure 1B). An additional control of an empty well without cells containing only FITC-Dextran leakage was used. There was significantly increased amounts of FITC-Dextran leakage in the empty well compared to the transwell with Vk2 cells grown in media only (KSFM) ($p<0.01$), (Figure 1B) Based on this result all leakage experiments were conducted with 20 ng/mL of DMPA as a positive control.

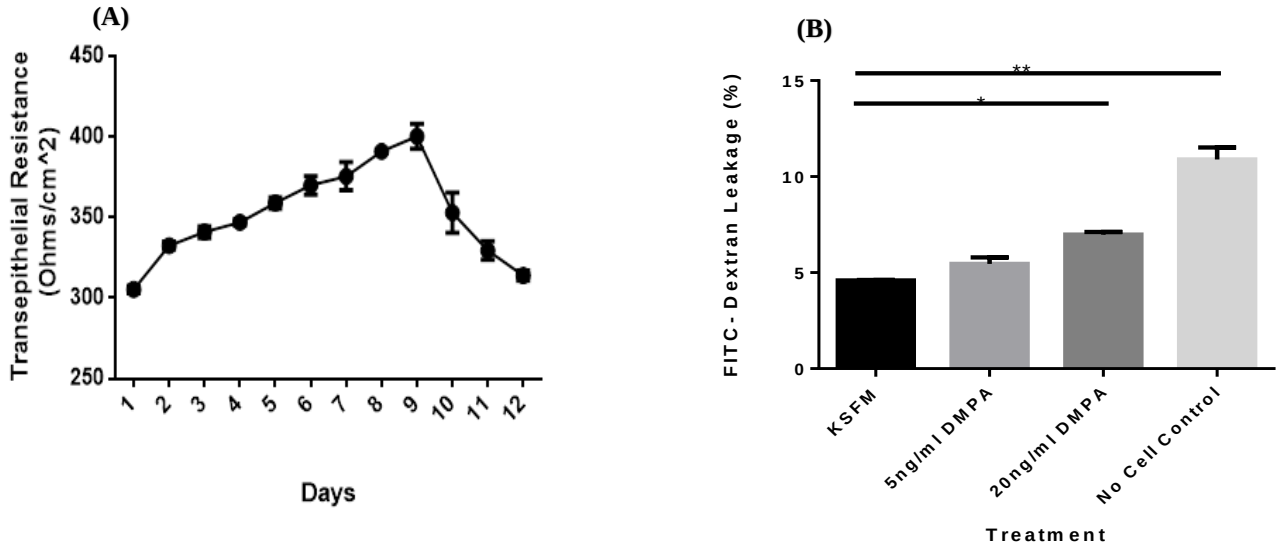


Figure 1: Optimization of Vk2 ALI culture TER measurements and FITC-Dextran Leakage assay. **(A)** TER measurements were taken for three separate well inserts on different culture days of 1 through 12. **(B)** FITC-Dextran leakage of Vk2 in the presence and absence of media containing DMPA. KSFM is media treated cells, and no cell control is an empty well. Data shown represents two pooled experiments, performed in duplicate. Data was analyzed using one-way ANOVA, with Tukey's test to correct for multiple comparisons. *$p<0.05$, **$p<0.01$. Error bars represent SEM.

4.1.2 Assess Barrier Function in ALI and LLI Vk2 Cell Cultures

Previous studies have identified the ability of HIV-1 to impair the epithelial barrier allowing viral and bacterial translocation[70, 163]. To assess if HIV-1 was impairing barrier function in Vk2 cells, TER measurements, FITC-Dextran leakage, and confocal microscopy was employed. Both X4 and R5 strains of HIV-1 were added to Vk2 cells. In ALI cultures, the barrier was not disrupted as indicated by no change in FITC-Dextran leakage ($p>0.05$), (Figure 2A), and/or TER measurements ($p>0.05$), (Figure 2B) compared to mock (media only) treated. In ALI cultures DMPA resulted in a significantly increased amount of leakage compared to mock (media) treated

cells only ($p<0.05$), (Figure 2A). However, no decrease in TERs was observed in DMPA compared to mock treated (Figure 2B).

As the stratified squamous epithelium of the vagina has been proposed to be robust against HIV-1 entry, and after determining that Vk2 cells grown in ALI cultures exposed to HIV-1 did not alter barrier function we also tested Vk2 cells in LLI cultures to determine if the absence of barrier disruption was due to the multiple layers of epithelium or if vaginal epithelium was not affected by HIV-1. Exposure of LLI cultures to HIV-1 resulted in increased leakage in Vk2 cells in the presence of both R5 ($p<0.01$) and X4 strains of HIV-1 as indicated by an increase in FITC-Dextran leakage (Figure 3A) and decreased TER measurements ($p<0.001$), (Figure 3B). DMPA also significantly increased leakage in LLI cultures compared to mock (non-hormone containing media), ($p<0.05$), (Figure 3A), however, there were no significant differences in DMPA treated Vk2 cell TER measurements compared to mock Vk2 cells. Thus, the Vk2 cell barrier functions are influenced by HIV-1. But not when multiple epithelial layers are present.

ZO-1 tight junction protein has been implicated in barrier function of epithelial cells[70]. Optimization of ZO-1 localization and imaging was determined in Vk2 cells before the addition of HIV-1 (Figure 4). Following the optimization of visualization of ZO-1 in Vk2 cells, both X4 and R5 strains of HIV-1 was added to Vk2 cells in LLI cultures on day 7 for 24 hours. Exposure to R5 strain of HIV-1 to Vk2 cells showed a significant decrease in ZO-1 fluorescence, compared to mock (media) treated Vk2 cells ($p<0.01$), (Figure 5). Interestingly, X4 exposed Vk2 cells did not show a significant change in the amount of florescence compared to mock Vk2 cells, (Figure 5). Florescence intensity was measured by imageJ.

Overall, in line with other *in-vitro* analysis our results indicate that a monolayer of cells exposed to HIV-1 results in the disruption of the epithelial barrier. Additionally, DMPA results in

a significant increase in leakage but does not impact TER measurements. Moreover, Vk2 cells grown in multilayers *in-vitro* provide a robust barrier against HIV-1.

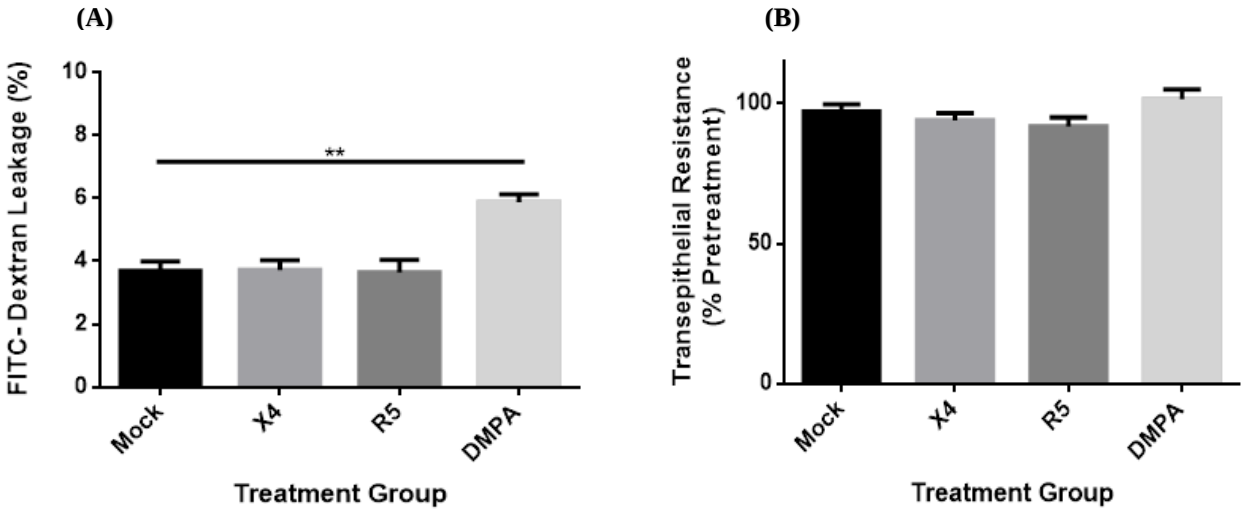


Figure 2: Barrier function of Vk2 Cells remains unchanged in ALI culture conditions following exposure to HIV-1. Vk2 cells were grown in ALI culture conditions as described in material and methods and exposed to HIV-1 for 24 hours. **(A)** FITC-Dextran leakage of Vk2 cells after exposure to HIV-1 for 24 hours. **(B)** Transepithelial resistance of Vk2 cells after exposure to HIV-1 for 24 hours. Data shown represents data pooled from two separate experiments with at least duplicates per group. Data was analyzed using one-way ANOVA with Tukey's test to correct for multiple comparisons. **$p<0.01$ Error bars represent SEM.

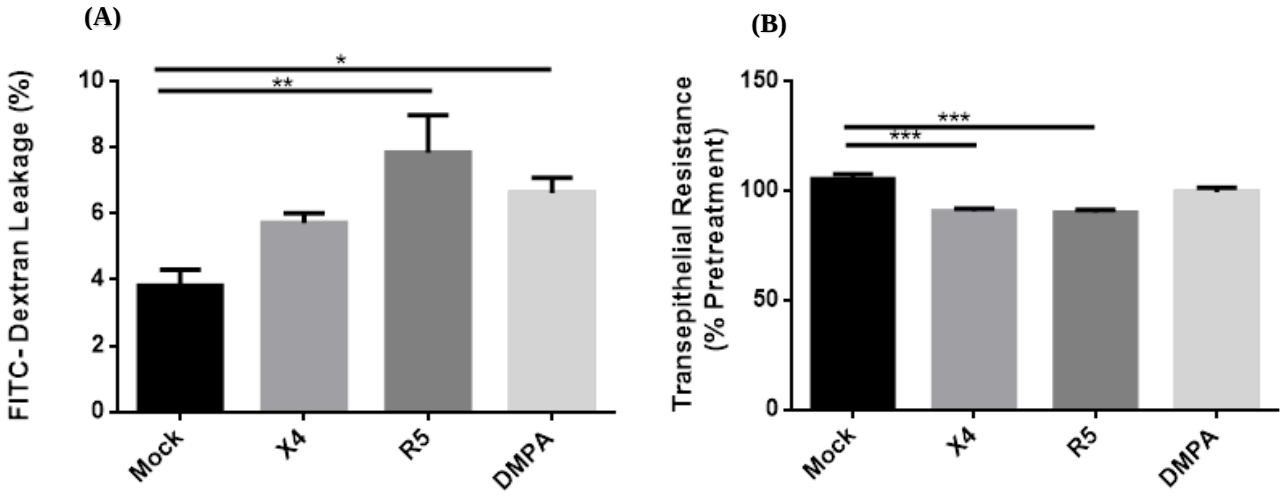


Figure 3: Barrier function of Vk2 cells is disrupted is LLI cultures following exposure to HIV-1. **(A)** FITC-Dextran leakage of Vk2 cells after exposure to HIV-1 for 24 hours. **(B)** TER of Vk2 cells after exposure to HIV-1 for 24 hours. Data shown represents data pooled from two separate experiments with at least duplicates per group. Data was analyzed using one-way ANOVA, with Tukey's test to correct for multiple comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Error bars represent SEM.

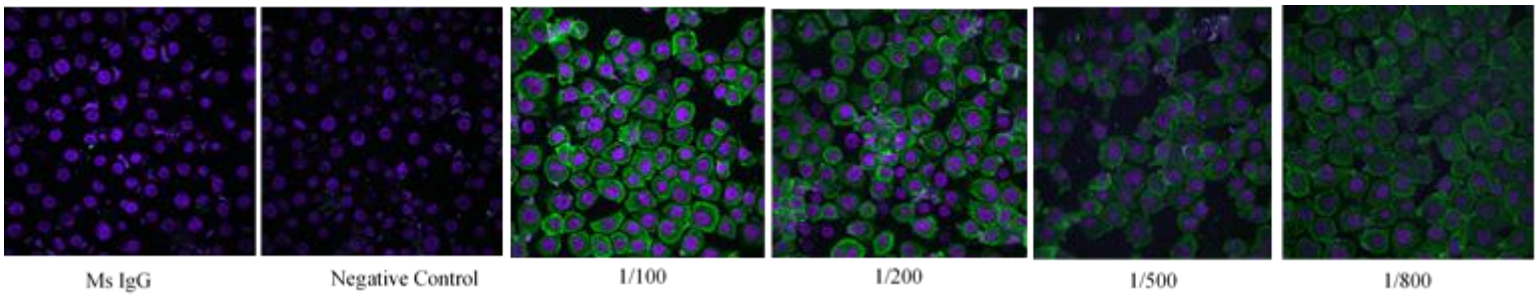


Figure 4: Optimization of barrier staining protein ZO-1 in Vk2 cells. Vk2 cells were grown for 7 days in LLI cultures as described in materials and methods and incubated with varying dilutions of rabbit anti-ZO-1 polyclonal antibody. ZO-1 was stained using a FITC conjugated mouse-anti-ZO-1-FITC Monoclonal Antibody, as shown in green. Nuclei were stained with DAPI as shown in purple. Ms IgG is mouse serum. Images are representative of one experiment with triplicates

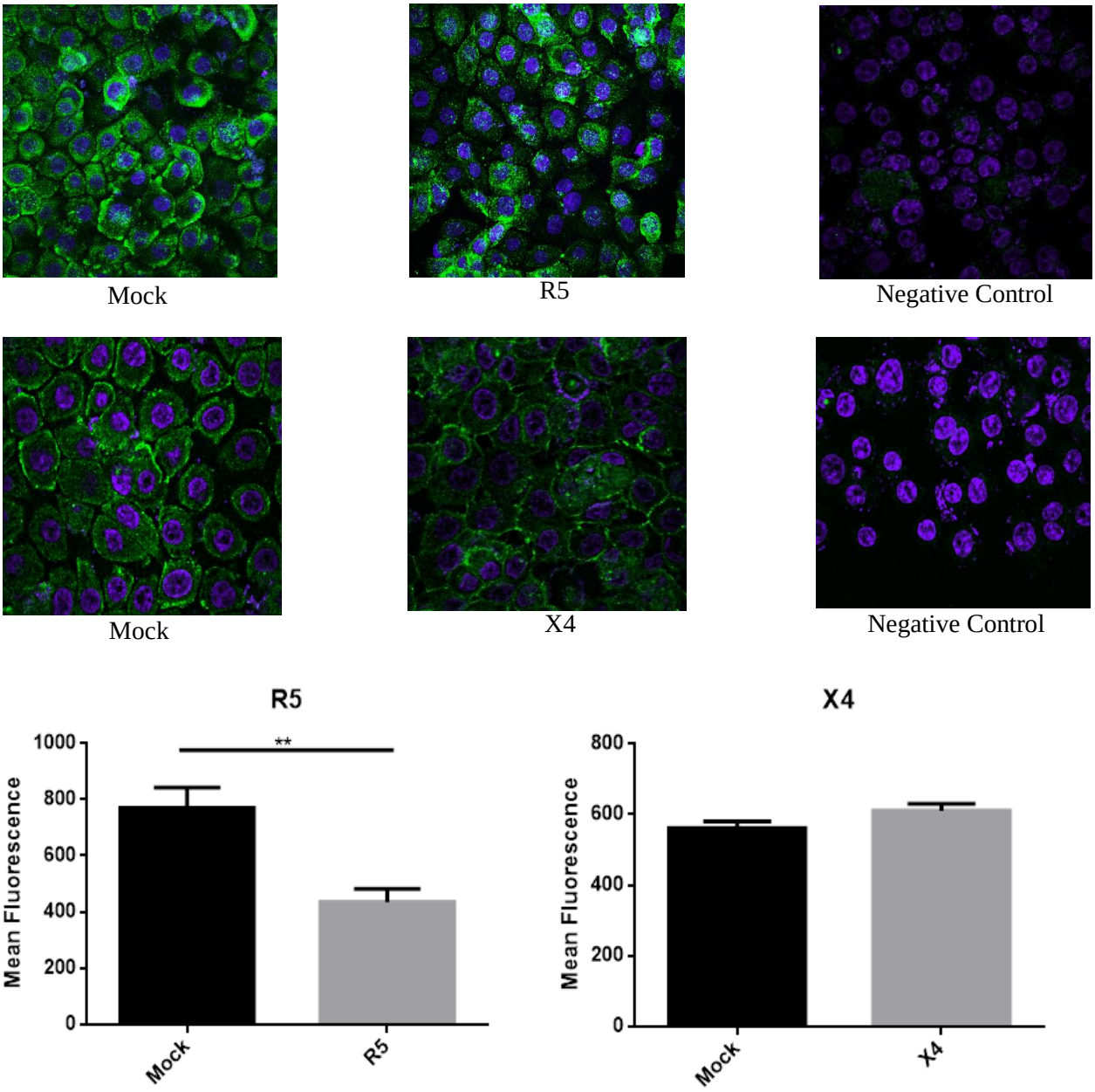


Figure 5: Tight junction protein ZO-1 is disrupted following exposure to R5 strain of HIV-1, however X4 strain of HIV-1 appears to have no discernible effect. Vk2 cells were grown in LLI cultures and exposed to R5 strain of HIV-1 for 24 hours. Cells were fixed and stained as described in materials and methods and subjected to confocal microscopy. ZO-1 was stained using a 1/200

dilution of mouse anti-ZO-1-FITC monoclonal antibody, as shown in green, and measured by imageJ. Nuclei were stained with DAPI as shown in purple. Experiment has been done twice with at least duplicate with R5, and once in triplicates with X4. **(A)** Images shown are representative of one image from one experiment, X4 and R5 experiments were run separately and taken on different days. **(B)** Fluorescent measurements of barrier protein ZO-1 measured using imageJ. Data was analyzed using an unpaired T-test **$p<0.01$.

4.2 Study Endocytic uptake and transport of HIV-1 in WT Vk2 cells

HIV-1 has been reported to use endocytosis for entry into epithelial cells[143, 164, 165]. Therefore, after determining that barrier function was not altered in ALI cultures, we sought to determine the precise mechanism of how HIV-1 crosses Vk2 cells.

Vk2 cells were grown in ALI conditions and exposed to $6x10^5$ IU/mL of both X4 and R5 HIV-1. To understand endocytic uptake and transport of HIV-1 in Vk2 cells, p24 ELISAs and TZM-bl assays were performed. The p24 ELISA is a well-documented technique used for assessing the presence of HIV-1 by measuring the viral capsid protein p24. A drawback of this technique is that it can measure dead or inactive virus as long as HIV-1 contains a p24 capsid protein. Therefore in addition to p24 ELISA, TZM-bl assays were employed to measure infectious virus in supernatant. Although there was no indication of barrier impairment in ALI cultures, both X4 and R5 strain of HIV-1 were present in basolateral compartments in Vk2 cells as indicated by p24 ELISA (Figure 6A) indicating that HIV-1 most likely crossed the vaginal epithelium. Interestingly, a significantly increased amount of R5 virus was observed in basolateral supernatants of Vk2 cells compared to X4 virus ($p<0.01$), (Figure 6A). Similarly, infectious virus was also present in basolateral supernatants of Vk2 cells in ALI cultures as indicated by TZM-bl

assay (Figure 6B). Again, a significantly increased amount of R5 virus observed in the basolateral compartments inVk2 cells compared to X4 as indicated by TZM-bl assay ($p<0.01$), (Figure 6B).

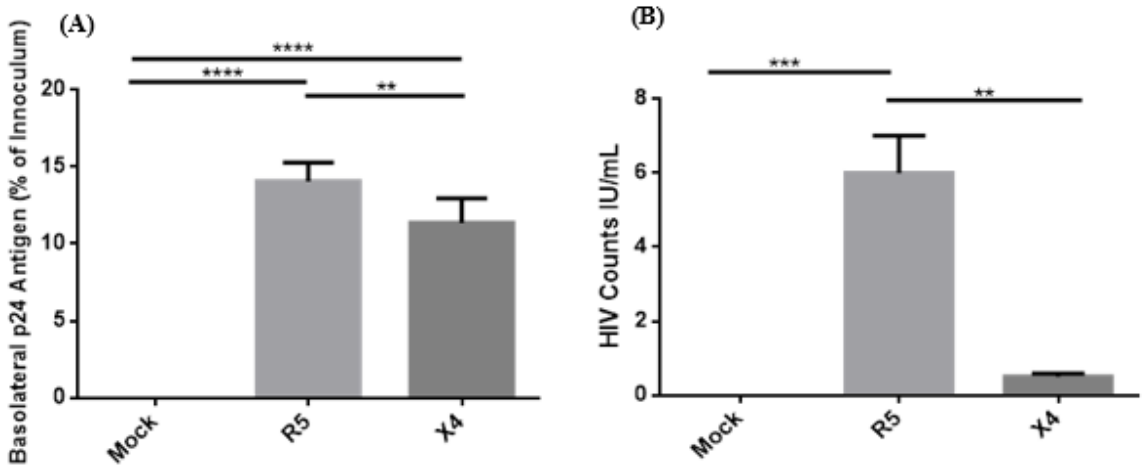


Figure 6: Presence of HIV-1 in basolateral compartments of Vk2 ALI cultures. On day 7 Vk2 Cells were exposed to either X4 or R5 strain of HIV-1 for 4 hours. After 4 hours, cells were washed with room temperature trypsin for 3 minutes to remove any non-adhered virus. 24 hours later supernatants were collected and assessed for HIV-1. **(A)** p24 ELISA of X4 and R5 virus in basolateral compartments of WT Vk2 cells. **(B)** Infectious HIV-1 counted in the basolateral compartment of WT-Vk2 cells measured by TZM-bl assay. (A) Experiment has been done twice. Data is representative of two experiments done in triplicates (B) Experiment has been done twice. Data is representative of one experiment done in triplicates. Data was analyzed using one-way ANOVA with Tukey's test to correct for multiple comparisons. **$p<0.01$, ***$p<0.001$, ****$<p<0.00001$. Error bars represent SEM.

4.2.1 Investigate the Method of Entry into Vk2 Cells by HIV-1

After determining that HIV-1 could potentially cross Vk2 cells in ALI cultures, we wanted to understand how HIV-1 may be entering Vk2 cells. To assess how HIV-1 might be entering epithelial cells, dynasore, an inhibitor of clathrin-mediated endocytosis was added to Vk2 cell as a pretreatment for 1 hour before the addition of HIV-1. Transcytosed virus was measured using a TZM-bl assay. Vk2 cells pretreated with dynasore exposed to R5 virus resulted in significantly less infectious HIV-1 crossing Vk2 cells ($p<0.05$), (Figure 7A). While X4 showed a reduction in the amount of HIV-1 that transcytosed after treatment with dynasore, these results did not reach significance (Figure 7B). To ensure that a 100uM concentration of dynasore was not damaging Vk2 cells, cytotoxicity measurements were measured by lactate dehydrogenase assay. A confluent layer of Vk2 cells grown in transwells was lysed and used as a positive control. 100uM of dynasore did not induce cytotoxicity compared to WT-untreated (media only) ($p>0.05$), (Figure 8). Whereas the positive control of lysed Vk2 cells resulted in significantly more LDH levels compared to both dynasore treated and media only treated Vk2 cells ($p<0.0001$), (Figure 8).

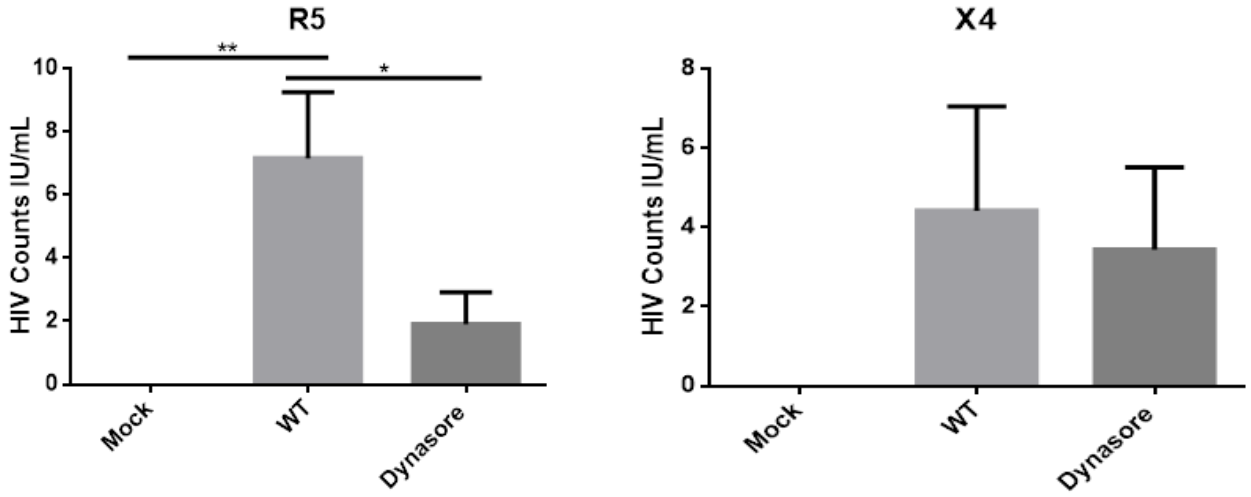


Figure 7: Dynasore treatment leads to decreased HIV-1 present in basolateral compartments in Vk2 cells grown in ALI culture conditions. On day 7 Vk2 Cells were exposed to either X4 or R5 strain of HIV-1 for 4 hours. After 4 hours, cells were washed with room temperature trypsin for 3 minutes to remove any non-adhered virus. 24 hours later supernatants were collected and assessed for infectious HIV-1 using a TZM-bl assay. Experiment has been done twice. Data is pooled from two separate experiments done in triplicates. Data was analyzed using one-way ANOVA with Tukey's test to correct for multiple comparisons. *p<0.05, **p<0.01, Error bars represent SEM.

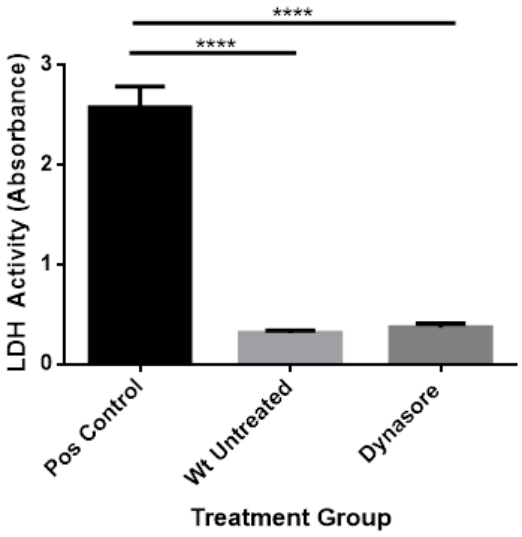
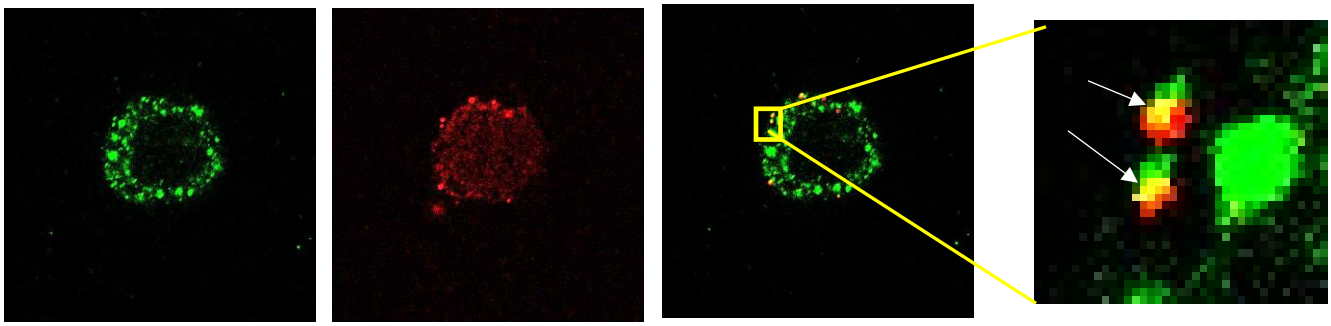


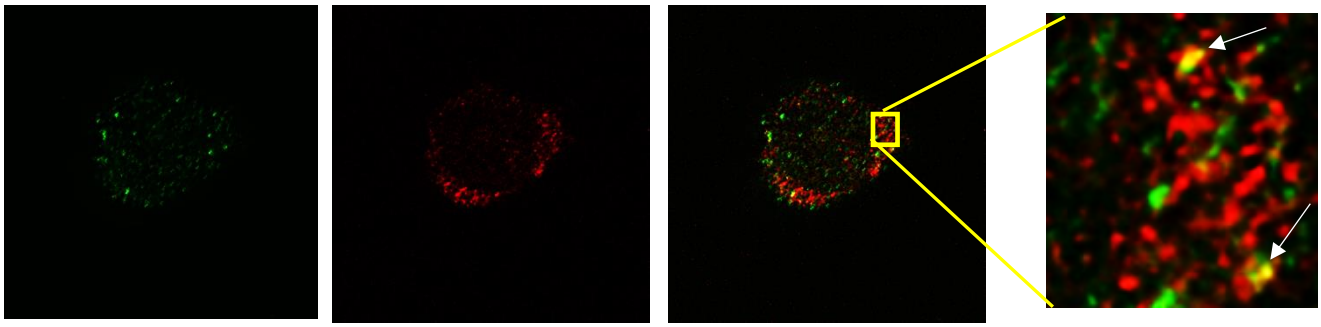
Figure 8: Cytotoxicity of Vk2 cells in ALI cultures with dynasore as assessed by lactate dehydrogenase (LDH) assay. Vk2 cells were exposed to dynasore for 1 hour, after which apical media was removed and subjected to LDH assay. Positive control is lysed Vk2 cells. Data shown represents two pooled experiment done in duplicates or triplicates. Data was analyzed using one-way ANOVA, with Tukey's test to correct for multiple comparisons. ****p<0.0001. Error bars represent SEM.

4.2.2 Visualize HIV-1 Trafficking in Vk2 Cells

After determining that dynasore, a clathrin pit inhibitor, decreased HIV-1 entry and that may enter Vk2 cells by endocytosis, we wanted to visually assess trafficking of HIV-1 in Vk2 cells. $6x10^6$ IU/mL of X4 or R5 strains of HIV-1 was added to LLI cultures of Vk2 for 4 hours for early endosomal staining and 24 hours for late endosomal staining. Colocalization of HIV-1 within early endosomal and late endosomal compartments is indicated by yellow. Both X4 and R5 strains of HIV-1 were found to colocalize with early endosomal marker EEA1 (Figure 9) and late endosomal marker RAB7 (Figure 10). Indicating HIV-1 uses the endosomal pathway to enter and cross Vk2 cells.



EEA1 R5 Merge Enlarged



EEA1 X4 Merge Enlarged

Figure 9: HIV-1 co-localizes with early endosomal marker EEA1. Vk2 cells were grown until 30-50% confluency, exposed to HIV-1 for 4 hours, stained and subjected to confocal microscopy as described in materials and methods. EEA1 was stained using a rabbit anti-human EEA1 polyclonal antibody as primary, andgoat anti-rabbit IgG Alexa fluor 488 as secondary. . HIV-1 was stained using a goat anti-human HIV-1 polyclonal antibody as primary and donkey anti-goat Cy5 as secondary (pseudocoloured) as shown in red. Yellow indicates colocalization. Magnification is 60x. Experiment has been done once in triplicate. Images shown are representative of one image..

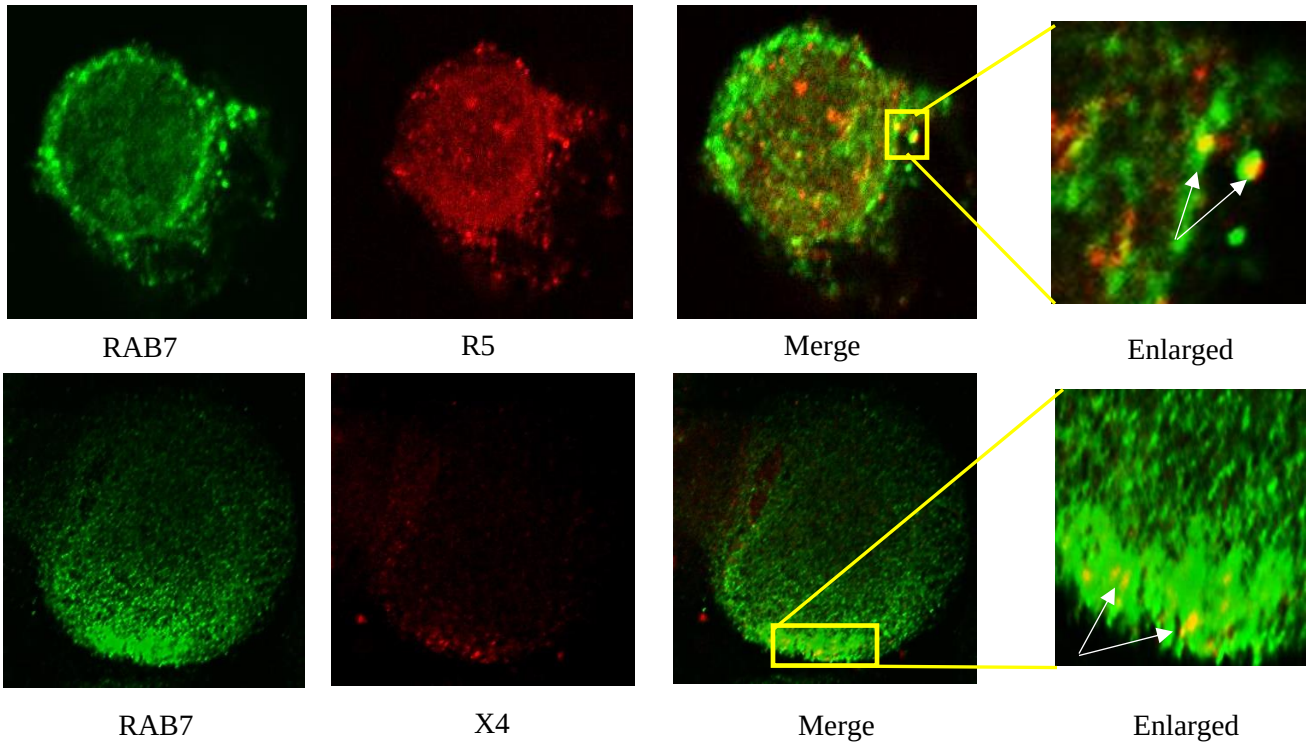


Figure 10: HIV-1 colocalizes with late endosomal marker RAB7. Vk2 cells were grown until 30%-50% confluency, exposed to HIV-1 for 24 hours, stained and subjected to confocal microscopy as described in materials and methods. RAB7 was stained using a rabbit anti-human RAB7 polyclonal antibody as primary, and goat anti-rabbit IgG Alexa fluor 488 as secondary. HIV-1 was stained using a goat anti-human HIV-1 polyclonal antibody as primary and donkey anti-goat Cy5 as secondary (pseudocoloured) as shown in red. Yellow indicates colocalization.

Experiment has been done once in triplicate. Magnification is 60x. Images shown are representative of one image.

4.3 Determine the Role of LAMP3 in HIV-1 Uptake and Transcytosis Across Vaginal Epithelial cells

Vaginal epithelial cells are the first line of defense against HIV-1 in the lower FRT. HIV-1 has been proposed to utilize multiple endocytic pathways of entry into cells including clathrin-mediated, macro- and/or micropinocytosis, caveolae dependent, and clathrin/caveolae independent[98, 143, 166, 167]. While in the cell, HIV-1 can be trafficked to different areas such as recycled to the cell surface, sequestered inside of the cell, trafficked to the lysosome for degradation or released onto the other side of the cell. However, the mechanisms that result in the different trafficking routes of HIV-1 is not well understood. LAMP3, a lysosomal associated membrane protein, has been implicated in the endosomal and lysosomal pathways, and trafficking of viruses[157, 160, 168]. To our knowledge, LAMP3 has not being investigated in transmission and trafficking of HIV-1 in vaginal epithelial cells. Therefore, we decided to investigate the potential role of LAMP3 in the uptake and transcytosis of HIV-1 in Vk2 cells.

4.3.1 Validation of LAMP3 Vk2 Overexpression (OE) and LAMP3 Vk2 Knockdown (KD) cell lines

To investigate the potential involvement of LAMP3 in HIV-1 uptake and transcytosis across vaginal epithelial cells, LAMP3 KD and LAMP3 OE cell lines were created and validated by Dr. Atif Zahoor and Ryan Chow. For validation of LAMP3 KD and LAMP3 OE cell lines, WT-Vk2, LAMP3 KD and LAMP3 OE cell lines were subjected to qRT-PCR to assess mRNA expression of LAMP3, and western blotting, and confocal microscopy were used to assess LAMP3

protein expression. The qPCR indicated that LAMP3 KD did not show change in relative gene expression compared to WT-Vk2 cells (Figure 11A). This is most likely due to the CRISPR knock-out system only targeting the gene at exon 1. This results in truncated forms of LAMP3 mRNA being expressed, but no functional protein being produced. Overexpression of LAMP3 mRNA resulted in significantly increased amounts of LAMP3 gene expression compared to WT-Vk2 cells ($p<0.0001$), (Figure 11A). Western blots of LAMP3 KD demonstrated a fainter band compared to WT (Figure 11B). In contrast, LAMP3 OE shows a very thick band (Figure 11B) of LAMP3 protein confirming the expression of LAMP3 KD and LAMP3 OE cell lines.

Following the validation of LAMP3 KD and LAMP3 OE cell lines, prior to experiments, single cell clones of LAMP3 KD and LAMP3 OE cells were isolated in order to obtain a population of cells expressing similar expression of LAMP3, which was confirmed by confocal microscopy (Figure 12A). Florescence levels were measured by imageJ. LAMP3 KD cell lines expressed significantly less protein than WT Vk2 cell lines($p<0.05$), and LAMP3 OE cell lines ($p<0.001$). In contrast, LAMP3 OE cell lines expressed significantly more protein than both WT Vk2 cell lines ($p<0.01$), and LAMP3 KD cell lines ($p<0.001$), (Figure 12B).

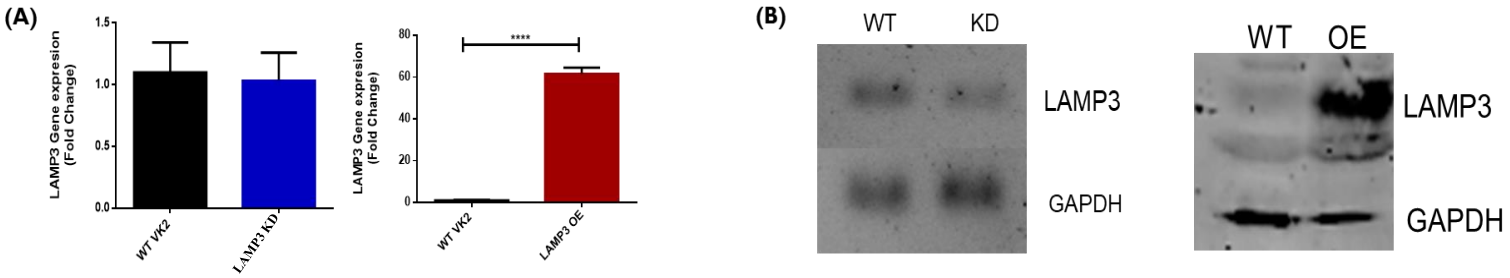


Figure 11: Validation of LAMP3 overexpression and LAMP3 knockdown cell lines performed by Dr Atif Zahoor and Ryan Chow. **(A)** WT Vk2s, LAMP3 OE and LAMP3 KD cell lines were grown

for 7 days at which RNA from was extracted and subjected to qPCR. Data shown represents 3 pooled experiments each done in triplicates **(B)** Cell lysates were prepared from Vk2 WT LAMP3 OE and LAMP3 KD cells and subjected to western blot. GAPDH was used as a loading control. The glycosylated, active form of LAMP3 is shown to be upregulated. Data shown is representative of one experiment. Error bars represent SEM. ****p<0.0001

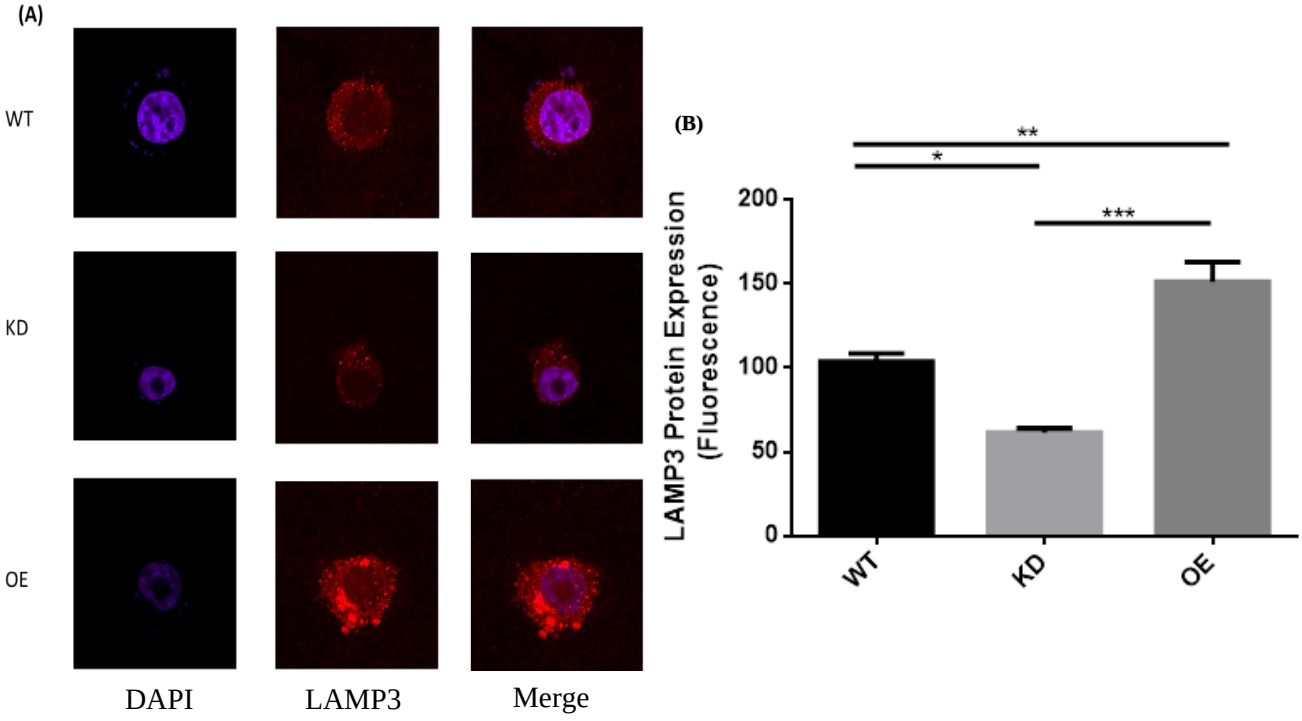


Figure 12: LAMP3 protein expression in single cell clone isolates of WT-Vk2, LAMP3 KD and LAMP3 OE cell lines. **(A)** WT-Vk2, LAMP3 KD Vk2 and LAMP3 OE Vk2 cells were grown until 30-50% confluency in LLI cultures, fixed and subjected to confocal microscopy as described in materials and methods. LAMP3 was stained using a mouse anti-human LAMP3 polyclonal antibody as primary and goat anti-mouse IgG Alexa Fluor 549 as secondary as shown in red. **(B)** Quantification of LAMP3 expression in single cell clone isolates measured by image J. Data represents three different pooled experiments done in triplicates and was analyzed by one-way

ANOVA with Tukey's test for multiple comparisons. *p=0.05, **p= 0.01, ***p= 0.001. Error bars represent SEM.

4.3.2 Assess Barrier Function in LAMP3 KD and LAMP3 OE cell lines

LAMP3 has been suggested to be involved in virus transport, but, has also been documented to be involved in other processes such as cell growth, cell communication, and cell adhesion. Thus, we wanted to ensure that LAMP3 KD and LAMP3 OE epithelial barriers functioned similarly to that of WT-Vk2 cells. To do this, we used TER measurements and FITC-Dextran leakage assays in both ALI and LLI cultures and exposed them to R5 and X4 strains of HIV-1. Before the addition of HIV-1, LAMP3 KD and LAMP3 OE cell line barrier function was assessed. Growth of LAMP3 KD and LAMP3 OE cell lines were assessed using TER measurements, and baseline leakage was assessed using a FITC-Dextran leakage assay. LAMP3 KD and LAMP3 OE cell lines were not significantly different in their levels of baseline leakage compared to WT-Vk2 cells (Figure 13A). DMPA was used as a positive control and significantly increased leakage compared to WT-Vk2 cells ($p<0.01$), (Figure 13A). Similarly, growth of LAMP3 KD and LAMP3 OE cells was not significantly different to that of WT-Vk2 cells as indicated by TER measurements (Figure 13B). Indicating that alterations in LAMP3 expression does not alter barrier function.

After determining that alterations in LAMP3 gene expression does not alter barrier function, we exposed LAMP3 KD and LAMP3 OE cell lines to HIV-1. Exposure to both R5 and X4 virus to WT-Vk2 cells, LAMP3 KD, and LAMP3 OE grown in ALI cultures did not alter barrier function as there was no reduction in leakage (Figure 14A and Figure 15A) as well no change in TER measurements (Figure 14B and Figure 15B). After transitioning to LLI cultures there was a significant increase in leakage of WT-Vk2 cells and LAMP3 KD cells to X4 strain of

HIV-1 ($p<0.05$), (Figure 14C). While exposure of X4 to LAMP3 OE cell lines was not significantly different to LAMP3 OE mock we do see a slight increase in the amount of leakage compared to mock LAMP3 OE cells (Figure 14C). However, there was a significant reduction in TER measurements of WT-Vk2 cells, LAMP3KD and LAMP3 OE exposed to HIV-1 compared to their respective mocks (Figure 14D). Similarly, ALI cultures of WT-Vk2 cells, LAMP3 KD, and LAMP3 OE cells exposed to R5 did not significantly change leakage compared to mock treated (Figure 15A) or alter TER measurements (Figure 15B). However, exposure of R5 to WT-Vk2 cells, LAMP3 KD, and LAMP3 OE cell lines grown in LLI cultures resulted in a significant increase in leakage compared to mock treated groups (Figure 15C) and a significant reduction in TER measurements (Figure 15D). Although LAMP3 has been document to be involved in cell-cell adhesion it does not alter barrier function in Vk2 cells.

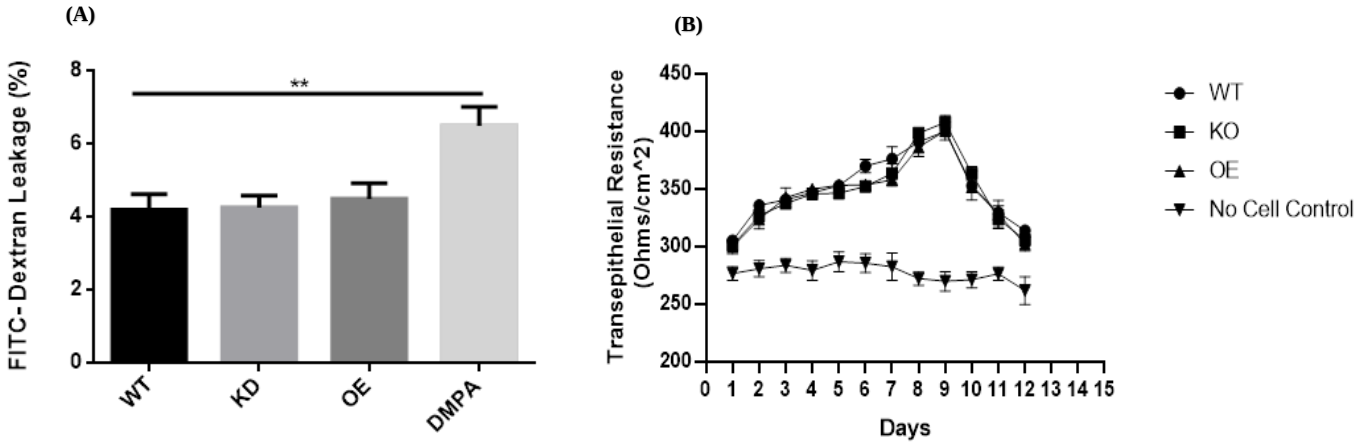


Figure 13: Alterations in LAMP3 expression does not impact barrier function in Vk2 cells. WTVk2 cells, LAMP3 KD and LAMP3 OE cells were grown in ALI culture conditions as described in Materials and Methods. **(A)** Baseline FITC-dextran leakage of WT-Vk2 cells, LAMP3 KD and LAMP3 OE cell lines. DMPA is WT-Vk2 treated cells. Data shown represents two pooled

experiments done in duplicates or triplicates. **(B)** TER measurements were taken for three separate well inserts from three experiments on different days from 1 through 12. Data was analyzed by two-way ANOVA (B), and one-way ANOVA (A) with Tukey's test for multiple comparisons. ** $p<0.01$. Error bars show SEM.

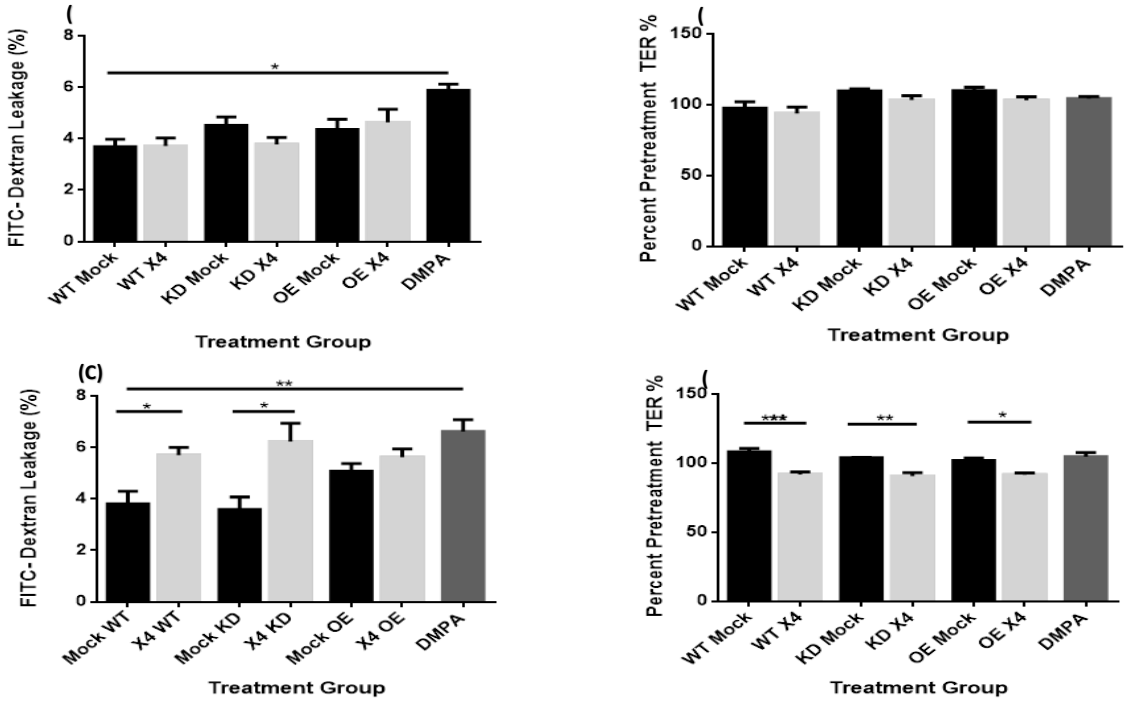


Figure 14: X4 Strain of HIV-1 increases leakage and decreases transepithelial resistance in LLI cultures but not in ALI cultures. WT-Vk2, LAMP3 KD and LAMP3 OE cells were grown in either ALI or LLI cultures as described in Materials and Methods. On day 7 R5 strain of HIV-1 was added to cells and barrier function was assessed using a FITC-Dextran leakage assay and transepithelial resistance. **(A)** FITC-dextran leakage of WT-Vk2, LAMP3 KD and LAMP3 OE in ALI cultures exposed to X4 strain of HIV-1. **(B)** TER measurements of WT-Vk2, LAMP3 KD, and LAMP3 OE cell lines in ALI cultures after exposure to X4 strain of HIV-1. **(C)** FITC-dextran leakage of WT-Vk2, LAMP3 KD, and LAMP3 OE cell lines in LLI cultures after exposure to

HIV-1. **(D)** TER measurement of LLI cultures in WT-Vk2, LAMP3 KD, and LAMP3 OE cell lines after exposure to HIV-1. Data shown is representative of two pooled experiments with at least duplicates in each group. Data was analyzed by one-way ANOVA with Tukey's test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM.

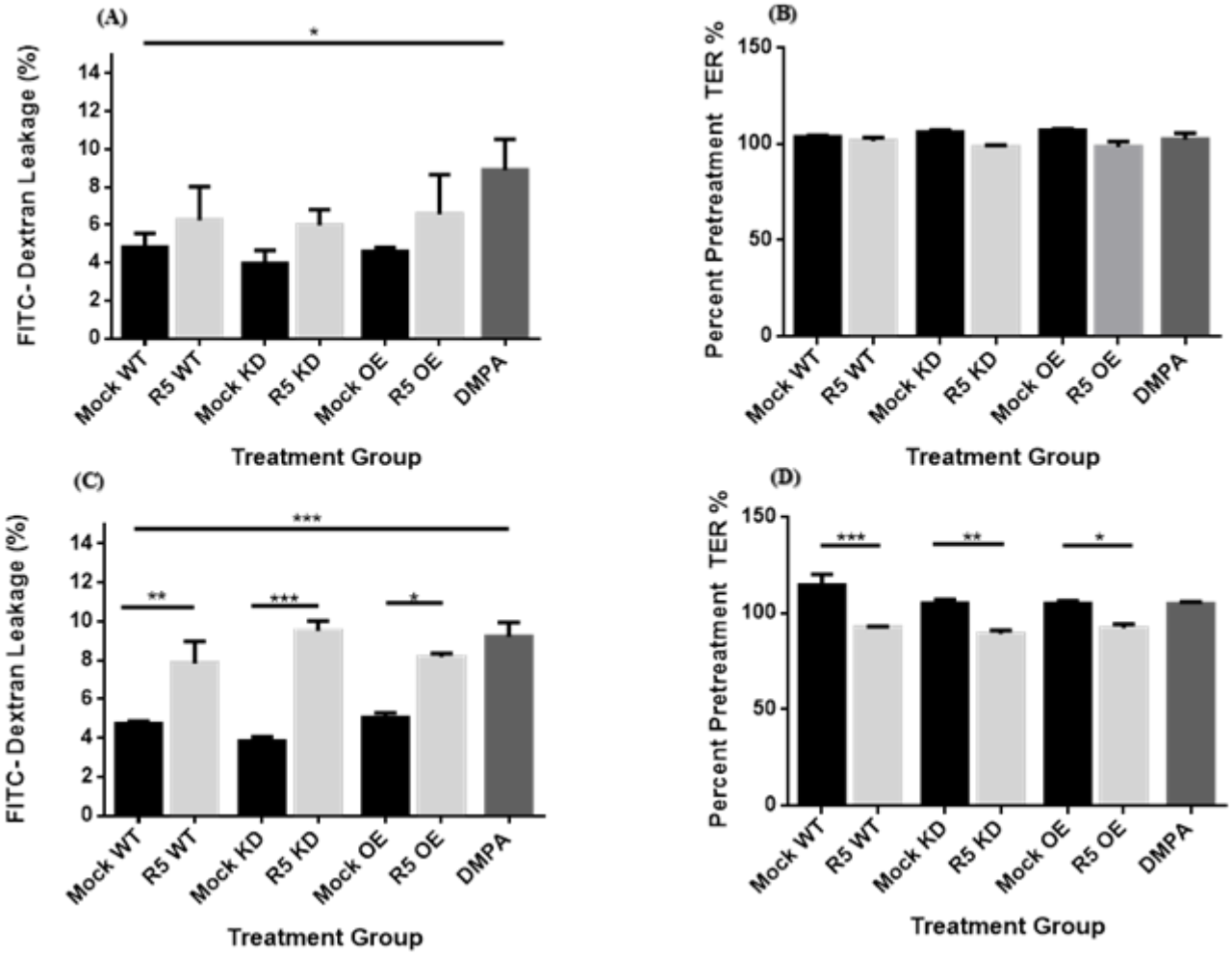


Figure 15: R5 strain of HIV-1 significantly increases leakage and decreases transepithelial resistance in LLI cultures but not in ALI cultures. WT-Vk2, LAMP3 KD and LAMP3 OE cells were grown in either ALI or LLI cultures as described in Materials and Methods. On day 7 R5 strain of HIV-1 was added to cells and barrier function was assessed using a FITC-Dextran leakage assay and transepithelial resistance. **(A)** FITC-dextran leakage of WT-Vk2, LAMP3 KD and

LAMP3 OE lines in ALI cultures exposed to R5 strain of HIV-1. **(B)** TER measurement in ALI cultures after exposure to R4 strain of HIV-1. **(C)** FITC-dextran leakage of LLI cultures in all three cells lines after exposure to HIV-1. **(D)** TER change of LLI cultures in all three cell lines after exposure to HIV-1. Data shown is representative of two pooled experiments with at least duplicates in each group. Data was analyzed by one-way ANOVA with Tukey's test to correct for multiple comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Error bars represent SEM.

4.3.3 Assess Location of LAMP3, and Gene Expression of LAMP3 After Exposure to HIV-1 in WT-Vk2 Cells

To assess the involvement of LAMP3 in HIV-1 uptake and transcytosis across vaginal epithelial cells several techniques were employed. qRT-PCR was used to measure the change in LAMP3 gene expression in WT-Vk2 cells after the addition of X4 and R5 strains of HIV-1 at varying time points. Significant upregulation of gene expression was seen in X4 exposed cells compared to mock treated ($p<0.05$), (Figure 16). While not significant, LAMP3 was upregulated at 2, 24 and 48 hours in R5 treated cells compared to mock treated Vk2 cells. Similarly, LAMP3 was upregulated at 2, 4, and 48 hours, and significantly upregulated at 6 hours after exposure to X4 strain of HIV-1 (figure 16). Indicating LAMP3 is induced in Vk2 cells after exposure to HIV-1.

After ascertaining LAMP3 gene expression is induced upon exposure to HIV-1, we were curious to understand the location of LAMP3 in Vk2 cells. As HIV-1 uses the endosomal pathway to cross epithelial cells, and LAMP3 is associated with endosomal pathway as well, we wanted to determine the location of LAMP3 within Vk2 cells. LAMP3 protein expression was visualized by confocal microscopy in both early and late endosomal compartments. Colocalization of LAMP3 and early endosomal marker EEA1 was observed as indicated by white (Figure 17). Similarly, LAMP3 and late endosomal marker RAB7 colocalized (Figure 18). These results show a potential interaction of LAMP3 to the endosomal pathway.

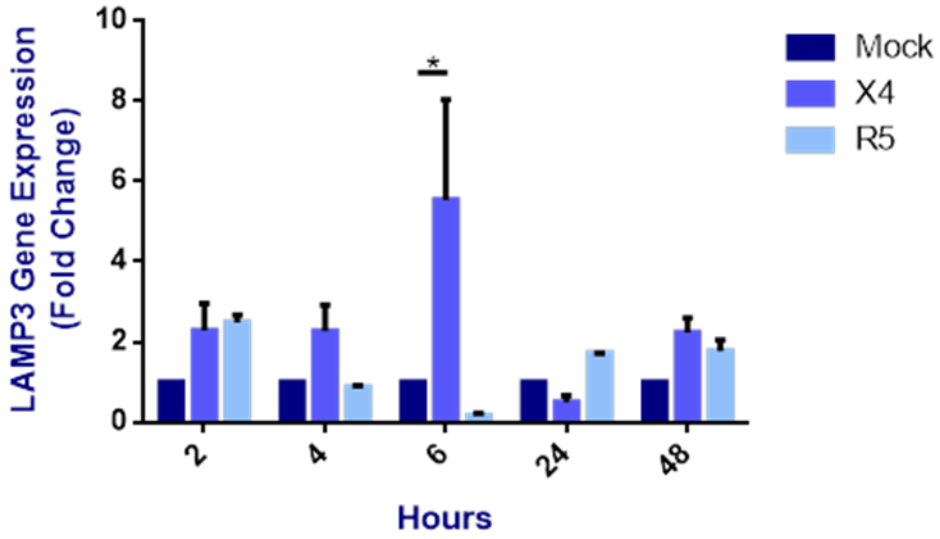


Figure 16: LAMP3 gene expression is induced in Vk2 cells upon exposure to HIV-1. Vk2 cells were grown in ALI cultures and exposed to R5 or X4 HIV-1 strains. After 2,4,6,24, and 48 hours, total cellular RNA was extracted and subjected to qRT-PCR using primers specific for LAMP3 as explained in materials and methods. Experiment has been done three times. Data shown represents 2 pooled experiments each done in triplicates and analyzed by two-way ANOVA with Tukey's test to correct for multiple comparisons. *$p<0.05$, Error bars show SEM.

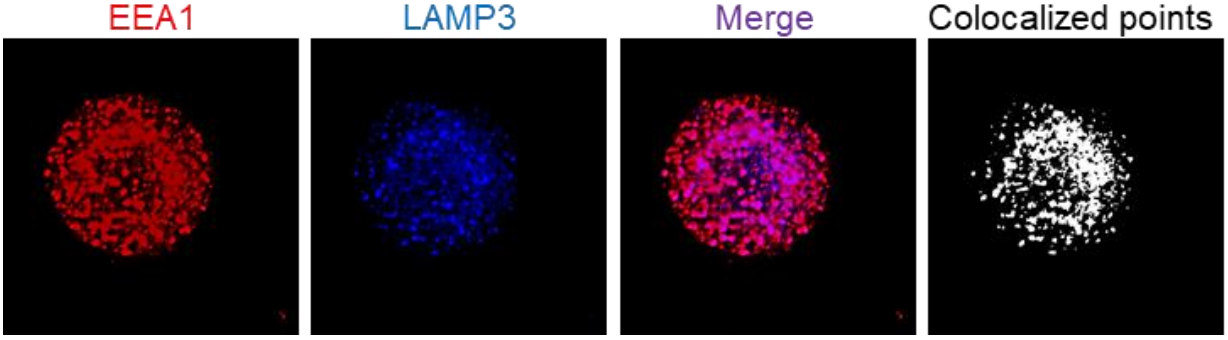


Figure 17: LAMP3 protein co-localizes with early endosomal marker EEA1. Vk2 cells were grown until 30%-50% confluency in LLI cultures, stained and subjected to confocal microscopy as described in materials and methods. EEA1 was stained using a rabbit anti-human EEA1 polyclonal antibody as primary, and goat anti-rabbit IgG Alexa fluor 488 as secondary. (pseudocoloured) as shown in red. LAMP3 was stained using a mouse anti-human LAMP3 polyclonal antibody as primary and goat anti-mouse IgG Alexa Fluor 549 as secondary (pseudocoloured) as shown in blue. White indicates colocalization. Experiment has been done once with triplicates. Experiment was performed by me. Images were taken by Dr. Nazli.

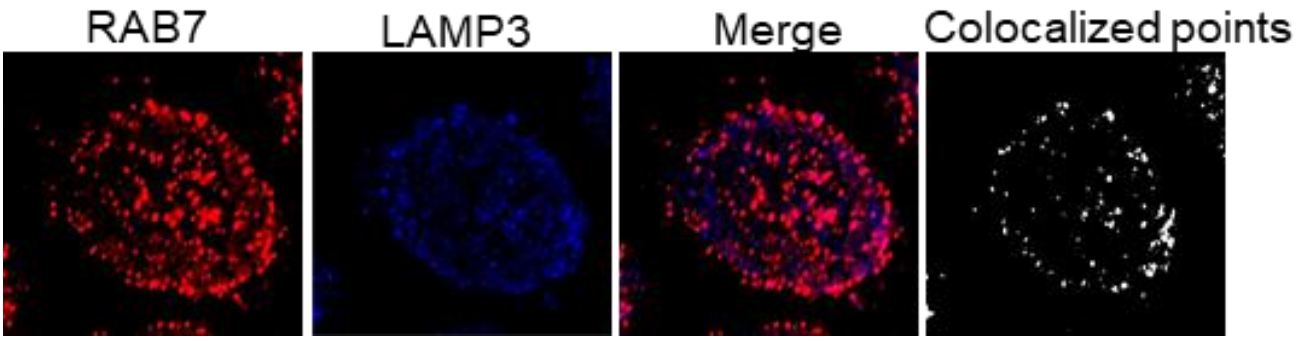


Figure 18: LAMP3 protein co-localizes with late endosomal marker RAB7. Vk2 cells were grown until 30%-50% confluency in LLI cultures, stained and subjected to confocal microscopy as described in materials and methods. RAB7 was stained using a rabbit anti-human RAB7 polyclonal antibody as primary, and goat anti-rabbit IgG Alexa fluor 488 as secondary. (pseudocoloured) as shown in red. LAMP3 was stained using a mouse anti-human LAMP3 polyclonal antibody as primary and goat anti-mouse IgG Alexa Fluor 549 as secondary

(pseudocoloured) as shown in blue. White indicates colocalization. Experiment has been done once with triplicates. Images were taken by Dr. Nazli.

4.3.4 Investigate the role of LAMP3 in uptake and transcytosis of HIV-1 in Vk2 cells using LAMP3 KD and LAMP3 OE cell lines

After determining that LAMP3 colocalizes with the endosomal pathway, we wanted to assess any potential involvement of LAMP3 with HIV-1 in Vk2 cells. To do this, confocal microscopy in LLI cultures was used to indicate any colocalization and potential interactions between HIV-1 and LAMP3. Additionally, LAMP3 has been implicated in the early entry of viruses, thus, intracellular virus was also measured in LLI cultures in the early hours of exposure using confocal microscopy[159, 162]. p24 ELISAs and TZM-bl assays were used to assess the amount of virus crossing Vk2 cells into the basolateral compartments. Only a few points of colocalization of Both X4 and R5 strains of HIV-1 were observed with LAMP3 in Vk2 cells, indicating a potential interaction (Figure 19A and 19B). Following this, intracellular virus was measured using confocal microscopy at 4 hours post-exposure to HIV-1. Exposure of LAMP3 OE cell line to R5 resulted in significantly more HIV-1 accumulating inside the cell at 4 hours compared to LAMP3 KD and WT-Vk2 cells ($p<0.01$), (Figure 20A). While not significant, LAMP3 KD cell line did show less HIV-1 compared to WT-Vk2 cells (Figure 20A). Similarly, LAMP3 OE cells exposed to X4 resulted in significantly increased amount of virus accumulation compared to WT-Vk2 cells ($p<0.01$), and LAMP3 KD cell lines ($p<0.001$), (Figure 20B). Again, while not significant, there was a reduction in the amount of X4 virus in LAMP3 KD cell lines compared to WT-Vk2 cells (Figure 20B).

To understand if LAMP3 was assisting in the trafficking of HIV-1, WT-Vk2 cells, LAMP3 OE and LAMP3 KD cell lines were grown in ALI and LLI cultures. On day 8, basolateral

supernatants were collected. p24 ELISAs were used to assess total virus present in basolateral compartments, and TZM-bl assays were used to measure infectious HIV-1 in basolateral compartments. According to the p24 ELISA both X4 and R5 resulted the presence of HIV-1 in basolateral compartments in ALI cultures in WT-Vk2, LAMP3 KD, and LAMP3 OE cell lines (Figure 21A and Figure 21C). While not significant, R5 appeared to show a slight increase in levels of HIV-1 present in LAMP3 KD basolateral compartments compared to both WT-Vk2 cells and LAMP3 OE cell lines and LAMP3 OE showed a decrease in HIV-1 compared to WT-Vk2 and LAMP3 KD cells (Figure 21 C). Although these trends were not seen in X4 (Figure 21A). Similar patterns were seen in the amount of infectious virus that was present in basolateral compartments grown in ALI cultures. While not significant, LAMP3 KD cells exposed to R5 showed a slight increase in the amount of infectious virus in basolateral compartments compared to WT-Vk2 cells, and a reduction in the amount of infectious virus present in LAMP3 OE cell line compared to WT-Vk2 cells (Figure 21D). There were no significant differences or trends seen in the amount of X4 present in basolateral compartments in LAMP3 KD and LAMP3 OE cell lines compared to WT-Vk2 cells (Figure 21B). This led us to question if cells grown in ALI culture conditions resulted in less interaction with the most active basal cells. Upon transitioning to LLI cultures, again, in the TZM-bl assays there were no differences seen in the amount of X4 present in LAMP3 KD and LAMP3 OE cells compared to WT-Vk2 cells (Figure 22A). However, LAMP3 KD cells exposed to R5 resulted in significantly increased amounts of HIV-1 present in basolateral compartments compared to WT-Vk2 cells ($p<0.05$), (Figure 22B). In contrast, LAMP3 OE had significantly less HIV-1 present in basolateral compartments compared to LAMP3 KD cells ($p<0.01$), (Figure 22B). Overall, these results indicate that R5 is likely sequestered in cells that overexpress LAMP3 and

released in higher amounts when LAMP3 is knocked down. However, no clear effect is seen on trafficking of X4.

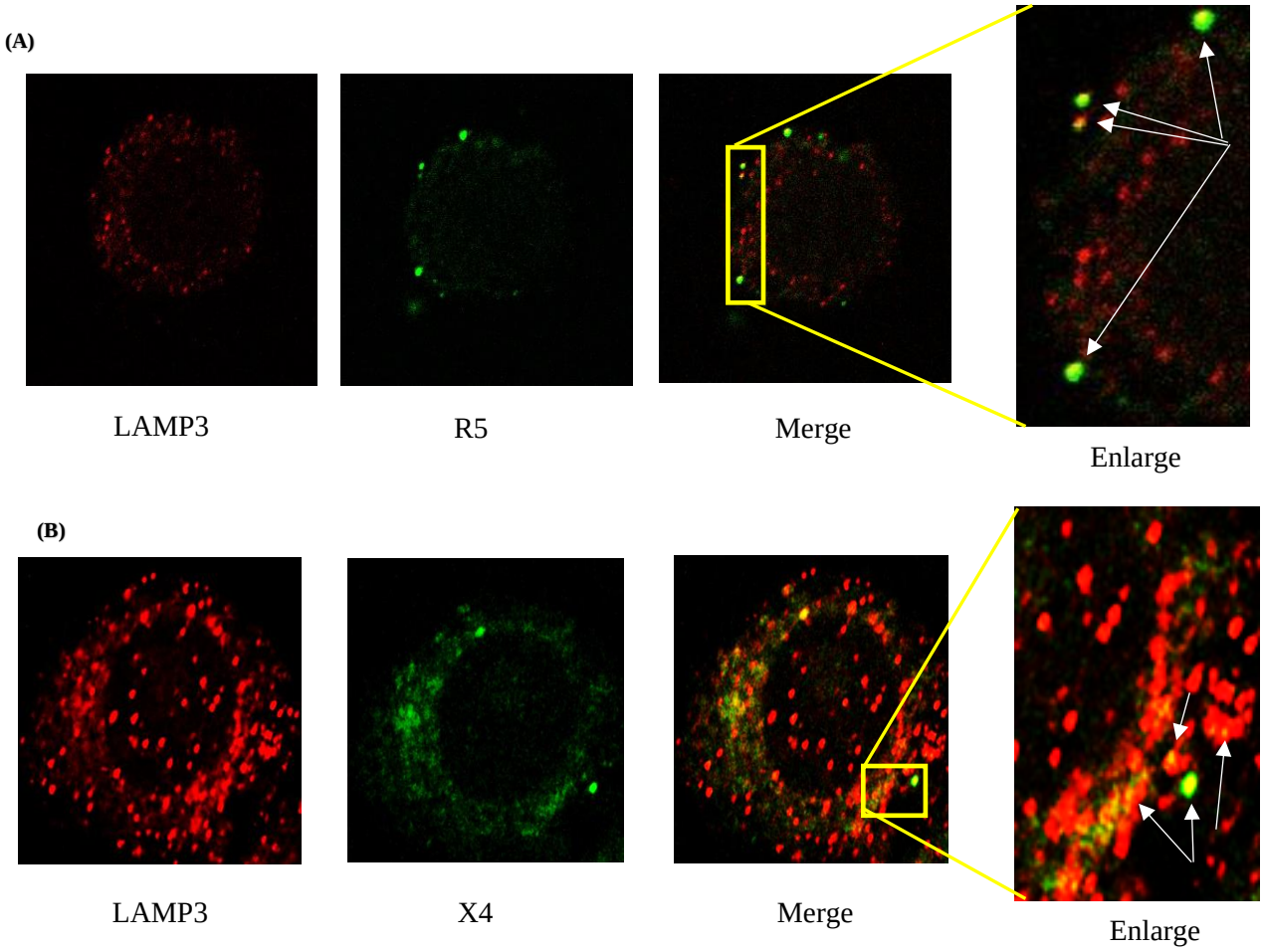


Figure 19: LAMP3 co-localizes with HIV-1. Vk2 cells were grown until 30%-50% confluency in LLI cultures, exposed to R5 and X4 strain of HIV-1 for 4 hours, stained and subjected to confocal microscopy as described in materials and methods. LAMP3 was stained with mouse anti-human LAMP3 polyclonal antibody as primary and goat anti-mouse IgG Alexa Fluor 549 as secondary as shown in red. HIV-1 was stained using goat anti-human HIV-1 polyclonal antibody as primary

and donkey anti-goat Cy5 as secondary (pseudocoloured) as shown in green. Yellow indicates colocalization. Experiment has been done once in triplicate.

Overall, these findings suggest that LAMP3 does not impact barrier function in Vk2 cells and that there is indeed an interaction with HIV-1 in vaginal epithelial cells. Specifically, LAMP3 is induced upon exposure to HIV-1 in vaginal epithelial cells as shown by qRT-PCR, and that LAMP3 is assisting in the early entry of HIV-1 into Vk2 cells in both X4 and R5 strains as seen by confocal analysis. Moreover, LAMP3 KD is assisting in the trafficking of R5 but not X4 in Vk2 cells as seen by p24 and TZM-bl analysis.

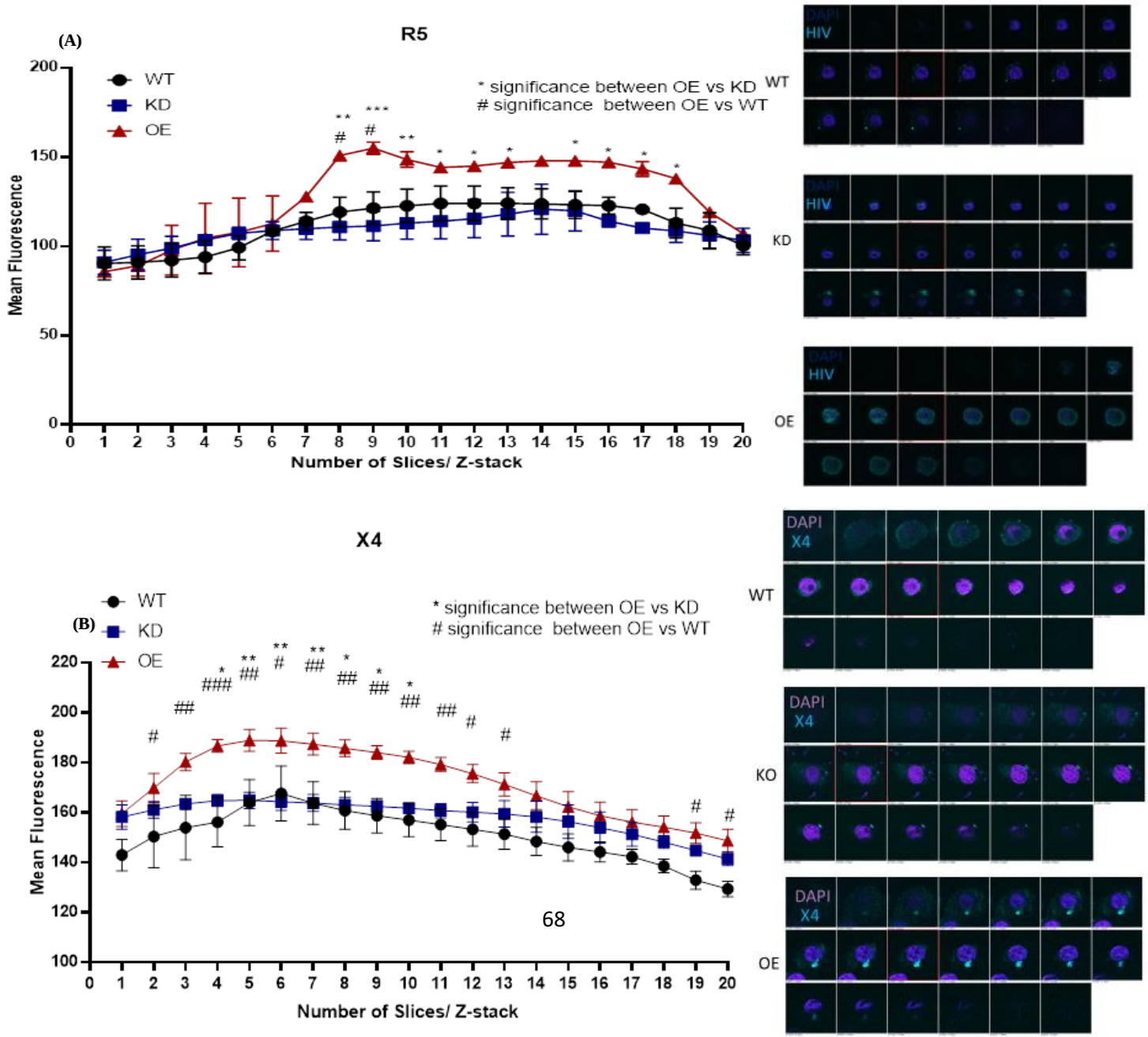


Figure 20: HIV-1 accumulates inside LAMP3 OE Cell Lines at 4 hours. Vk2 cells were grown until 30%-50% confluency in LLI cultures, exposed to R5 (A) or X4 (B) strain of HIV-1 for 4 hours, stained and subjected to confocal microscopy as described in materials and methods. HIV-1 was stained using goat anti-human HIV-1 polyclonal antibody as primary and donkey anti-goat Cy5 as secondary as shown in blue. Quantitation of HIV-1 in in WT Vk2, LAMP3 KD Vk2 and LAMP3 OE Vk2 Cell lines was done using image J. Data was analyzed by two-way ANOVA with Tukey's test to correct for multiple comparisons. Data shown is representative of one experiment done in triplicates. Error bars represent SEM. *p<0.05 ** p<0.01. *** p<0.0001

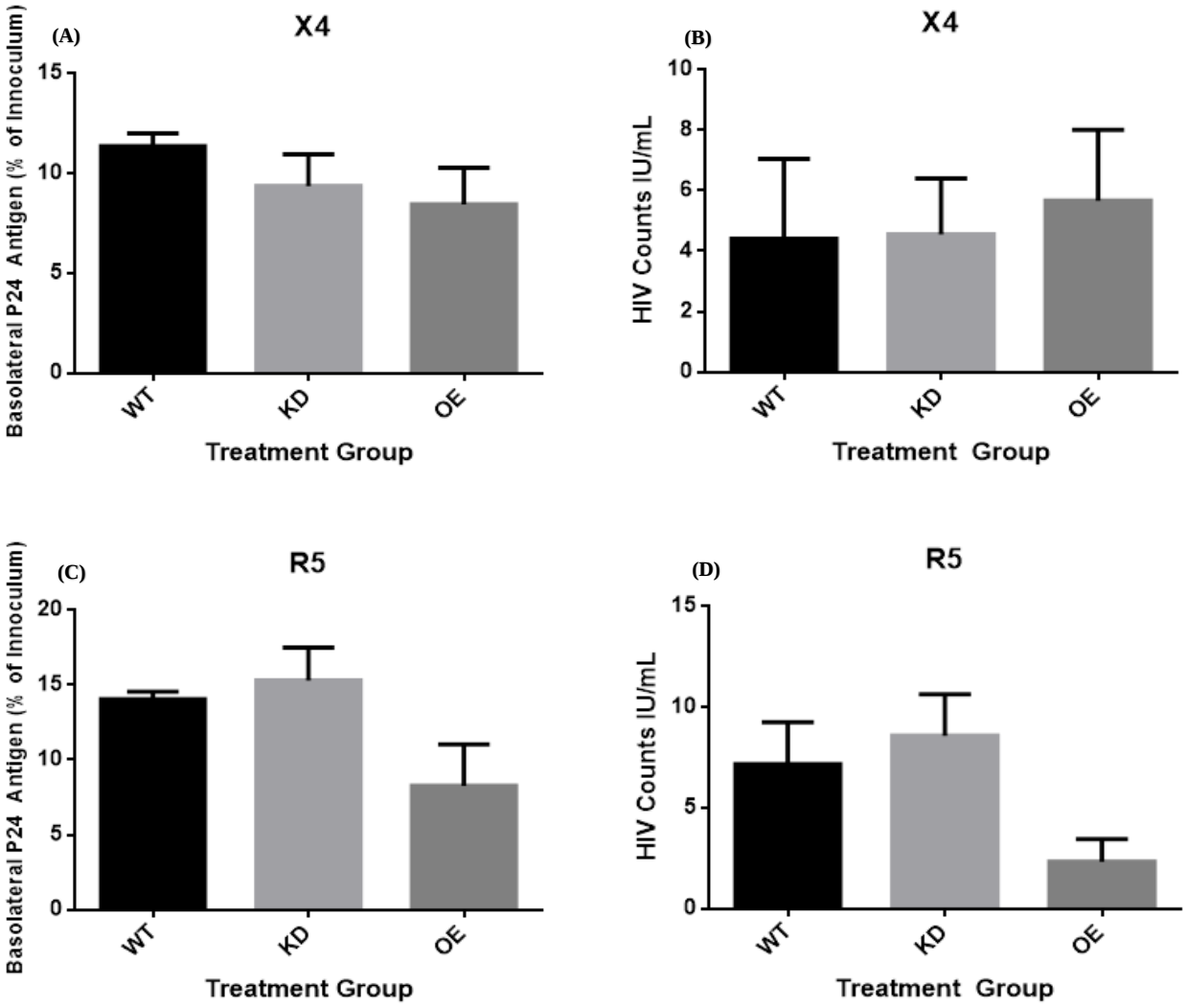


Figure 21: KD of LAMP3 expression appears to show a trend towards increased amount of R5 HIV-1 present in basolateral supernatants in ALI culture conditions but does not alter X4. WT-Vk2, LAMP3 KD and LAMP3 OE Vk2 cells were grown for 7 days in ALI culture conditions as described in materials and methods on day 7 cells were exposed to either X4 or R5 strain of HIV-1 for 4 hours. After 4 hours, cells were washed with room temperature trypsin for 3 minutes remove of any non-adhered virus. 24 hours later basolateral supernatants were collected and assessed for HIV-1 using a p24 ELISA and TZM-bl assay. **(A)** Basolateral measurement of p24 ELISA of LAMP3 KD LAMP3 OE and WT Vk2 cells exposed to X4 strain of HIV-1. **(B)** Infectious HIV-1 counted in the basolateral compartment of X4 exposed cells in ALI cultures. **(C)** Basolateral measurement of p24 ELISA of LAMP3 KD LAMP3 OE and WT Vk2 cells exposed to R5 strain of HIV-1. **(D)** Infectious HIV-1 counted in the basolateral compartment of R5 exposed cells in ALI cultures. Data is representative of two pooled experiments done in triplicates (A and C). Data is representative of two pooled experiments done in triplicates (B and D). Data was analyzed by one-way ANOVA with Tukey's test to correct for multiple comparisons. Error bars represent SEM.

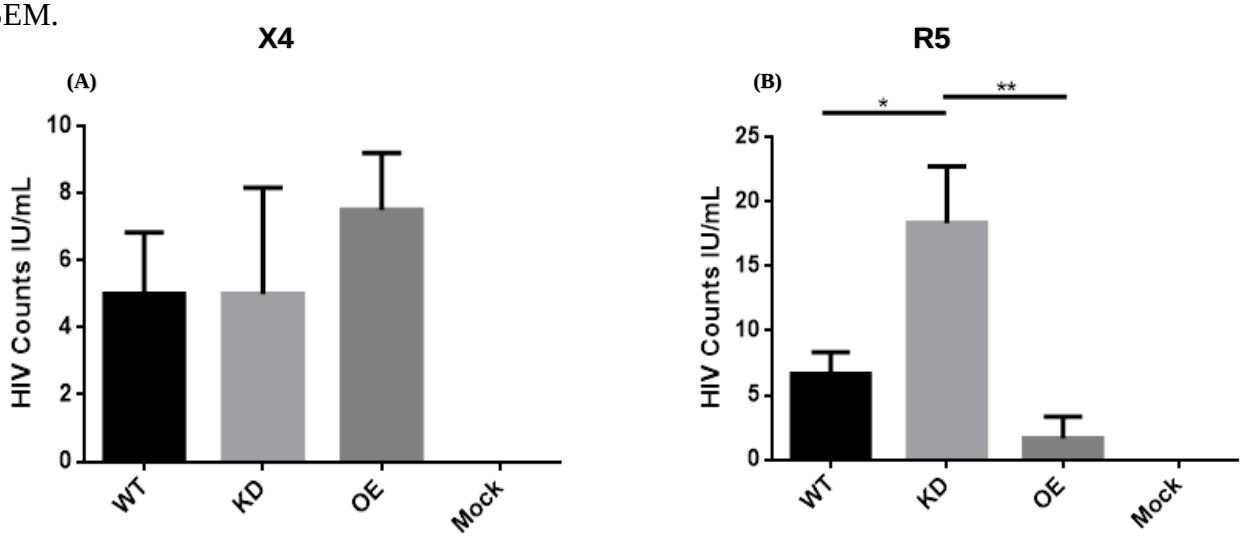
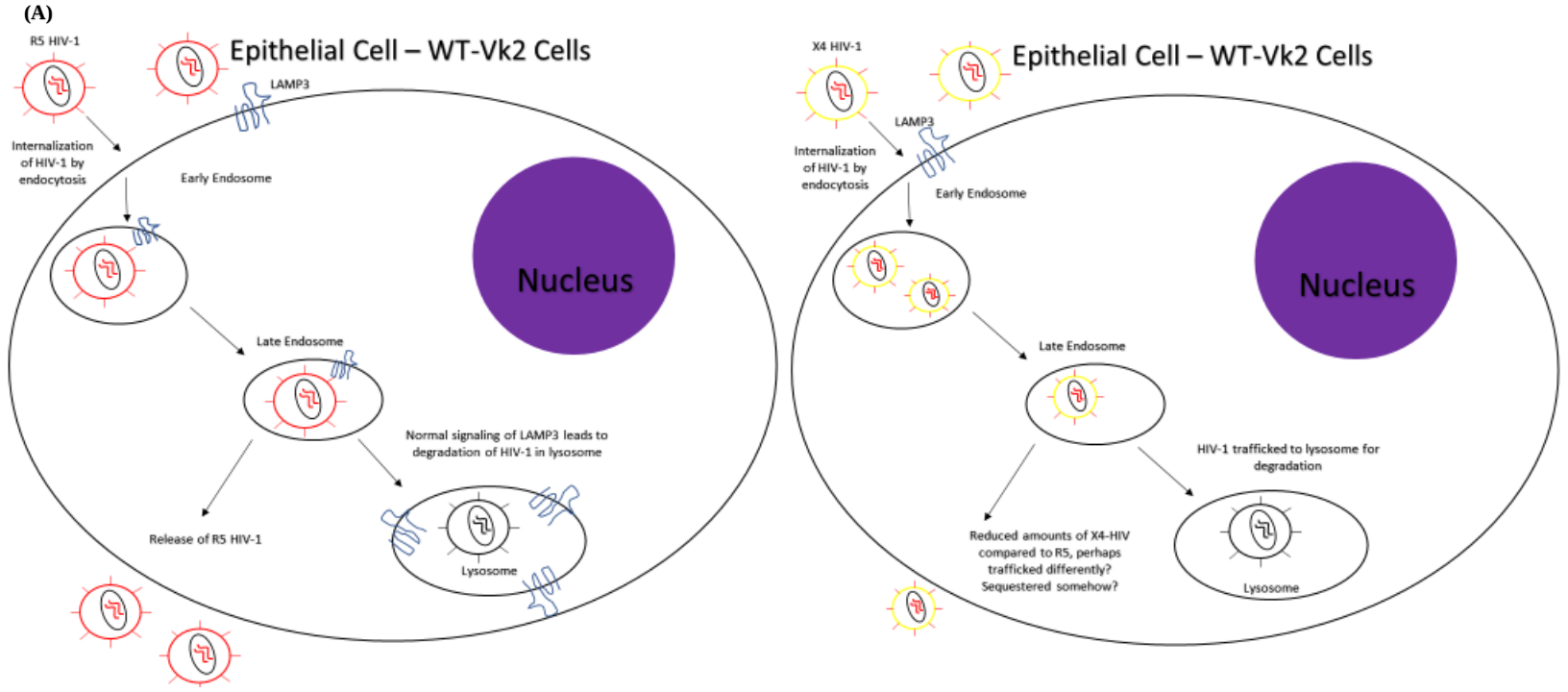


Figure 22: KD of LAMP3 expression significantly enhances the presence of infectious R5 HIV-1 in basolateral supernatants of LLI cultures but does not alter X4. WT-Vk2, LAMP3 KD and

LAMP3 OE Vk2 cells were grown for 7 days in LLI culture conditions as described in materials and methods. On day 7 cells were exposed to $6x10^5$ IU/mL of R5 or X4 strain of HIV-1 for 4 hours. After 4 hours, cells were washed with room temperature trypsin for 3 minutes to removeany non-adhered virus. 24 hours later basolateral supernatants were collected and assessed for HIV-1 using a TZM-bl assay. **(A)** Infectious HIV-1 counted in the basolateral compartment of X4 exposed cells in LLI cultures. Data is representative of two pooled experiments done in triplicates. **(B)**. Infectious HIV-1 counted in the basolateral compartment of R5 exposed cells in LLI cultures. Experiment has been done three times in duplicates or triplicates; data is representative of one experiment in triplicates. Data was analyzed by one-way ANOVA with Tukey's test to correct for multiple comparisons. Error bars show SEM. *<p0.05 ** p<0.01.



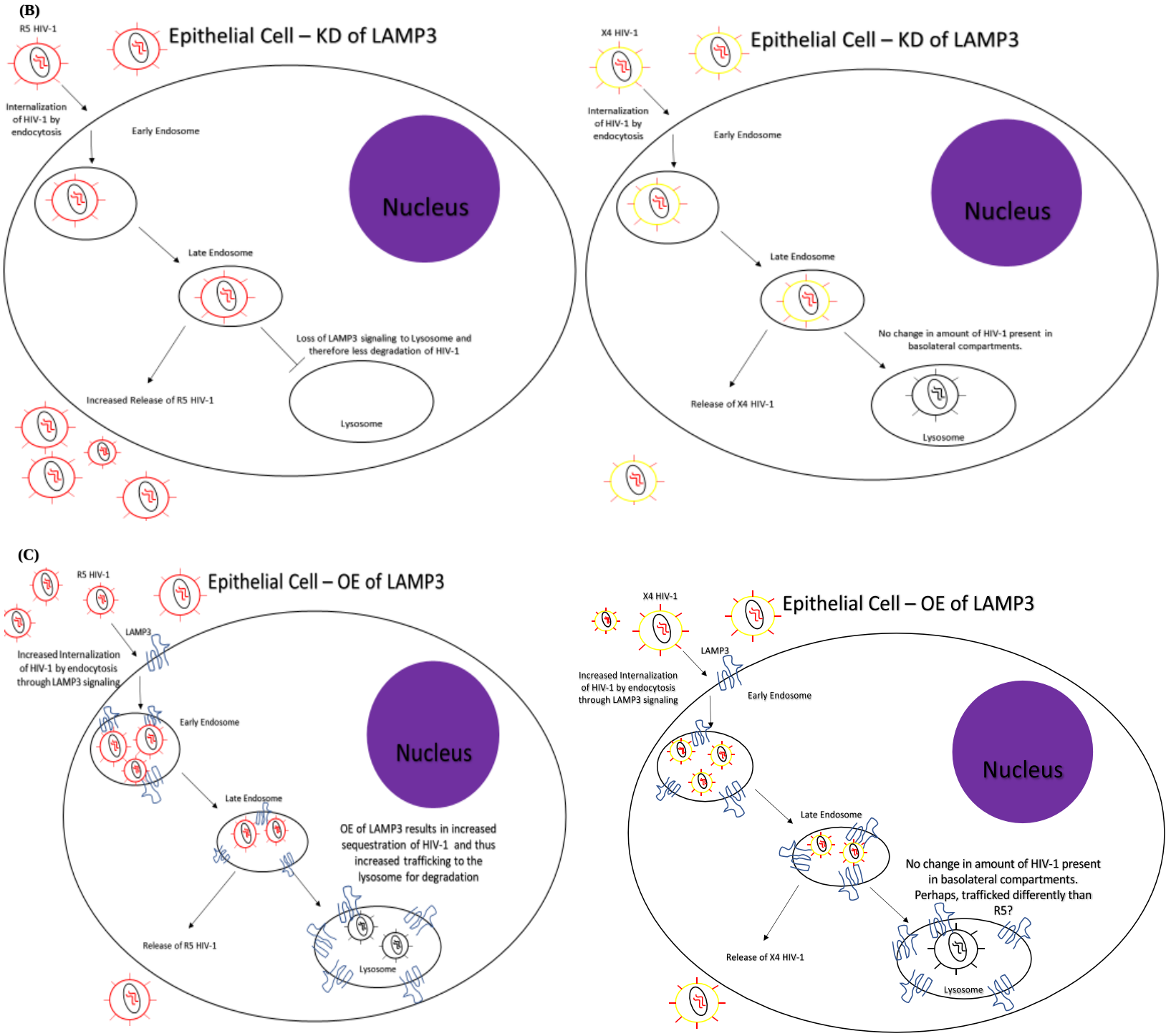


Figure 23: Study Summary. **(A)** Our study found that in WT-Vk2 cells there was significantly more R5 HIV-1 present in basolateral compartments compared to X4. **(B)** Our study also found

that KD of LAMP3 resulted in significantly increased amounts of R5 HIV-1 present in basolateral compartments compared to WT-Vk2 cells, but this difference was not observed in X4 HIV-1 treated groups. **(C)** Lastly our study also showed there were no significant differences in transcytosis of HIV-1 in LAMP3 OE cell lines compared to WT-Vk2 cells in both X4 HIV-1 and R5 HIV-1 exposed cells, although R5 HIV-1 exposed LAMP3 OE Vk2 cells showed slightly less HIV-1 present in basolateral compartments. Interestingly, in LAMP3 OE cell lines, both X4 HIV-1 and R5 HIV-1 appeared to enter Vk2 cells at a higher rate than WT-Vk2 cells and LAMP3 KD cells indicating LAMP3 may be assisting with entry of HIV-1 as well. These results demonstrate there may be different trafficking mechanisms of X4 HIV-1 and R5 HIV-1 in Vk2 cells that requires further investigation.

Chapter 5: Discussion

The vagina and ectocervix comprise the largest surface area within the FRT, and the vaginal epithelial cells that line the lower FRT are the first point of contact with infectious HIV-1 during heterosexual transmission. In order for HIV-1 to successfully infect CD4+ expressing T cells and macrophages, HIV-1 must traverse the vaginal epithelial barrier and gain access to the underlying lamina propria. Despite advanced treatment options for those suffering with HIV-1, there is still no effective cure or vaccine to prevent new HIV-1 infections. While there is a consensus that vaginal epithelial cells do not become productively infected with HIV-1, little is known about the interactions of HIV-1 and vaginal epithelial cells. Specifically, barrier function and trafficking of HIV-1, as well as proteins involved within epithelial cells that may be involved with HIV-1 trafficking. These gaps in knowledge represent a major obstacle in the development of effective strategies to prevent HIV-1 acquisition in women. Therefore, the aim of this thesis was to better understand how HIV-1 crosses vaginal epithelial cells and investigate the role of LAMP3 in HIV-1 uptake and transcytosis of HIV-1 across vaginal epithelial cells *in-vitro*. The overall hypothesis was that LAMP3 will assist in HIV-1 uptake and transcytosis of HIV-1 across vaginal epithelial cells and was addressed in two specific aims. First, to determine how HIV-1 crosses vaginal epithelial cells. Specifically, its effects on barrier function and endocytic uptake and transport. Secondly, to determine the role of LAMP3 in uptake and transcytosis of HIV-1 across vaginal epithelial cells using WT-Vk2 cells, LAMP3 KD Vk2 cells and LAMP3 OE Vk2 cells.

In summary, the results from the study demonstrates that knocking down LAMP3 resulted in increased levels of R5 HIV-1 present in basolateral compartments (Figure 22 B). Moreover, no observable effect was seen in basolateral compartments of cells treated with X4, thus contradicting our hypothesis (Figure 22 A). KD of LAMP3 in lung epithelial cells results in less production of influenza A virus[157] . Also, treatment of macrophages with anti-LAMP3 Mab inhibited infection by several R5 and dual tropic strains of HIV-1[159]. Therefore, based on these results we expected to see an increase of HIV-1 in LAMP3 OE Vk2 cells, so it was surprising to see KD had increased levels of HIV-1. LAMP3 has been implicated in the endosomal pathway, which HIV-1 uses therefore we hypothesized that enhancing LAMP3 expression would allow increased amounts of virus to cross Vk2 cells. However, as HIV-1 does not infect epithelial cells there may be an alternate role of LAMP3 in HIV-1 trafficking in epithelial cells. In addition, Vk2 cells grown in multilayered structures, that more physiologically and accurately reflects conditions of the lower FRT, provided a robust barrier against HIV-1(Figure 2). Conversely, cells grown in a monolayer exposed to HIV-1 resulted in barrier disruption compared to mock treated Vk2 cells (Figure 3). We also found that HIV-1 entered Vk2 cells through the endocytic pathway (Figure 9 and 10), and significantly more R5 HIV-1 crossed Vk2 cells compared to Vk2 cells exposed to X4 HIV-1 (figure 6). Alterations (KD or OE) of LAMP3 expression did not affect barrier function compared to WT-Vk2 cells. LAMP3 was specifically induced in Vk2 cells upon exposure to X4 strain of HIV-1 at 6 hours but induced at other timepoints (Figure 16) and KD of LAMP3 significantly increased the amount of infectious R5 HIV-1 present in basolateral compartments of Vk2 cells compared to WT-Vk2 cells grown in LLI cultures (Figure 22 A).

5.1 How HIV-1 crosses vaginal epithelial cells

HIV-1 has been documented to cross epithelial cells through endocytosis and impair barrier function allowing microbial translocation [12, 23, 98, 143]. However, a lack of understanding regarding the early transmission events in the lower FRT warrant further investigation. While no model flawlessly recapitulates the same structural and biological conditions in the human FRT, using a simplified *in-vitro* model that physiologically mimics conditions of the lower FRT provides a foundation before moving to *in-vivo* studies. For our study we used an immortalized cell line that contains characteristics similar to human vaginal epithelial cells that line the lower FRT and grew these cells in an ALI culture system that promotes the growth of multilayered and terminally differentiated cells reflective of the squamous epithelium in the vaginal tract.

Maintenance of a robust epithelial barrier is imperative to prevent pathogens, including HIV-1, from penetrating the underlying tissue resulting in infection[9]. By establishing and maintaining barrier function we can more accurately assess interactions of epithelial cells and HIV-1 to mimic conditions *in-vivo*. Before exposing Vk2 cells to HIV-1 we first established baseline growth using transepithelial resistance measurements and assessed barrier function after 7 days using FITC-Dextran leakage assays (Figure 1A and Figure 1B). To establish a positive control for leakage we used two different concentrations of DMPA and found that 20 ng/mL resulted in significantly increased leakage compared to mock treated (Figure 1B). DMPA was used a positive control for leakage as previous work in the lab has established DMPA resulted in increased leakage in Vk2 cells (Lam, Master's Thesis 2019, Macsphere). Moreover, transcriptomic data in Vk2 cells grown in ALI cultures treated with DMPA had decreased expression of genes associated with cell-cell adhesion (Woods et al., unpublished). This was further complimented by research in the upper genital tract. Microarray analysis of DMPA treated primary human epithelial cells showed an

increase in increased gene expression related to inflammation[169]. Therefore, it is possible that cells treated with DMPA results in prolonged levels of inflammation, resulting in increased leakage and microbial translocation, thus escalating risk of HIV-1 acquisition[169]. While not *in-vitro,* this also mirrors work done by Calla et al., where they found that DMPA promoted HSV-2 susceptibility in humanized mice by reducing genital tissue expression of the cell-cell adhesion molecule desmogelin-1 and increasing mucosal barrier permeability[170]. Furthermore, a clinical study comparing women receiving DMPA vs an oral contraceptive found that the DMPA group resulted in significant downregulation of genes involved in the maintenance of mucosal barrier functions, cell proliferation, and cell to cell signaling[171]. Thus, DMPA treatment was concluded to be an effective positive control when assessing leakage in Vk2 cells.

After establishing the best timeline to conduct experiments and a positive control for leakage, Vk2 cells were grown in ALI and LLI culture conditions and exposed to HIV-1. Barrier function was assessed in cells exposed to HIV-1 by using a combination of both ALI and LLI cultures, FITC-Dextran leakage assays, TER measurements, and confocal imaging in LLI cultures was done. LLI cultures were chosen for confocal assessment as ALI cultures resulted in unclear images due to multiple cell layers. Previous studies exploring the ability of HIV-1 to impair the epithelial barrier has been investigated in primary genital epithelial cells, intestinal cells, and oral epithelial cells[70, 122, 172-174]. However, this has yet to be established in vaginal epithelial cells. Vk2 cells grown in ALI culture condition showed no significant differences in the epithelial barrier after exposure to HIV-1 (Figure 2A and Figure 2B). However, when LLI cultures were used there was significantly increased leakage in R5 HIV-1 treated Vk2 cells compared to mock (Figure 3A) and significantly reduced TER measurements in both X4 and R5 treated groups (Figure 3B). While not *in-vitro,* this mirrors results of Sankapal et al., who investigated tight junction disruption in

ectocervical organ explants after 24 hours of exposure to HIV-1 indicating HIV-1 may impair the vaginal barrier grown in a monolater of cells[175]. Visually, we saw a significant reduction of ZO-1 protein expression measured by fluorescence in R5 treated Vk2 cells indicating barrier impairment. Interestingly, there were no differences seen in fluorescence in X4 treated groups. However, the barrier may still be impaired in X4 treated cells through other mechanisms. Experiments in ectocervical epithelial cells grown in monolayers exposed to either HIV-1 IIB or HIV-1 Bal have been documented to show a decrease in tight junction expression[127]. This has further been demonstrated in endocervical cells and oral epithelial cells further supporting our results[70, 174]. Mechanistically, there may also be other factors contributing to increased susceptibility and tight junction disruption leading to microbial translation in vaginal epithelial cells or increased leakage after exposure to HIV-1 that we did not investigate in our study, including the production of cytokines and chemokines. Indeed, previous studies investigating cytokine production after HIV-1 exposure showed increases in CXCL1 and CXCL10 in culture supernatants of HIV-1 exposed ectocervical tissues[175, 176]. CXCL10 and CXCL11 have been reported to be involved in recruitment of T cells and macrophages to HIV-1 infected lymph nodes, which could in turn result in recruitment of target cells towards to epithelial region resulting in enhanced transmission[177]. Moreover, significant upregulation of inflammatory cytokines such as TNF-α, IL-6, and IL-1β was observed 24 hours after HIV-1 exposure in endometrial cells[177]. TNF-α is a cytokine documented to disrupt epithelial tight junction assembly. Although in our LLI culture system, while we observed decreased TER and a slight increase in leakage of fluorescent dye in X4 HIV-1 treated Vk2 cells, the barrier may still be impaired through other mechanisms. Thus, our results indicate that HIV-1 is able to disrupt the epithelial barrier in vaginal epithelial cells grown in a monolayer upon exposure to HIV-1, and that vaginal epithelial cells grown in a

multilayered structure may provide better protection against HIV-1. While our study did not investigate potential mechanisms as to why increases in leakage occurred, an analysis of the relevant inflammatory cytokines and their ability to decrease barrier integrity may be an important avenue for future investigation[178].

Several studies have documented the ability of HIV-1 to transcytose across an in-tact cervico-vaginal epithelial layer[23, 143]. However, the precise trafficking mechanism in Vk2 cells has yet to be investigated. In our system, to assess trafficking of HIV-1 across a tight epithelial layer cells were grown in ALI cultures and exposed to HIV-1 on the apical side. Basolateral HIV-1 was measured using a p24 ELISA and TZM-bl assay. Moreover, the precise intracellular trafficking route of HIV-1 was assessed by colocalization of HIV-1 with EEA1 and RAB7 using confocal microscopy. Both X4 and R5 were present in basolateral supernatants of Vk2 cells, however, a significantly increased amount of R5 HIV-1 was present in basolateral supernatants in Vk2 cells compared to X4 treated cells (Figure 6A and Figure 6B). Although the mechanism is not clear, there have been documented reports of selective R5 HIV-1 transmission over X4 HIV-1 in epithelial cells, and that X4 HIV-1 appears to become sequestered inside of cells[179, 180]. There may be several factors that can influence selective transmission of R5 HIV-1 over X4 HIV-1 across the vaginal epithelium, including, mucus, defensins, and chemokines. In fact, some epithelial models, including the ectocervical epithelial cell line ECT/E6E7 have been shown to produce baseline levels of SDF-1, a CXCR4-binding chemokine, which could potentially block X4 HIV-1 transmission[181, 182]. Moreover, as previously mentioned, defensins are commonly released by epithelial cells and may be more restrictive against X4. For example, human beta-defensins 2 and 3 were found to inhibit CXCR4 tropic viruses greater than CCR5 tropic[183]. Although we did not investigate this in our model, human beta-defensins 2 is produced by the epithelial cell line

Vk2/E6E7 and has been documented to be induced by pathogenic *Candida albicans*[184]. This may be a worthwhile avenue to investigate in the future. Furthermore, R5 HIV-1 may be favourably transcytosed over X4 HIV-1 due to surface changes between the two strains. The V3 loop in gp120 of X4 HIV-1 has a higher cationic charge than R5 HIV-1, which could produce a stronger affinity for binding of X4 HIV-1 to polyanionic mucin and clearance of X4 HIV-1 over R5 HIV-1. Mucin is however, not present in our system, and this speculation requires further experimentation[185]. Moreover, while not in vaginal cells, primary intestinal epithelial cells have been shown to selectively transfer R5 HIV-1 over X4 HIV-1 through galactosylceramide, an alternative receptor for CCR5 but not CXCR4[186]. Perhaps there is an alternative receptor present in vaginal epithelial cells that may be facilitating transfer of R5 HIV-1 over X4 HIV-1. While these are all interesting possibilities, we did not investigate this in our system, but the observations made in my research raise the similar question whether there is a potential "gatekeeper" function for selective transmission of R5 HIV-1 over X4 HIV-1 in vaginal epithelial cells.

Since we observed HIV-1 present in the basolateral supernatants of Vk2 cells grown in ALI cultures, we were curious to assess how HIV-1 could be entering and crossing Vk2 cells. Literature has suggested that HIV-1 commonly uses endocytosis as a means to cross epithelial cells[12, 98, 187]. Our results support this for vaginal epithelial cells, as dynasore, an inhibitor of clathrin-mediated endocytosis resulted in less HIV-1 present in the basolateral compartments of Vk2 cells, indicating that HIV-1 may enter cells using clathrin mediated endocytosis. In both R5 HIV-1 and X4 HIV-1 exposed cells treated with dynasore, we saw a reduction in the amount of virus present in basolateral compartments compared to mock treated, however, only R5 reached significance (Figure 7A and Figure 7B). These results were also seen by Kinlock et al., who found that increasing concentrations of dynasore resulted in less dual tropic HIV-1 inside Vk2 cells,

indicating that HIV-1 enters Vk2 cells by endocytosis[143]. Dynasore inhibits the GTPase of dynamin, preventing clathrin coated endocytosis, interestingly, while both X4 and R5 treated Vk2 cells saw reduced HIV-1 in the basolateral compartments X4 was not found to be significantly reduced. This could be also due to different methods of trafficking of each strain into the cell, or that X4 was sequestered resulting in only a miniscule amount of HIV-1 crossing Vk2 cells. Of course, this observation needs to be further investigated.

Confocal analysis further supports the notion of HIV-1 crossing Vk2 cells through endocytosis as colocalization signals of both X4 and R5 was seen in early endosomal marker EEA1 (Figure 9), and late endosomal marker RAB7 (Figure 10) containing cellular compartments. This was not surprising as other studies have documented endocytosis of HIV-1 in cervical and foreskin epithelial cells[98]. While we did not investigate recycling markers such as transferrin in vaginal epithelial cells, which is also part of the endocytic pathway, colocalization of transferrin and HIV-1 has been observed further supporting our results that HIV-1 enters Vk2 cells through endocytosis[143]. This could also be indicative of a potential biological interaction between HIV-1 and the endocytic pathway. EEA1 is an early endosomal protein that is implicated as the main sorting station in the endocytic pathway and the docking of incoming endocytic cargo before fusing with early endosomes which is then delivered to late endosomes[188]. Interestingly, HIV-1 is documented to enhance the activity of master regulator protein synthesis- associated late endosomes/lysosomes (mTOR), which in turn, results in a signaling cascade and causes repositioning of late endosomes and repositions lysosomes to the periphery resulting in successful HIV-1 replication in HeLa cells[189]. A loss of mTOR function leads to a blockade of viral particle assembly and release. Perhaps HIV-1 co-opts proteins involved in endocytic machinery in epithelial cells to aid in crossing to the basolateral compartment in Vk2 cells[189].

In summary, to address the first aim outlined in the thesis, we determined that indeed, the stratified barrier is a robust defense against HIV-1. However, HIV-1 can still cross an intact stratified layer of Vk2 cells as virus was present in basolateral supernatants. Moreover, HIV-1 uses the endocytic pathway to cross Vk2 cells *in-vitro.*

5.2 Determine the Role of LAMP3 in HIV-1 Uptake and Transcytosis Across Vaginal Epithelial Cells

It is important to elucidate host-factors exploited by HIV-1 in order to develop better prevention strategies. LAMP3 is a virus inducible gene and has been documented to be involved in the entry and exit of several viruses[157, 162]. Previous studies have suggested that LAMP3 is involved in the endosomal and lysosomal pathway and sorting of cargo into the lysosome and extracellular vesicles [168]. However, the early events and proteins involved in HIV-1 transmission in vaginal epithelial cells remain poorly defined. Therefore, for the second aim of this thesis we decided to investigate the role of LAMP3 in HIV-1 uptake and transcytosis across vaginal epithelial cells.

To investigate the potential involvement of LAMP3 in HIV-1 uptake we first investigated if LAMP3 gene expression was induced upon HIV-1 exposure. While not significant at all timepoints, except 6 hours, for X4, we did see upregulation at various timepoints in both R5 and X4 (Figure 16). This is in agreement with the literature, as LAMP3 is documented to be a virus inducible gene. For instance, Zhou et al., found that LAMP3 is specifically induced upon influenza A virus infection at 12, 24, and 48 hours in in human lung epithelial (A549) cells[157]. Host factors can play an important role in HIV-1 progression and transmission. For example, the host factor TRIM5α, a cytoplasmic protein, has been implicated in the inhibition of the early steps of HIV-1 replication[190]. Specifically, upon interaction with the HIV-1 capsid protein, TRIM5α forms a

hexameric lattice structure causing an increase in E3 ubiquitin ligase activity, resulting in premature uncoating of the viral core and reverse transcriptional blocking[191]. It could be possible that HIV-1 is using LAMP3 to somehow modulate the efficiency of transport within Vk2 cells[192].

We also found that LAMP3 is localized in endosomal compartments in Vk2 cells (Figure 18) indicating a potential biological interaction. To further investigate this, intracellular and basolateral HIV-1 was assessed using LAMP3 KD and LAMP3 OE cell lines. Interestingly, we found that OE of LAMP3 resulted in more HIV-1 intracellularly in both X4 HIV-1 and R5 HIV-1 treated cells compared to WT-Vk2 and LAMP3 KD cell lines according to confocal microscopy, although no differences were seen when comparing X4 HIV-1 and R5 HIV (Figure 20). However, when assessing basolateral HIV-1, a significantly increased amount of R5 virus was seen in basolateral supernatants of LAMP3 KD cell lines compared to basolateral supernatants in WT-Vk2 cells (Figure 22B). Basolateral supernatant in LAMP3 OE cell lines, while not significant, contained less R5 virus compared to basolateral supernatants of WT-Vk2 cells (Figure 22 B). However, no differences were seen in basolateral supernatants of LAMP3 KD, LAMP3 OE or WT-Vk2 cell lines in X4 treated cells (Figure 22 A). LAMP3 belongs to the tetraspanin superfamily and is reported to incorporate into HIV-1 virions and colocalize with HIV-1 proteins, Env and gag[193-195]. LAMP3 is also reported to be associated with tetraspanin enriched microdomains that act as a gateway for HIV-1 budding at the plasma membrane in HeLa and Jurkat cells[196]. We also saw colocalization of LAMP3 and HIV-1 in both X4 HIV-1 and R5 HIV-1 treated cells (Figure 19), which may act as a gateway for HIV-1 entry in Vk2 cells, however, further experimentation needs to be conducted on LAMP3 and its role in HIV-1 entry. While no literature has been conducted on the interactions of LAMP3 and HIV-1 in epithelial cells, some research has been done on the role of LAMP3 in HIV-1 infection in macrophages which may shed

some insight into our results. Interestingly, there were slight differences in the MFI between X4 and R5 (Figure 20), documentation of CXCR4 downregulation on host cells from mutations in LAMP3 have been reported and thus less entry of X4 was observed[197]. Although epithelial cells lack CD4 they contain both CXCR4 and CCR5 receptors[13, 198]. Perhaps alterations of LAMP3 expression changes surface expression of CXCR4 in LAMP3 KD cell lines compared to LAMP3 OE cell lines. Confocal microscopy assessing CXCR4 and CCR5 expression in KD and OE cell lines could help answer this question. Another possible explanation as to why we see increased HIV-1 present in OE cell lines compared to both KD and WT-Vk2 cells is through autophagic processes. LAMP3 has been implicated in autophagic processes. Specifically, it has been shown that knockdown of LAMP3 reduces the ability of cells to complete the autophagic process, and cells with high LAMP3 expression show increased basal autophagy levels[199]. Autophagy is a tool that host cells use to defend against viral infection, specifically, autophagosomes deliver trapped cargo to the lysosome for degradation. The process of viral autophagy results from the innate immune system and PRR signaling to induce interferon production and selectively degrade immune components associated with viral particles[200]. In fact, several lines of evidence support beneficial effects of the stimulation of autophagy-mediated lysosomal degradation to potentiate the immune response to HIV-1[201]. Moreover, inhibition of autophagy by HIV-1 is necessary to prevent the sequestration of HIV-1 proteins within autophagosomes and subsequently their lysosomal degradation[201]. It could be that basal autophagy levels are higher in LAMP3 OE cell lines resulting in the trapping of HIV-1, however, this needs to be further investigated.

Another observation we saw was the presence of significantly more HIV-1 in basolateral supernatants of LAMP3 KD cells than WT-Vk2 cells in R5 treated groups in LLI cultures 24 hours after exposure to HIV-1, but this same observation was not seen in X4 treated Vk2 cells. This was

surprising as literature has shown KD of LAMP3 in macrophages resulted in less HIV-1 production in macrophages[158, 159]. This could indicate a potential role of LAMP3 in the trafficking of R5 isolates of HIV-1 but not X4. As HIV-1 uses the endocytic compartment, from the late endosomes, LAMP3 delivers its cargo to either the lysosomes for degradation or in intraluminal vesicles which are released as exosomes after fusion of multivesicular bodies to the plasma membrane[202]. Since X4 has been documented to be selectively sequestered over R5 it is possible that LAMP3 is required for signaling of R5 to the lysosomal compartments for degradation and that a loss of LAMP3 allows R5 strains to escape degradation by the lysosome. Although X4 also colocalized with LAMP3 there could be other host-factors that may recognize X4 over R5 resulting in the sequestration of X4 isolates. However, this is speculation and requires further investigation. This could explain differences in basolateral virus and why we do not see these same differences in X4 and R5 treated cells. LAMP3 been proposed to act a molecular facilitator, stabilizing membrane proteins required for signaling events or other cellular functions[203]. Although not an HIV-1 study, it was found that in immortalized human embryonic lung fibroblasts, and HEK 293T cells that depletion of LAMP3 resulted in increased HSV-1 yields and that LAMP3 in HSV-1 extracellular vesicles could control dissemination of the virus in the host[204]. Using various trafficking inhibitors may shed some light into the mechanism by which the KD of LAMP3 is exerting its effect on increased trafficking of HIV-1.

5.3 Limitations

While every model has its strengths, there are also limitations. It is important to recognize the limitations and the subsequent impacts it could have on the interpretation and translatability of our study. Our model allows the foundational study of potential host-factors that could influence HIV-1 susceptibility. However, there are several fundamental features of the lower FRT that are not

present including a layer of mucus, presence of microbiota, and immune cells[205]. The impact that this would have on our results is not known. However, one could speculate that we may see increased levels of transmission if Langerhans cells (LC) and T cells were present within our system. HIV-1 could penetrate through the stratified squamous epithelium and contact LC by passing between epithelial cells, LC could then migrate and transfer HIV-1 to resting T cells resulting in infection[206]. On the other hand, having a layer of mucus and bacteria could slow HIV-1 transcytosis or decrease infection measured in TZM-bl cells by trapping and inactivating HIV-1. For instance, enhanced trapping of HIV-1 by mucus is seen with a more Lactobacillus dominant microbiota[207]. Another important limitation is that the observation of significantly increased HIV-1 present in LAMP3 KD was seen using a monolayer of cells. While this adds to our knowledge of host-factors that may influence HIV-1 susceptibility, the translatability of this unclear. The vaginal epithelial cells that line the vaginal tract are stratified, not a single monolayer. It could be possible that when HIV-1 is added to a stratified multilayer of cells, less HIV-1 is interacting with LAMP3 which results in a reduced response. Therefore, testing this in animal models would be beneficial to assess whether similar results are observed. Moreover, off target effects using CRISPR/Cas9 to alter gene expression is often observed[208]. For example, overexpression of LAMP3 was found to promote metastasis in uterine cervical cells[209]. If cell structures are altered in OE or KD such as signaling to the lysosome for degradation of essential proteins, not just HIV-1, this could have implications in the breakdown of multiple macromolecules for the natural functioning of the biological system. Together, there are various biological factors in our system that could potentially distort the accuracy of our findings and additional experiments both *in-vitro* and *in-vivo* are necessary to confirm the results.

5.4 Future Directions

Using an ALI and LLI culture system allowed a foundational analysis of how HIV-1 crosses Vk2 cells and the involvement of LAMP3 in HIV-1 uptake and transcytosis in Vk2 cells. While this provided insights into the mechanism of how HIV1 crosses Vk2 cells there were several factors that may influence susceptibility in the FRT that were not present in our system. Thus, future efforts focusing on more accurately representing biological conditions in the FRT should be developed to gain a more comprehensive understanding in future studies. Including immune cells, a mucus layer, and the presence of microbiota commonly found in the vaginal tract would allow this model to be more characteristic of *in-vivo* systems. Additionally, we used cell-free virus in our culture system, including cell-associated HIV-1 with relevant cell populations present in the vaginal tract would provide an understanding of cell-cell transmission and if that influences transcytosis of HIV-1 across vaginal epithelial cells. Another avenue to exposure would be to assess the potential selective transmission of R5 HIV-1 over X4 HIV-1 and why there appears to be increased levels of R5 virus crossing the vaginal epithelium. Lastly, using inhibitors to delineate the precise mechanism of LAMP3 trafficking in KD cell lines would assist in understand its role in HIV-1 trafficking. Overall, our model system provides a solid foundation to exposure multiple areas of future investigation and advance our understanding of HIV-1 transmission in the FRT.

5.5 Conclusion:

Women are at an increased risk of HIV-1 acquisition, especially in sub-Saharan Africa, therefore it is imperative to understand early transmission events that occur in the FRT to develop better prevention strategies. The stratified barrier is robust against HIV-1 entry; however, HIV-1 can still penetrate these layers and gain access to the underlying target immune cell populations. Therefore, the goal of this study was to gain a deeper understanding trafficking of HIV-1 in the

lower FRT and LAMP3's potential involvement in the trafficking of HIV-1 in vaginal epithelial cells. To do this we used both ALI and LLI culture systems and examined barrier function, HIV-1 crossing the epithelium into the basolateral compartment and assessed intracellular compartments HIV-1 uses to cross Vk2 cells using both X4 and R5 strains of HIV-1. Next, we utilized LAMP3 KD and LAMP3 OE cell lines to investigate the potential role of LAMP3 in HIV-1 uptake and transcytosis across Vk2 cells.

The main findings of this study were that 1) HIV-1 does not impair barrier function in ALI culture systems *in-vitro*, however, in a monolayer of Vk2 cells, HIV-1 exposure results in decreased barrier function as demonstrated through a significant decrease in TER's and an increase in FITC dextran leakage. 2) HIV-1 utilizes the endocytic pathway to cross Vk2 cells shown by inhibitor dynasore, as well as colocalization in endocytic compartments EEA1 and RAB7. 3) Increased amounts of R5 virus were present in basolateral compartments compared to X4 virus. 4) LAMP3 is specifically induced in Vk2 cells upon HIV-1 exposure and may influence HIV-1 trafficking as seen by decreased levels of HIV-1 in LAMP3 OE cell lines and more HIV-1 present in basolateral supernatants of LAMP3 KD cell lines as seen by TZM-bl assays. This study successfully lays the foundational assessment of understanding how HIV-1 crosses Vk2 cells and the role of LAMP3 in HIV-1 uptake and transcytosis across Vk2 cells. This lays the groundwork for future functional studies which should be focused on understanding why increased amounts of R5 HIV-1 are present in basolateral compartments in Vk2 cells compared to X4 HIV-1 and the mechanism by which LAMP3 may be exerting its effect upon HIV-1 exposure in Vk2 cells.

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